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thesis entitled

Extrusion Processing of Cereals and Legumes:
Protein-Phytate-Iron Interactions in Relation
to Iron Bioavailability

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Human Nutrition

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Date November 11, 1994

EXTRUSION PROCESSING OF CEREALS AND LEGUMES: PROTEIN-PHYTATE-IRON INTERACTIONS IN RELATION TO IRON BIOAVAILABILITY

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1994

ABSTRACT

EXTRUSION PROCESSING OF CEREALS AND LEGUMES: PROTEIN-PHYTATE-IRON INTERACTIONS IN RELATION TO IRON BIOAVAILABILITY

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The effects of extrusion processing on availability of iron from cereals and legumes was investigated. Legume flours extruded at 110°C had higher in vitro iron dialyzability compared to those extruded at 135°C or to boiled legume flours. Durum wheat semolina extruded at 50°C or 96°C had higher amounts of dialyzable iron than raw semolina. In vivo iron bioavailability, measured by rat hemoglobin regeneration efficiency, was lower in durum wheat pasta extruded at 205°C compared to semolina or a standard source of iron, ferrous sulfate. The negative effect of high temperature extrusion on iron availability was diminished by navy bean supplementation. Durum wheat (85%)/navy bean flour (15%) pasta extruded at the same temperature (205°C) had higher iron bioavailability than durum wheat pasta.

The mechanisms involved in the effects of extrusion processing on iron availability were studied. Although extrusion resulted in extensive degradation of phytic acid and tannins, two known inhibitors of iron bioavailability, these factors alone could not explain the varying extrusion effects

on iron dialyzability. A more complex system involving the associations of protein, phytate, and iron was investigated by in vitro assays. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of reduced and unreduced durum wheat protein fractions indicate that the formation of disulfide linkages during extrusion may be responsible for the greater than two-fold increase in insoluble protein fraction of semolina after extrusion at 50°C or 96°C. Extrusion at both experimental temperatures also increased the amount of iron and phytate associated with the albumin and globulin fractions of semolina. An iron binding polypeptide (MW:16,500 Da) was identified in the albumin, globulin and insoluble protein fractions of durum wheat. It is proposed that an increase in the iron bound to the soluble and low molecular weight proteins, albumins and globulins may explain the increased dialyzability of iron after low temperature extrusion.

ACKNOWLEDGMENTS

I gratefully acknowledge the financial support of the John Harvey Kellogg Fellowship which enabled me to complete my doctoral program.

I would like to express my gratitude to my major professor, Dr. Wanda Chenoweth for her thoughtful guidance, encouragement and advice throughout my graduate study.

I also would like to thank my committee members, Dr. Maurice Bennink, Dr. Perry Ng, Dr. Mark Uebersax and Dr. Irvin Widders for serving on my committee and offering their technical expertise.

Thanks to my labmates and all my friends at Michigan State University for making my stay here exciting and memorable.

My love and gratitude to my parents and family members for inspiring and stimulating my interest in learning.

Finally, my deepest appreciation is extended to a special person in my life, Rishi Nalubola for his confidence in my abilities and his unflinching support towards my graduate education.

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CHAPTER 1. INTRODUCTION

Iron is a trace element that is of continuing public health concern. Iron deficiency is a common disorder with incidence varying widely with age, sex, race and economic status (Fairbanks et al., 1971; DeMaeyer and Tegman, 1985; Stoskman, 1987; Arthur and Isbister, 1987; Skikne, 1988). The prevalence of anemia is estimated at 30% of the world population (DeMaeyer and Tegman, 1985). Nutritionists have routinely expressed concern that the levels of iron intake are not adequate and marginally deficient intakes may exist (Wolf, 1982; Arthur and Isbister, 1987).

Food iron may be classified as either heme iron or nonheme iron. Although the percent absorption of nonheme iron is much lower than that of heme iron, a normal diet is higher in nonheme iron than in heme iron. Thus, the major contribution of available iron is made by nonheme iron from sources such as grains, legumes, fruits and vegetables. Heme iron sources include animal foods such as beef, pork, lamb, chicken, fish and liver.

A meal is a complex, chemical mixture containing compounds which interact with minerals and either inhibit or facilitate their absorption. Total mineral content in a food or meal, therefore, provides only a rough approximation of the amount available for absorption (Monsen, 1980).

Bioavailability is defined as the proportion of the total mineral in a food, meal or diet that is utilized for normal body functions (Fairweather-Tait, 1992). Variations in dietary levels and forms of proteins, peptides, and amino acids can affect the bioavailability of a variety of minerals including iron and zinc. In addition, during food processing, interactions that take place between minerals and other components of food such as proteins and phytates may have either a positive or negative effect on mineral bioavailability.

LITERATURE REVIEW

EXTRUSION PROCESSING

The food extruder is considered a high-temperature short-time bioreactor (Harper, 1989) that is being used increasingly to manufacture a variety of products. These include increasing varieties of ready-to-eat cereals, salty and sweet snacks, croutons for soups and salads, dry and soft-moist pet foods, pasta and macaroni products, texturized meat analogs made from vegetable protein, precooked food mixtures for infant feeding, soup and drink bases, and pregelatinized starch.

The operation of an extruder has been summarized by Harper (1989). Ingredients are released from the feed hopper into the preconditioner at a controlled rate. The preconditioner is a pressurized chamber in which raw granular food ingredients are uniformly moistened or heated by contact with water or steam before entering the extruder. When the food enters the extruder, the extrusion screw sequentially conveys and heats food ingredients and works them into a continuous plasticized mass while rotating in a tightly fitting barrel. As the flights on the extruder screw convey the food materials down the barrel, the mechanical energy used to turn the screw is dissipated causing a rapid rise in the temperature of the food ingredients. The resulting plasticized feed ingredients are then forced through a die.

The pressure drop across the die rapidly converts the high-temperature water in the product to steam and causes puffing to occur.

The use of twin-screw extruders is relatively new and has the advantages of improved conveying and mixing capabilities and an extended range of applications compared to the single-screw extruder (Smith and Ben-Gera, 1980). The advantages of extrusion processing over conventional cooking procedures are its relatively low cost, high productivity, energy efficiency, ability to produce high quality products, ability to process dry, viscous materials, and ease of production of new foods and product shapes (Harper, 1989).

CHEMICAL INTERACTIONS OF MINERALS IN FOODS

There are many reactions that minerals might undergo in foods to effect a change in solubility and/or charge that subsequently alters their bioavailability. Metals exist in water not as ions but as hydrates. Hydrolysis of the hydrates may occur as the pH is raised, causing them to lose protons and forming less soluble or insoluble hydroxides, which may precipitate and thus become unavailable (Clydesdale, 1988).

Various types of bonds can effectively tie up and precipitate or solubilize a mineral in a complex. Chelation is one form of complex formation where the ligand forms more than one bond with the mineral. Mineral complexes can also be formed through ionic and covalent bonds. Often, these bonds are responsible for the chemical properties of the complex. In determining the physical properties, including solubility of a complex, intermolecular forces such as dipoles, hydrogen bonds, and dispersion or London forces play a more important role than bonding (Clydesdale, 1988).

The mechanisms involved in mineral interactions in foods which have the potential to affect bioavailability have been broadly defined into four categories by Clydesdale (1989):

1. Mineral Displacement - Displacement of a mineral from a complex with another mineral to form a soluble (available) or insoluble (unavailable) complex, eg. mineral reactions with fibers or phytate.

2. Polymineral-Ligand Complexes - The addition of a second or third mineral to a soluble mineral-ligand complex causing precipitation by forming a polymineral-ligand complex, eg. calcium-zinc-phytate complex.

3. Polymineral-Polyligand Complexes - The addition of a mineral causing a mineral-ligand complex to form one or more minerals binding to more than one substrate (ligand) and forming a polymineral-polyligand complex, eg. iron-zinc-phytate-protein complex, protein-phytic acid-zinc complex.

4. Enzyme Susceptibility of Complexes - The formation of a polymineral-ligand complex which changes the susceptibility of the mineral-ligand bonds to cleavage by digestive enzymes.

CHARACTERIZATION OF IRON IN CEREALS AND LEGUMES

The form in which iron exists in wheat has been studied. In wheat bran, approximately 60% of the total iron is present in a salt extractable form identified as monoferric phytate. The biological availability of iron from monoferric phytate, either isolated from wheat bran or the synthetic product, determined by a hemoglobin depletion-repletion bioassay, was seen to be equal to a reference compound, ferrous ammonium sulfate (Morris and Ellis, 1976). Another form of iron, ferric phytate (3 to 4 moles iron/mole phytate), thought to be present in the insoluble bran residue, had significantly lower biological availability than monoferric phytate or the reference compound. Because of its salt-extractability and water soluble properties, it was proposed that monoferric phytate in bran may be bound to cationic sites of proteins or other cellular components and the utilization of the iron is through solubilization of the monoferric phytate by an ion exchange mechanism (Morris and Ellis, 1976).

In another study, May et al. (1980) examined the nature of the endogenous iron in wheat grains and bran using ^{57}Fe Mössbauer spectroscopy. The spectra of seeds and bran from wheat grown in ^{57}Fe-enriched culture indicated that most of the iron in wheat is present in the form of monoferric phytate with a high-spin configuration.

Iron in different legumes has also been isolated and characterized. Crichton et al. (1978) isolated phytoferritin, a storage form of iron, from pea and lentil seeds. Subunits with molecular weights of 20,300 and 21,400 Da were identified in peas and lentils, respectively. Phytoferritins were seen to have a larger cavity in the interior of the molecule than mammalian ferritin, thus enabling them to store 1.2 to 1.4 times as much iron. Although the quartenary structure of the phytoferritins was similar to mammalian ferritin, there were large differences in primary structure, phytoferritins having a higher asparagine/aspartic acid content and a higher isoelectric point than mammalian ferritin. Phytoferritin was identified by Lynch and Covell (1987) as the major iron-containing protein fraction in commercial soybean flour.

Kojima et al. (1981) studied iron distribution in pinto beans in terms of ease of solubilization. They reported that about 25% of the iron in pinto beans is readily soluble upon incubation, 45% of the iron can be mobilized by chelating or reducing agents and 30% of the iron is firmly bound to the insoluble bean residue.

Chidambaram et al. (1987) reported fractionation and partial characterization of iron binding components of digested pinto beans. In vitro enzymatic digestion and dialysis (molecular weight cutoff of 14,500 Da) showed that 58% of the iron binding components of pinto beans are dialyzable. Using ^{59}Fe, a low MW (14,500 Da) protein fraction was identified in the dialysate. The predominant non-dialyzable protein fraction, which retains 42% of iron was found to have a MW of 49,000 Da. One of the weaknesses of this study is the use of ^{59}Fe to identify iron binding proteins which requires the assumption of equilibration between the added radioactive iron and the naturally present iron.

The major hindrance to studies identifying or quantifying iron binding protein fractions from foods is the lack of appropriate techniques. One alternative to using radio-iron is the use of reagents that can specifically stain iron binding proteins separated on a polyacrylamide gel. Chung (1985) developed a simple and fast staining procedure using a Ferene S/thioglycolic acid reagent that can be used to specifically stain iron binding proteins on polyacrylamide gels. The method is based on the reaction between Ferene S and iron atoms present in the proteins with thioglycolic acid acting as a reducing agent (converting Fe^{+++} to Fe^{++}) and an anion labilizer (facilitates iron release from the proteins). The reagent was shown to detect transferrin, lactoferrin, ferritin, hemoglobin and cytochrome *c* with good sensitivity.

DETERMINING IRON BIOAVAILABILITY

Several approaches including in vitro, animal and clinical studies have been used to estimate the bioavailability of iron in foods. One in vitro method is based on the assumption that a soluble form of iron is likely to be absorbed. It includes the measurement of iron soluble in dilute hydrochloric acid (Shah et al., 1977; Lee and Clydesdale, 1979). The most common in vitro procedure is the iron dialyzability assay (Miller et al., 1981). Dialyzable iron passes from an in vitro enzymatic (pepsin-pancreatin) digestion of a food or test meal into the interior of a dialysis bag containing a bicarbonate buffer. The most widely used animal method compares the hemoglobin response in anemic rats given graded quantities of the iron source with that obtained from comparable amounts of ferrous sulfate. Two variations of the method are possible -- the slope ratio assay (official method of AOAC) which measures the relative biological value of an iron source, and the hemoglobin regeneration efficiency (Mahoney and Hendricks, 1982) which measures the percentage of iron ingested that is incorporated into hemoglobin. Clinical studies in humans to determine the availability of iron in foods commonly involves feeding of a food with extrinsic or intrinsic radioiron tags. At the end of the experimental period, blood is drawn to measure hematocrit and serum ferritin, and to determine the absorption of iron (Cook et al., 1972; Hallberg and Bjorn-Rasmussen, 1972; Forbes et al., 1989).

EFFECT OF PROTEIN ON IRON AND ZINC BIOAVAILABILITY

Protein-Iron Interactions

A great deal of literature is available on the interactions between proteins and iron during absorption and their effects on the availability of the mineral. A proposed mechanism for nonheme iron absorption suggests that free iron released during digestion chelates with a variety of substances, including proteins. Some of these substances bind iron tightly and are excreted along with the bound iron, while other substances release the iron at the mucosal surface (Monsen, 1988).

Results from studies using extrinsic radioactive iron labels added to single foods or to meals have shown that iron absorption differs depending on the type of protein in food. Noncellular proteins such as egg albumen, milk, cheese, and soy protein tend to depress nonheme iron absorption in humans (Cook and Monsen, 1976; Lynch et al., 1985) while cellular proteins such as beef, liver, lamb, pork, chicken, fish are known to enhance nonheme iron absorption in humans (Hallberg et al., 1979; Cook and Monsen, 1976). In addition to their effects on non-heme iron, cellular proteins also have been shown to enhance heme iron absorption in humans (Hallberg et al., 1979).

The Meat Factor. The facilitation of nonheme iron absorption by meat is not a general property of all animal tissues but is specific to certain animal proteins (Hurrell et al., 1988). The identity of this iron absorption enhancing "factor" in meat is not known in spite of considerable research. It is suggested that the meat factor may be the cysteine-containing peptides released during digestion of beef

(Martinez-Torres et al., 1981; Taylor et al., 1986). Cysteine containing peptides produced during meat digestion were seen to enhance absorption of nonheme iron in humans. In contrast, oxidizing these products reduced the absorption of nonheme iron by 63% (Taylor et al., 1986). Thus it appears that free sulfhydryl groups aid in the absorption of nonheme iron.

Meat may enhance iron bioavailability by keeping iron in a soluble form. The iron solubilization effect of meat may be due to a particular peptide fragment(s) and/or profile of amino acids. In vitro pepsin digestion products with MW <10,000 solubilized significantly more iron than those with MW >10,000 (Clydesdale, 1988). Both raw and cooked meat appear to increase nonheme iron absorption. A water extract of cooked beef has been reported to increase nonheme iron absorption to a lesser extent than the residue (Bjorn-Rasmussen and Hallberg, 1979). Monsen (1988) reported that addition of a mixture of amino acids that mimic beef increased the absorption of nonheme iron.

<u>The Milk Factor.</u> Based on in vitro studies conducted using a mixture of milk with iron in cereals (Clydesdale and Nadeau, 1984) and with iron in model systems (Platt et al., 1987), Clydesdale (1988) proposed that there is a "milk factor" which exerts a protective effect against the fiber- and phytate-induced precipitation of iron and thus increases its potential bioavailability. Clydesdale and Nadeau (1984) evaluated the ability of milk and several of its fractions to

solubilize iron in corn, wheat and oats. There was more total soluble iron in the presence of whole milk, lactose-free milk and nonfat dry milk than a water control. When deproteinized milk was used, the solubilization effect largely disappeared in wheat and in a mixture of the three-grains. They concluded that the milk factor seemed to be in the protein fraction.

In vivo studies have also demonstrated the iron absorption enhancing effect of milk. Randhawa and Kawatra (1993) found that a habitually consumed diet supplemented with 190 ml milk (equivalent of 8 g protein) per day greatly improved the apparent absorption and retention of iron, zinc, copper and manganese in pre-adolescent girls.

Egg Proteins. Peters et al. (1971) reported low bioavailability of egg yolk iron in humans as well as in vitro. Phosvitin, the principal phosphoprotein of the yolk, contains about 0.4% iron, accounting for most of the iron present (Taborsky, 1983). It has been shown that 50% or more of the amino acid residues of this protein are phosphoserine, accounting for the high iron-binding capacity (Taborsky, 1983). Sato et al. (1984) showed that the phosvitin-iron complex could promote iron precipitation in the intestine. Albright et al. (1984) reported that iron from the phosvitin-iron complex is not released by heating or treating with citric acid or NaCl. Only EDTA released iron from phosvitin without heating. Phosphopeptides released from phosvitin during luminal digestion of egg yolk protein have the ability to strongly bind ferric iron in the lumen (Sato et al., 1985). Thus, phosvitin is presumed to be responsible for the poor availability of egg yolk iron.

Soy Proteins. Legume proteins are generally acknowledged to adversely affect iron bioavailability (Bothwell et al., 1982). Soybeans are rich in iron, but the bioavailability of iron in humans is low. One explanation may be that a major fraction of the iron in soybeans is present in the form of phytoferritin which is known to have low bioavailability (Lynch and Covell, 1987). Substitution of a portion (30% to 50%) of meat in a hamburger meal by soy flour reduced nonheme iron bioavailability in humans (Hallberg and Rossander, 1982; Lynch et al., 1985). In vitro studies suggest that the inhibitory effect of soybean products on nonheme iron bioavailability may be related to the protein component of soy products (Schricker et al., 1982). In vitro digestion of soybeans showed iron trapped within large peptide aggregates; iron was released only upon dissociation of the aggregates (Schnepf and Satterlee, 1985). A soybean protein isolate reduced nonheme iron absorption to a greater extent than the whole beans and extensive enzymic hydrolysis of the soybean protein with papain removed most of the inhibitory effect (Lynch and Covell, 1987).

The influence of soy protein on iron nutrition, however, is not entirely negative. When soy flour was partially substituted for beef, there was 30-60% improvement in heme iron absorption compared to a beef control. This increase in the absorption of heme iron was seen when soy flour was incorporated with ground beef into a meat patty and fed to human subjects solely as a patty or as part of a mixed meal (Lynch et al., 1985).

Not only the iron naturally present in foods, but also the iron added to foods can be affected by the type of protein in the food. It has been shown that bioavailability of added iron can be influenced by the formation of iron complexes with protein digestion products. In a study measuring iron dialyzability in the presence of selected proteins and fractionated protein digestion products, Kane and Miller (1984) found that dialyzability of added iron (ferric chloride) was higher for bovine serum albumin and beef, than for egg albumin, gelatin, casein, soy protein isolate or gluten. Low molecular weight (<6000 to 8000 Da) digestion product fractions from bovine serum albumin and beef were seen to enhance iron dialysis; similar fractions from casein and soy protein isolate did not affect iron dialysis. The authors speculated that the influence of proteins on iron bioavailability may be related to the affinity of undigested or partially digested protein for iron and to the stability of small molecular weight soluble iron complexes formed from protein digestion products.

Another study conducted by Nelson and Potter (1979) determined, in vitro, the ability of five protein sources (wheat gluten, soy protein isolate, zein, albumin, and casein) to bind added ferrous and ferric iron as well as the effects of pH, time and temperature on this binding. More than 50% of the added iron (ferrous or ferric iron) was bound to the insoluble fraction of the proteins. In vitro digestion of these complexes in an HCl-pepsin-pancreatin system released 64-97% of the iron suggesting that protein-bound iron may be readily freed for absorption within the gastrointestinal tract.

Subsequent research using the hemoglobin repletion assay in rats showed that protein-bound ferrous iron was as biologically available as ferrous sulfate. In contrast, the bioavailability of protein-ferric complexes was lower than the standard, ferric pyrophosphate (Nelson and Potter, 1980).

Protein-Zinc Interactions

Changes in dietary protein levels affect the absorption of a number of minerals including zinc. However, the effect of dietary protein on zinc absorption in humans is controversial. Greger and Snedeker (1980) observed that human subjects absorbed zinc more efficiently when fed a high protein (24.1g N/day) diet than a low protein (8.1g N/day) diet. Other investigators have observed either improvements in zinc absorption and/or retention (Price et al., 1970) or no changes (Colin et al., 1983) when dietary protein levels were elevated.

Several individual amino acids, particularly histidine, cysteine, and tryptophan, have been found to increase absorption and/or retention of zinc in tissues of rats (Greger and Mulvaney, 1985; Wapnir and Stiel, 1986). In humans the bioavailability of zinc from zinc:histidine complexes mixed in a ratio of 1:2 or 1:12 was shown to be higher when compared to zinc sulfate (Scholmerich et al., 1987). Theories on how amino acids may improve zinc absorption have been put forth by many investigators. Greger (1988) suggested that amino acids may improve absorption by forming soluble complexes with zinc and thus prevent formation of insoluble hydroxides. Wapnir and Stiel (1986) studied, in vivo, the structural characteristics of amino acids that could influence the facilitation of zinc intestinal absorption. Greater zinc absorption from zinc-amino acid complexes than from zinc-non amino acid homologues in the small intestine of rats suggests that amino acid transport systems may be involved.

Dietary proteins can also affect mineral utilization when minerals are "trapped" inside protein or peptide complexes that are resistant to proteolysis. Lonnerdal (1987) suggested that incomplete hydrolysis of casein in cow's milk by infants may lead to decreased absorption of zinc. Browning reaction products (amino acid-carbohydrate complexes) in toasted and other heat-treated foods which are resistant to hydrolysis in the gut can complex metals and have been shown to reduce zinc absorption (Lykken et al., 1986). Similarly, it has been hypothesized that when protein-phytate-zinc complexes are incompletely digested, mineral absorption is decreased (Erdman et al., 1980).

Although a great deal of research has focused on the effects of specific proteins on iron utilization, the effects of egg and meat proteins on zinc availability have not been studied in depth. The effect of milk protein on the absorption of zinc from sources other than milk has not been studied. However, much research has been done on the absorption of the zinc present in milk itself. Studies comparing human milk and cow milk have consistently shown that zinc is absorbed more efficiently from human milk (Sandstrom et al., 1983). Bobilya et al. (1991) found that in neonatal pigs the bioavailability of zinc in non fat dry milk and low fat plain yogurt was very high and similar to that in zinc carbonate. Wood and Hanssen (1988) reported that compared to water, cow's milk and lactose-free milk significantly reduced zinc absorption in postmenopausal women. Pecoud et al. (1975) showed that when single doses of zinc sulfate were given along with milk to healthy young volunteers, zinc absorption was decreased as indicated by a significant drop in serum zinc levels.

Zinc is known to form complexes with soy proteins also. In a study of zinc complexes, the major portion of extractable zinc from defatted soy flour was found to be either free or associated with very low molecular weight proteins, peptides or their complexes with phytic acid rather than the major proteins of soybean (Clydesdale, 1989). The interactions between zinc, proteins and phytic acid are discussed further in the section below dealing with phytate-protein-zinc complexes.

EFFECT OF PHYTATE AND PHYTATE-PROTEIN COMPLEXES ON IRON AND ZINC BIOAVAILABILITY

Phytate [myoinositol 1,2,3,5/4,6-hexakis (dihydrogen phosphate)] is a naturally occurring organic compound found in plants. It constitutes 1-6% by weight of most nutritionally important legume, cereal, and oilseeds, where it serves as a storage phosphate that becomes available during germination (Lasztity and Lasztity, 1990).

Phytic acid strongly interacts with proteins in a pH-dependent manner. At low pH, phytic acid forms electrostatic linkages with the basic lysine, arginine, and histidine residues, resulting in neutral, insoluble complexes that dissolve only below pH 3. At neutral and basic pH, both phytate and most proteins have a net negative charge which leads to their virtually complete dissociation from each other. In the presence of multivalent cations, however, protein-cation-phytate complexes may occur. These complexes tend to dissociate at extremely high pH (>10) and the phytic acid becomes insoluble, while the protein remains in solution (Cheryan, 1980).

O'Dell and deBoland (1976) developed a method for the identification of protein-phytate complexes on polyacrylamide gels via the precipitation of phytate as a white band of ferric phytate. Recently, DiLollo et al. (1991) showed that the method developed by O'Dell and deBoland is not specific for protein-phytate complexes. They evaluated the use of a chromogen, cobalt hexaammine chloride solution believed to be specific for iron, as a means to improve the visualization of the ferric phytate on the gels. Results suggest that the white bands which are believed to indicate protein-phytate complexes could be the result of non-specific protein iron interaction in addition to phytate iron interaction. These findings were supported by experiments in which proteins containing no phytate gave positive staining for the reaction believed to be specific for a phytate-iron complex. A procedure to accurately identify protein-phytate or protein-phytate-iron complexes separated on polyacrylamide gels is yet to be developed.

Phytate has been suggested to influence protein digestibility. Ritter et al. (1987) reported that in vitro digestibility (determined by the pH stat procedure and the dialysis equilibrium method) of soy protein with lower phytate content (0.07%) was greater than soy protein with a higher phytate content (1.41%). In vitro kinetic studies were conducted with a range of soy protein and phytate concentrations to determine the velocity of the hydrolysis reaction in the presence of the enzyme pronase. Results of the kinetic studies indicated that differences in the digestibility of the soy proteins were not due to stearic hindrance by phytate at enzyme-substrate reaction sites but probably due to accumulation of end products.

In addition to its interactions with protein, phytate is known to have an adverse effect on mineral nutrition. Under favorable conditions, phytic acid may precipitate all polyvalent cations. The insolubility of metal-phytate complexes has been claimed to interfere with the bioavailability of calcium, zinc, and iron in humans (Morris, 1985; Graf and Dintzis, 1982; Graf and Eaton, 1984).

Phytate also forms complexes with more than one mineral in a complex, eg. calcium-zinc-phytate complex. Due to the higher affinity of zinc than of calcium for phytate, calcium exhibits a bimodal effect on the solubility of zinc, i.e. low concentrations of calcium increase the solubility of zinc whereas high concentrations potentiate the precipitation of zinc by phytate (Graf and Eaton, 1984).

Interactions with Iron

In mature plant seeds, the inositol phosphates occur mainly as hexaphosphate, but during food processes involving prolonged heat treatment, it is likely that lower forms of inositol phosphates are formed. All inositol phosphates from di- to hexaphosphates were found to form insoluble iron complexes (de Boland et al., 1975).

Complete hydrolysis of inositol hexa- and pentaphosphates through activation of endogenous phytase led to a strong increase in the availability of iron estimated in vitro (Sandberg and Svanberg, 1991). Presence of low inositol phosphates was found to induce an increase in iron solubility through the formation of small soluble iron complexes. During digestion of phytate in the stomach and small intestine it is likely that phytate is degraded to lower inositol phosphate forms (Sandberg et al., 1987).

The beneficial effects of partial phytate removal and heat on iron bioavailability from soy protein-based diets, studied by chick hemoglobin repletion, were reported by Rodriguez et al. (1985). The authors attributed the beneficial effects to promotion of protein-iron-phytate complex digestion and the release of endogenous or added iron. Recently, a protein-related moiety in the conglycinin fraction of soybean protein isolate has been demonstrated to be one of the major inhibitors of iron absorption in humans (Lynch et al., 1994). Although it is known that phytate modifies iron dialyzability/bioavailability (Brune et al., 1992; Sandberg and Svanberg, 1991; Sandberg et al., 1989; Hallberg et al., 1987), it is difficult to identify a quantitative relationship between phytate content and iron dialyzability (Lombardi-Boccia et al., 1991).

Interactions with Zinc

Although the soluble zinc in soybean apparently is not associated with phytate, phytic acid is thought to be the primary inhibitory factor in soybean products that results in reduced zinc bioavailability. A reduction in phytate is reported to improve the bioavailability of soybean zinc (Ellis and Morris, 1981; Forbes et al., 1979; Lonnerdal et al., 1988; Zhou et al., 1992). Lei et al. (1993) reported that supplementation of corn-soybean meal diets with microbial phytase improved the bioavailability of zinc to weanling pigs.

Some of the earliest experimental evidence that a zinc-phytate-protein complex was responsible for the increased requirement of zinc in an animal diet containing soybeans compared to a diet not containing soybeans was provided by O'Dell and Savage (1960). Allred et al. (1964) found that soybean protein from which phytic acid had been removed was no longer capable of binding zinc in vitro. Studies also show that zinc in particular is poorly utilized from soy products as compared to other minerals, although the presence of soy protein per se has little effect on the bioavailability of zinc from other sources in the diet (Forbes et al., 1979; Forbes and Parker, 1977).

Changes in protein phytate interactions during processing may influence zinc bioavailability. Studies show that zinc utilization from certain soy protein isolates is much lower than from soybean meals (Rackis and Anderson, 1977). One explanation may be that isoelectric precipitation used in the manufacture of soy protein isolate causes the formation of phytate-protein complexes, which result in the formation of phytate-protein-mineral complexes in the final product, thus rendering the mineral unavailable (Cheryan, 1980).

EFFECT OF EXTRUSION PROCESSING ON MINERAL BIOAVAILABILITY

Chemical changes during extrusion that have been suggested to play a role in influencing mineral bioavailability are: 1) formation of Maillard products, 2) increase in lignin fraction due to heat treatment and 3) formation of amylose-lipid complexes (Fairweather-Tait et al., 1989). An increase in in vitro iron dialyzability was reported in extruded maize based snack foods (Hazell and Johnson, 1989) and defatted soy flour (Latunde-Dada, 1991). It has been speculated that during extrusion some of the high molecular weight compounds (including phytate) are degraded, thus releasing iron (Latunde-Dada, 1991). A reduction in the phytic acid content of maize and potato after extrusion cooking was reported by Fairweather-Tait et al. (1987) and its degradation may be responsible for increases in iron availability.

Some researchers have reported that extrusion decreased the amount of dialyzable iron. Lombardi-Boccia et al. (1991) found a slight decrease in iron dialyzability after extrusion cooking of legumes. This loss in iron dialyzability could be due to interactions between minerals and tannins, interactions that are probably promoted by the extrusion process. Another possibility is that extrusion cooking deactivates the phytase naturally present in cereals and legumes (Kivisto et al., 1986). Deactivation of phytase prevents degradation of phytate and thus, a decrease in the amount of dialyzable iron may be seen.

Other researchers reported no changes in iron absorption in humans caused by extrusion of maize and potato products (Fairweather-Tait et al., 1987) and high fiber cereal product (Kivistö et al., 1986). The equipment used in the extrusion process considerably increased the iron content of products and this added iron appeared to be as available for absorption as the endogenous iron in the food. However, apparent zinc absorption decreased in humans fed extruded maize and potato products (Fairweather-Tait et al., 1987).

The effect of extrusion cooking of a bran-flour mixture on body retention of iron and zinc in normal adults was demonstrated. Stable isotopes ^{58}Fe and ^{67}Zn were administered with nonextruded or extruded cereal with milk and isotopic retention was measured from fecal excretion. It was observed that extrusion cooking had no effect on isotope retention (Fairweather-Tait et al., 1989). It could be argued that the enhancement effect of milk on nonheme iron and zinc absorption (Randhawa and Kawatra, 1993) may have masked any changes in iron and zinc availability caused by extrusion. Also, the conflicting results on the effects of extrusion on iron and zinc availability reported by various researchers may be partially explained by the differences in temperature, pressure, and moisture conditions used during extrusion processing.

EFFECT OF EXTRUSION PROCESSING ON PHYSIOCHEMICAL PROPERTIES OF PROTEINS

Protein Digestibility

Specific reactions during extrusion that modify the digestibility of proteins and the availability and identity of amino acids, as summarized by Phillips (1989) and Stanley (1989), are the following:

1. Disrupt plant structures - Intact plant structures represent a significant barrier to digestive enzymes, and the combination of heat and shear during extrusion may represent a very efficient way of releasing the nutrients.

2. Break/form non-covalent bonds - In practice, extrusion cooking usually produces complete denaturation of proteins measured as a reduction in solubility and enzymatic activity and the formation of extended unfolded protein networks (Jeunink and Cheftel, 1979; Rhee et al., 1981).

3. Break/form disulfide bonds - Various workers (Rhee et al., 1981; Hager, 1984) have shown that disulfide bonds contribute to the new, extended protein and networks produced by extrusion. Although reformation of extensive disulfide bonded

networks could prevent digestion, the low content of cysteine/cystine in plant proteins makes such an event unlikely.

4. Break/form peptide bonds - There seems to be little evidence to document a major role of peptide bond scission or formation during extrusion cooking.

5. Promote side-chain reactions with reducing sugars - Nonenzymatic browning, the Maillard reaction, is probably the most thoroughly studied side-chain reaction that degrades protein nutritional quality. This type of reaction leads to a decrease in protein quality both by lowering digestibility and by producing nonutilizable and toxic products.

The chemical properties of protein are changed by the Maillard browning reaction even under mild conditions. In general, animal proteins are known to have higher Maillard browning than the plant proteins because of their high content of amino acids such as lysine, methionine, arginine, histidine etc., which readily react with glucose in animal proteins (Yen et al., 1989).

6. Inactivate protease inhibitors - The destruction of protease inhibitors is seen to increase with extrusion temperature and moisture content. At constant temperature, inactivation of trypsin inhibitors in soybeans increased with product residence time and moisture content (Mustakas et al., 1970).

Protein Solubility

Heat treatment used in extrusion processing may alter the solubility of proteins. Solubility of native proteins may be explained by the charge and hydrophobicity of protein molecules; however, solubility of heated protein solutions depends also on the molecular size which is seen to increase through hydrophobic interactions and disulfide bond formation upon heating (Phillips, 1989). Upon heat treatment, initial denaturation of proteins may occur with little or no apparent loss in solubility; the denaturation step is usually followed by aggregation and coagulation or gelation (Nakai and Li-Chan, 1989). For salt-extractable beef muscle proteins, heating at 50°C or higher temperatures results in decreased solubility. In sharp contrast, heating of soy protein isolate solution results in increased solubility (Nakai and Li-Chan, 1989).

Gujska and Khan (1991) studied the effects of high temperature extrusion on protein solubility and distribution in navy and pinto beans. SDS-PAGE showed redistribution of protein fractions in both beans. A high degree of protein insolubility was found after extrusion, which resulted in a decrease in albumin and globulin fractions and an increase in the insoluble residue.

Soy Protein Texturization

The texturization of soy proteins during extrusion processing has been studied extensively because of its primary importance in the manufacture of meat analogs. In native soybean protein molecules, most amino acid residues responsible for the chemical reactions during processing of soybean protein foods - such as cysteine (-SH), cystine (S-S), and hydrophobic amino acid residues - are buried in the inside region of the molecule, inaccessible to water. These residues become reactable with each other through the exposure from the inside by heat denaturation during extrusion processing. The unique texture of texturized soybean products produced by extrusion is the result of both the intermolecular interchange reaction between the exposed -SH and S-S groups and the intermolecular hydrophobic reaction among the exposed hydrophobic amino acid residues (Fukushima, 1991).

Prudencio-Ferreira and Areas (1993) showed that disulfide linkages, and hydrophobic and electrostatic interactions were the main stabilizing mechanisms for the 3-dimensional structure of soy protein isolate extruded at various temperature and moisture contents. Infrared spectra showed the presence of ß-sheet anti-parallel structures. Peptide bonds were of negligible importance in extrusion texturization of soy protein.

WHEAT PROTEINS

Knowledge of the chemistry, composition and function of wheat grain proteins is needed to fully understand the effects of extrusion processing on wheat proteins. The traditional classes of wheat endosperm proteins as separated by fractional extraction using the procedure of Osborne (1907) include: 1) the gluten proteins (gliadins, LMW glutenins, and residue proteins or HMW glutenins) and 2) the nongluten or soluble proteins (albumins and globulins). In addition to these classes, other types of proteins known to be present in the wheat endosperm are lipid-binding purothionins (Redman and Fisher, 1968), ligolins (Frazier, 1983), a small portion (1%) of endosperm protein associated with starch granules (Greenwell et al., 1985), the chloroform-methanol extractable "CM" proteins (Aragoncillo et al., 1975) and the triticins (Singh and Shepherd, 1985).

Gluten Proteins

The traditional method of preparation of gluten involves gentle washing of a flour-water dough in an excess of water or a dilute salt solution to remove most of the starch and soluble material, until the gluten is obtained as a rubbery mass containing about 80% of the total protein of the flour (Wrigley and Bietz, 1988). In general, gliadins and glutenins can be extracted from wheat flour based on their solubility properties; gliadins are soluble in aqueous alcohol, whereas glutenins are soluble in dilute acid, usually acetic acid.

Gluten proteins, gliadins and glutenins, are generally characterized by having a high content of proline and glutamine. Overall, about one of every three amino acid residues is glutamine, and about one of every seven residues is proline (Wrigley and Bietz, 1988). The low content of amino acids with charged side chains, eg. lysine, histidine, arginine, aspartate results in a low ionic character for the gluten proteins. The gliadin and glutenin fractions of gluten proteins differ slightly in amino acid composition. Gliadins have higher amounts of proline, glutamine, cysteine, isoleucine and phenylalanine whereas glutenins have higher amounts of glycine, lysine and tryptophan (Wrigley and Bietz, 1988).

Gliadins and glutenins also differ in their physical properties, most notably in their viscoelasticity. Gliadin is cohesive, but with low elasticity, whereas glutenin is both cohesive and elastic. Gliadin is composed of proteins of relatively low molecular weight in comparison with the high molecular weight (HMW) proteins of the glutenin fraction. The gluten matrix is a major determinant of the unique rheological properties of wheat doughs essential to many of the food uses of wheat flour (MacRitchie et al., 1990).

Nongluten or Soluble Proteins

The soluble proteins of wheat, located in the embryo, aleurone layers and the endosperm account for about 20% of the total protein (Wrigley and Bietz, 1988). The soluble proteins consist primarily of albumins and globulins and include enzymes and enzyme inhibitors involved in metabolic activity

(Payne and Rhodes, 1982). Osborne (1907) described the extraction of albumins and globulins from wheat flour based on their solubility in water and dilute salt solution, respectively.

Albumins and globulins have significantly more lysine, aspartic acid, threonine, alanine and valine than do gliadin and glutenin fractions, but have less glutamine (Wrigley and Bietz, 1988). Because of their higher lysine contents, albumins and globulins have better nutritional values than most other wheat proteins (Lasztity, 1984). Isolation and characterization of albumins and globulins indicates great heterogeneity among the proteins; at least six different kinds of albumins and three different types of globulins were purified by Pence and Elder (1953).

Although the albumin and globulin proteins are presumed to be less important to breadmaking quality than gluten proteins (Wrigley and Bietz, 1988), studies have shown that the soluble protein content is significantly correlated with protein quality and functionality of the wheat flour (Pence et al., 1954; Campbell and Lee, 1982; Lasztity, 1984). In a review of wheat proteins and their technological significance, Schofield and Booth (1983) noted that the overall role of albumins and globulins in breadmaking is not yet clear.

JUSTIFICATION

Cereals and legumes are important components of a diet. Besides being a staple food in the diet of many populations, cereals provide calories, proteins and many essential minerals and vitamins. Legumes are a highly utilized crop due to the large number and diversity of genera and species. Legumes provide variety to the diet and supply needed protein for many populations lacking animal protein (Uebersax and Songyos, 1989). Legumes are also valuable sources of minerals, including iron and zinc.

Extrusion processing has been identified as a key food processing technology for the future (Harper, 1989). It would prove beneficial to expand the uses of extrusion processing to include legumes and legume supplemented cereals because of their significance to people in various parts of the world. The effect of extrusion processing of cereals and legumes on mineral bioavailability is not clear; researchers have reported both beneficial and adverse effects of extrusion processing. Such conflicting data justify further investigation on this topic.

The mechanism(s) by which extrusion affects mineral absorption are not known. Many mechanisms have been suggested - formation of amylose-lipid complexes, Maillard reaction products, lignin complexes. The potential for these complexes to significantly influence mineral availability is unclear.

Interactions between proteins, iron and phytate also could be altered during extrusion processing and influence iron availability. It is known that proteins play an important role in the absorption of minerals by forming complexes with minerals and/or phytates thus either enhancing or inhibiting the absorption of the mineral. It is also well-documented that extrusion results in protein denaturation and changes protein digestibility and solubility. Protein denaturation caused by extrusion processing could affect protein-iron and protein-iron-phytate complex formation and determine the release of the mineral for absorption. Thus, the interactions between protein, iron and phytate as affected by extrusion processing need to be investigated.

The objectives of this study were:

1. To assess the influence of different extrusion processing conditions on iron availability, phytate degradation and tannin content of legumes.
2. To evaluate the effect of extrusion processing of durum wheat and legume supplemented durum wheat on iron bioavailability using a rat model.
3. To study in vitro protein-iron-phytate and protein-zinc-phytate interactions in extruded durum wheat.
4. To determine the effects of extrusion processing at different temperatures on the solubility and distribution of proteins in durum wheat.

5. To investigate the effects of extrusion processing at different temperatures on the association of iron and phytate with the protein fractions in durum wheat; to identify the iron-binding polypeptides and determine their molecular weights.

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CHAPTER 2. THE INFLUENCE OF EXTRUSION PROCESSING ON IRON DIALYZABILITY, PHYTATES AND TANNINS IN LEGUMES

ABSTRACT

Iron dialyzability, chemical forms of iron, tannin content, and phytic acid degradation in boiled, low impact and high impact extruded navy beans, chickpeas, cowpeas and lentils were determined. In boiled legumes, the dialyzable iron was 1.2 to 2.7% of total iron. Dialyzable, soluble and ionic iron were highest in low impact extruded legume flours. Lower forms of inositol phosphates (inositol tri-, tetra- and penta-phosphates) increased to 51 to 71% of total phytate in legumes processed by boiling or extrusion compared to 21 to 33% in raw legumes. Tannin content was lower in extruded products compared to boiled or raw legumes. The influence of extrusion processing on iron dialyzability varied with processing conditions. Results suggest that the degradation of phytate and changes in tannin content may not be responsible for the increase in iron dialyzability and solubility associated with low impact extrusion processing.

INTRODUCTION

Extrusion processing is being used increasingly to process new cereal and legume products. Extrusion is a high temperature, short time (HTST) cooking process which uses high shear at elevated pressure and temperature. Interactions among food components during extrusion processing may have a positive or negative effect on the bioavailability of nutrients, including bioavailability of iron.

Bioavailability has been defined as the proportion of the total nutrient in a food, meal or diet that is utilized for normal body functions (Fairweather-Tait, 1992). Important chemical factors affecting the bioavailability of iron in foods include the valence, solubility and degree of chelation or complex formation of the iron (Lee and Clydesdale, 1979). Measurements of the percentage of soluble or dialyzable iron in a food are commonly used in vitro techniques to assess potential iron bioavailability (Tanner and Whittaker, 1989).

Several factors including phytates and tannins may contribute to the low bioavailability of iron in legumes which has been reported. Phytate has been identified as an inhibitor of iron bioavailability (Brune et al., 1992; Hallberg et al., 1987). During food processes involving heat treatment, naturally occurring phytate in plants may be degraded to lower forms of inositol phosphates which have been reported to induce an increase in iron solubility through the formation of small soluble iron complexes (Sandberg et al., 1989). Tannins, located mainly in the seed coat or testa of beans, are another factor that has been suggested to be responsible for the low bioavailability of iron in legumes (Torrance et al., 1982; Rao and Prabhavathi, 1982).

Due to the significance of dietary fiber and complex carbohydrate in nutrition and health, an increased consumption of legumes is being promoted (Morrow, 1991). Because legumes also are an important plant source of iron, the purpose of the present research was to assess the influence of extrusion processing on iron bioavailability by determining in vitro iron dialyzability and chemical forms of iron. The effect of extrusion processing on phytate degradation and tannin content of legumes also was determined.

MATERIALS AND METHODS

Legumes

Domestically grown navy beans, chickpeas, cowpeas and lentils were obtained from Morrice Grain and Bean Company, Morrice, MI. All dry bean samples were ground using a Fitzpatrick mill with a 3A 187 mesh screen to obtain legume flours.

Extrusion Processing

A Baker Perkins, MPF-50 (25:1 L/D) corotating twin-screw extruder was used to extrude the legume flours under low and high impact conditions. The major differences in the configuration of low and high impact processes include a) screw configuration, b) screw speed, c) moisture content and d) barrel zone temperatures. The specific conditions of the two extrusion processes are as follows:

	Low impact	High impact
Screw speed (rpm)	350	400
Added moisture (%)	16	10 - 14
Die temperature (°C)	105 - 117	131 - 137
Barrel temperature (°C)	82 - 93	101 - 108

The extruded products (moisture content - 5.8 to 13.6%) were ground using the Fitzpatrick mill with a 0.10 cm mesh screen prior to analyses.

Boiling

The non-extruded legumes were cooked using a conventional home cooking procedure. The legumes were soaked overnight in tap water (bean:water - 1:3), heated to boiling point and held at that temperature for 30 min by which time they were soft. The boiled legumes were homogenized to a smooth consistency. All the water used for soaking or boiling the legumes was used for homogenization. The samples were stored in the refrigerator (for 3 days or less) until they were analyzed.

Total Iron Analysis

Extruded and non-extruded samples were wet-ashed with concentrated nitric acid and 30% hydrogen peroxide. The ash was dissolved in 0.1 N hydrochloric acid and the solution was analyzed for iron using atomic absorption spectroscopy (AAS) (Perkin-Elmer Model 2380).

***In Vitro* Iron Dialyzability**

The iron dialyzability assay developed by Miller et al. (1981) was used as a measure of potential iron bioavailability in boiled and extruded legume products. The extruded legume flours were mixed with water in a ratio of 1:3 (flour:water), heated to boiling temperature and held for 15 min. The slurry was cooled to room temperature prior to analysis. Iron content of the dialysate was determined using ferrozine color reagent.

Quantification of the Chemical Forms of Iron

Boiled legumes were analyzed for elemental, total nonelemental, soluble and ionic iron according to the method proposed by Lee and Clydesdale (1979). The extruded legume flours that were prepared for the iron dialyzability assay were also analyzed for the chemical forms of iron. The complexed iron was measured as the difference between the soluble iron (determined by AAS) and ionic iron (determined using the ferrozine color reagent).

Determination of Inositol Phosphates

Phytic acid and its degradation products including inositol hexa-, penta-, tetra-, and tri-phosphates were determined using ion exchange chomatography and high pressure liquid chromatography techniques according to Graf and Dintzis (1982) and Sandberg and Ahderinne (1986) modified as follows. Sample Preparation - Raw legumes were milled using a Micro-Mill (Chemical Rubber Co., OH) to pass through a sieve equipped with a 60 mesh. Samples of 0.5 g raw, boiled or extruded legume flours were extracted under mechanical agitation with 20 ml 0.5 M HCl for 2 hours at 20°C. The extract was centrifuged and supernatant decanted, frozen overnight and filtered under pressure through a 0.47 μm membrane filter. The filtrate was diluted with 10 ml distilled deionized water (DDW) and passed through an ion exchange column containing 0.65 ml resin (AG 1-X8, 200-400 mesh) at 0.4 ml/min followed by 10 ml of 0.025 M HCl. Inositol phosphates were removed from the resin with ten 1 ml portions of 2 M HCl. The eluent was evaporated to dryness on a hot plate set at low temperature and diluted with 1 ml of double deionized water.

Mobile Phase - The mobile phase consisted of 0.05 M formic acid:methanol (46:54) to which 1.5 ml/100 ml of tetrabutylammonium hydroxide was added. The pH was adjusted to 4.3 by addition of 9 M sulfuric acid. The mobile phase was filtered through a Millipore filter (0.45 μm) under vacuum and degassed.

HPLC Procedure - Inositol phosphates were separated on a reverse phase Supelcosil LC-18 column (25.0 cm x 4.6 mm) with 5 micron particle size (Supelco, Inc., PA). Injections were made with a Rheodyne 7010 injector equipped with a 20 μl loop. The optimal flow rate was 1 ml/min. Inositol phosphates were detected using a differential refractometer (Waters, Model R401). Retention times and peak areas were measured with a Peak Simple II integrator (SRI Instruments, CA).

Determination of Tannin Content

Tannin content of legumes, expressed as catechin equivalents, was determined using a procedure described by Price et al. (1978). Ground sample (200 mg) was mixed with 10 ml of 1% concentrated HCl in methanol and incubated at room temperature for 20 min. The mixture was centrifuged at 1500 g for 10 minutes and filtered through a Whatman filter # 1, and the filtrate was analyzed for tannins using the vanillin-HCl reagent.

Statistical Analyses

Student's t test and one-way analysis of variance followed by the test for least significant difference were used to compare means at 95% confidence level.

RESULTS AND DISCUSSION

Iron Dialyzability

Total iron concentration was significantly higher in extruded legume flours (Table 1). A similar increase in iron content in extruded products was reported by other researchers (Fairweather-Tait et al., 1987; Lombardi-Boccia et al., 1991; Hazell and Johnson, 1989). The wear of certain parts of the extruder is presumed to be the cause for the contamination of iron in the extruded products. However, there was no detectable elemental iron in our extruded products. Thus, the equipment used in extrusion processing may not have been responsible for the increase in iron in the extruded products.

The amount of dialyzable iron in low impact extruded products was higher than in boiled legumes. However, the dialyzable iron content of high impact extruded and boiled legume products was similar except in navy beans. In navy beans, both low and high impact extruded products had higher dialyzable iron than the boiled legumes.

The increase in iron dialyzability in low impact extruded products cannot be fully explained. Speculations have been made that during extrusion some of the high molecular weight compounds (including phytate) are degraded, thus releasing iron (Fairweather-Tait et al., 1987). The reason for the differences in the effects of low and high impact extrusion processing on iron dialyzability is not readily apparent. The extent of protein denaturation during the extrusion process

Table 1. Total iron and dialyzable iron in boiled and extruded legume products[1].

	Total iron	Dialyzable iron	
	(μg/g dry wt)	(μg/g dry wt)	(% total)
	NAVY BEANS		
Boiled	61.51 ± 1.25	1.68 ± 0.15	2.74 ± 0.25
Ext. low[2]	70.83 ± 0.42*	2.85 ± 0.27*	4.03 ± 0.38*
Ext. high[3]	64.71 ± 1.56*	2.89 ± 0.34*	4.47 ± 0.53*
	CHICK PEAS		
Boiled	49.60 ± 0.92	1.34 ± 0.31	2.70 ± 0.10
Ext. low[2]	51.86 ± 0.26*	2.16 ± 0.25*	4.16 ± 0.48*
Ext. high[3]	51.94 ± 0.51*	1.43 ± 0.24	2.76 ± 0.47
	COWPEAS		
Boiled	47.90 ± 1.01	0.65 ± 0.07	1.36 ± 0.17
Ext. low[2]	53.87 ± 0.77*	1.00 ± 0.08*	1.86 ± 0.14*
Ext. high[3]	53.13 ± 1.26*	0.87 ± 0.07	1.63 ± 0.14
	LENTILS		
Boiled	57.15 ± 1.52	0.68 ± 0.11	1.18 ± 0.20
Ext. low[2]	61.82 ± 1.72*	1.60 ± 0.21*	2.59 ± 0.35*
Ext. high[3]	58.95 ± 2.09	0.70 ± 0.08	1.18 ± 0.14

[1]Each value represents the mean ± standard deviation of samples analyzed in triplicate.
[2]Legume flour extruded under low impact conditions.
[3]Legume flour extruded under high impact conditions.
*Student's t-test at 95% confidence indicates significant difference from values for respective boiled legumes.

and the resulting effect on protein solubility may produce variable effects on iron dialyzability.

Chemical Forms of Iron

Nonelemental iron in navy beans, chickpeas and cowpeas was significantly increased in the extruded products compared to their respective boiled legumes (Table 2). In lentils, an increase in nonelemental iron was seen only in the low impact extruded product compared to the boiled legume.

An increase in total soluble iron in low impact extruded products compared to the high impact extruded or boiled legume products was observed (Table 2). Iron solubility may be altered by extrusion processing due to the effects of heat, pressure and shear on protein solubility and distribution. Iron solubility may also be increased due to the degradation of phytate that occurs during extrusion processing. Since iron not only has to be soluble but also be in a low-molecular form to be dialyzable, total soluble iron (Table 2) in all legume products was higher than the dialyzable iron (Table 1).

Ionic iron was also higher in low impact extruded than high impact extruded or boiled navy beans and chickpeas (Table 2). In cowpeas and lentils, there were no significant differences in ionic iron between low impact extruded and high impact extruded products. However, the values were higher than the ionic iron in boiled legumes.

The results of the analyses of iron dialyzability and

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Table 2. Nonelemental, soluble and ionic iron in boiled and extruded legume products[1].

	Nonelemental (μg/g dry wt)	Soluble (% total)	Ionic (% total)
	NAVY BEANS		
Boiled	61.51 ± 1.25	7.29 ± 0.15	6.86 ± 0.26
Ext. low[2]	70.83 ± 0.42*	13.42 ± 0.51*	11.93 ± 0.18*
Ext. high[3]	64.71 ± 1.56*	7.33 ± 1.12	5.89 ± 0.33*
	CHICK PEAS		
Boiled	49.60 ± 0.92	5.33 ± 1.12	4.04 ± 0.92
Ext. low[2]	51.86 ± 0.26*	14.02 ± 1.00*	11.73 ± 1.48*
Ext. high[3]	51.94 ± 0.51*	5.04 ± 0.54	3.43 ± 0.27*
	COWPEAS		
Boiled	47.90 ± 1.01	6.87 ± 0.11	6.15 ± 0.14
Ext. low[2]	53.87 ± 0.77*	10.14 ± 0.48*	7.09 ± 0.14*
Ext. high[3]	53.61 ± 1.20*	7.33 ± 1.80	6.93 ± 0.12*
	LENTILS		
Boiled	56.62 ± 0.52	14.13 ± 2.71	5.14 ± 0.50
Ext. low[2]	59.14 ± 1.12*	21.32 ± 0.92*	7.58 ± 0.09*
Ext. high[3]	56.39 ± 0.67	13.80 ± 0.74	7.53 ± 0.19*

[1]Each value represents the mean ± standard deviation of samples analyzed in triplicate.
[2]Legume flour extruded under low impact conditions.
[3]Legume flour extruded under high impact conditions.
*Student's t-test at 95% confidence indicates significant difference from values for respective boiled legumes.

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chemical forms of iron suggest that low impact extrusion processing increased iron solubility and, as a consequence, iron dialyzability. High impact extrusion, on the other hand, did not change iron solubility compared to boiled legumes, hence no differences were seen in iron dialyzability of the two legume products. Ionic iron did not seem to accurately reflect iron dialyzability. The differences in the pH conditions used in the two assays may be responsible since a pH of 7.5 was used in determining iron dialyzability, whereas an acidic pH was used for the measurement of ionic iron.

Degradation of Phytate

The inositol tri-, tetra-, penta-, and hexaphosphate contents in raw, boiled, and extruded legume flours are presented in Table 3. Although no changes in total phytate content were observed, processing (either boiling or extrusion) increased the conversion of inositol hexaphosphate to its lower phosphate forms.

Phytic acid in raw legume flours ranged from 66 to 79% of the total inositol phosphate forms whereas, in boiled or extruded legume products, phytic acid content ranged from 20 to 50% only. Extrusion processing did not result in any consistent pattern of degradation of phytic acid. If iron dialyzability is directly correlated to the extent of phytate degradation, greatest conversion of phytate to lower inositol phosphates would be expected in low impact extruded legume

Table 3. Degradation products of phytic acid in raw, boiled, and extruded legume products[1].

	IP3	IP4	IP5	IP6	Total phytate
	-----% total phytate-------				(umole/g dry wt)
			NAVY BEAN		
Raw	0	5.4	15.4	79.3	14.0 ± 1.9
Boiled	8.4	16.5	28.1	47.0	14.0 ± 0.6
Ext.low[2]	9.5	21.5	37.1	37.4	15.4 ± 0.1
Ext.high[3]	10.8	13.6	25.9	49.8	14.6 ± 0.2
			CHICKPEA		
Raw	0	13.4	20.7	66.0	7.7 ± 0.1
Boiled	25.3	19.0	21.9	31.0	8.7 ± 0.1
Ext.low[2]	25.2	25.7	25.2	24.6	6.1 ± 0.4
Ext.high[3]	9.9	14.0	27.5	48.7	8.2 ± 0.1
			COWPEA		
Raw	0	7.1	20.8	72.3	11.5 ± 0.3
Boiled	27.1	19.7	22.2	31.0	11.1 ± 2.1
Ext.low[2]	14.5	24.6	29.4	31.6	9.6 ± 0.1
Ext.high[3]	21.3	31.9	27.1	19.7	9.8 ± 0.3
			LENTIL		
Raw	0	10.8	22.8	66.6	5.2 ± 0.1
Boiled	36.8	14.4	16.9	31.9	4.5 ± 0.4
Ext.low[2]	16.9	19.7	28.2	35.4	5.2 ± 0.6
Ext.high[3]	14.7	26.0	30.5	28.8	4.9 ± 0.1

[1]Each value represents the mean or mean ± standard deviation of samples analyzed in triplicate.
[2]Legume flour extruded under low impact conditions.
[3]Legume flour extruded under high impact conditions.

products since iron dialyzability was highest in these products. However, this pattern was observed only in navy beans and chickpeas and not in the case of cowpeas or lentils.

Previously, Sandberg et al. (1989) reported that only inositol hexa- and pentaphosphates decreased iron solubility at simulated physiological conditions and their degradation seemed to significantly reduce the inhibiting effect of phytate on iron availability. Sandberg and Svanberg (1991) reported that complete hydrolysis of inositol hexa- and pentaphosphates through activation of endogenous phytase led to a strong increase in in vitro iron availability. Our results suggest that although extrusion processing causes degradation of phytate, the presence of different forms of inositol phosphates does not appear to explain the varying effects of low impact and high impact extrusion on iron dialyzability.

Tannin Content

Extrusion processing decreased the tannin content of legume flours by about 31 to 76% compared to raw legumes (Table 4). The effects of low impact and high impact extrusion processing on iron dialyzability cannot be explained by the decrease in tannin content of extruded legume flours since the decrease was seen in both low impact and high impact extrusion processing. Boiling decreased tannin content by 20% in cowpeas only. No detectable tannins were seen in raw,

Table 4. Tannin content (mg catechin equivalents/g dry weight) of raw, boiled, and extruded legume products[1].

Legume	Raw	Boiled	Ext.low[2]	Ext.high[3]
Chickpea	2.32±0.3	2.64±0.3	1.10±0.3*	1.61±0.1*
Cowpea	2.91±0.1	2.31±0.1*	1.97±0.0*	1.91±0.0*
Lentil	7.65±0.2	7.98±0.8	2.22±0.1*	1.84±0.2*

[1]Each value represents the mean ± standard deviation of samples analyzed in triplicate; navy beans showed no detectable tannins.
[2]Legume flour extruded under low impact conditions.
[3]Legume flour extruded under high impact conditions.
*ANOVA test at 95% confidence level indicates significant difference from values for raw legumes.

boiled or extruded navy bean products.

Boiling and extrusion processing produced different effects on the tannin content of legumes. In the preparation of boiled legumes for analyses, the water used for soaking or boiling the legumes was used for homogenization. Thus, losses of tannins due to leaching during soaking or boiling were unlikely. Another speculation is that during extrusion, tannins may complex with other components of legumes, such as proteins or sugars, and may become undetectable by the vanillin assay.

In conclusion, the factors investigated in our study, phytates and tannins, do not seem to explain the varying effects of extrusion processing on iron dialyzability. Another component of legumes thought to interfere with iron bioavailability is fiber. The modification of fiber during extrusion processing is not fully elucidated; however, it has been shown that at mild or moderately severe conditions, extrusion does not significantly change dietary fiber content but solubilizes some fiber components. At more severe conditions, the dietary fiber content is seen to increase, mainly due to the formation of enzyme-resistant starch fractions (Asp and Björck, 1989; Theander and Westerlund, 1987). A redistribution of insoluble to soluble dietary fiber in extruded wheat flour also has been reported (Björck et al., 1984). The extent to which the effects of extrusion processing on iron dialyzability in legumes might be attributed to alterations in the distribution or characteristics of fiber components is yet to be determined. The role of other factors such as competing minerals, lignin complexes and the interactions between the various components in determining the effects of extrusion on iron bioavailability needs to be investigated.

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CHAPTER 3. BIOAVAILABILITY OF IRON IN EXTRUDED WHEAT PRODUCTS

ABSTRACT

The effect of extrusion processing of durum wheat and durum wheat (85%)/navy bean (15%) flours on bioavailability of iron was investigated. Dialyzable iron was 24.6, 7.3 and 16.8% in durum wheat flour, wheat pasta and wheat/bean pasta, respectively. In vivo iron bioavailability of semolina and the durum wheat pasta products was determined using a hemoglobin (Hb) repletion technique. The final Hb concentration, Hb-Fe gain and Hb regeneration efficiency (HRE) in the rats fed wheat pasta diets were significantly lower ($p<0.05$) than in rats fed semolina diets. However, no significant differences ($p>0.05$) were found between rats fed wheat/bean pasta diets and rats fed semolina diets.

INTRODUCTION

Extrusion processing has been identified as a key food processing technique for the future (Harper, 1989) and should be expanded to include legumes and legume supplemented cereals which are of importance to people in various parts of the world. Supplementation of cereals with legumes has significant nutritional benefits including improved protein quality. Navy bean supplementation of cereals may have great potential in the manufacture of extruded products because of its mild flavor, color and the ability to expand upon extrusion. However, the influence of legume supplementation of cereals on bioavailability of iron is not known.

Extrusion processing is unique in its potential ability to alter iron bioavailability due to the combination of heat, pressure and shear; therefore, the purpose of this study was to evaluate the effect of extrusion processing of durum wheat flour and navy bean supplemented durum wheat flour on iron bioavailability. The bioavailability of iron in durum wheat flour and extruded durum wheat pastas was measured in vitro and compared with an in vivo study using a rat bioassay.

MATERIALS AND METHODS

Extrusion Processing

Enriched durum wheat flour and raw navy bean (*Phaseolus vulgaris*) flour were used to make 100% durum wheat pasta and durum wheat (85%)/navy bean (15%) pasta. Durum wheat flour was sized to pass completely through a 30 mesh (US standard) and contained enrichment nutrients (thiamin, riboflavin, niacin, iron and calcium) as specified by USDA. Raw navy beans were hammer milled by passing through a Fitz Mill (Model D, Comminuting Machine, The W.J. Fitzpatric Co., Chicago) equipped with 0.07 cm sieve. Mixed dry flour ingredients were fed at the rate of 3.41 kg/min into the preconditioner where the materials were partially precooked with steam and water at 99°C. The preconditioned dough was deaerated (33 cm Hg) to achieve a smooth and uniform surface in the final product. The dough was passed through a co-rotating twin-screw extruder (Model TX-80, Wenger MFG, Sabetha, KS) at a mass temperature of 205 to 210°C with a screw speed of 154 rpm. Rotatory knives were used to length cut (ca. 2.4 cm) the pastas (macaroni) which were then dried in a dryer/cooler (Series IV, Wenger MFG, Sabetha, KS) maintained at a constant temperature of 71°C to a final moisture content of 9 to 10%.

In Vitro Iron Dialyzability

Enriched durum wheat flour, enriched semolina, wheat pasta or wheat/bean pasta were mixed with hot water (90°C) in a 1:3 ratio and allowed to stand for 10 min. The mixture was blended to a smooth consistency and freeze-dried. The in vitro iron dialyzability assay was carried out according to the procedure of Miller et al. (1981) as modified by Kane and Miller (1984). Dialyzable iron is considered a measure of iron bioavailability. Ferrozine color reagent was used to determine the total iron content of dialysate solutions (Stookey, 1970).

In Vivo Determination of Iron Bioavailability

Experimental Design

Fifty four male Sprague-Dawley weanling rats weighing 50 to 70 g (mean 62.7±4.6) were used. In the first 5 wk, the rats were fed an iron deficient diet containing 1.0 mg Fe/100 g diet. At the end of this depletion period, blood was collected from the tail artery for analysis of hemoglobin (Hb) (hemoglobin levels were 6 to 6.5 g/100 ml of blood), and body weights were recorded. The rats were divided into nine groups (six rats in each group) based on the value of hemoglobin (g/dl) times body weight (g), so that the means of hemoglobin times body weight in all groups were equal. Each group was randomly assigned to one of the nine diets described in the diet composition section. Food intake and body weights were recorded weekly. After 14 days on the test diets, the rats were anaesthetized and blood collected from the heart for analysis of hemoglobin. Iron concentration in livers was also determined. All procedures for handling the rats were approved by the All-University Committee on Animal Use and Care at Michigan State University.

Diet Composition

The nine diets fed to rats in this study were: control diet (1.0 mg Fe/100 g diet); control diet + ferrous sulfate (2.0 mg Fe/100 g diet); control diet + ferrous sulfate (3.0 mg Fe/100 g diet); semolina diet (2.0 mg Fe/100 g diet); semolina diet (3.0 mg Fe/100 g diet); durum wheat pasta diet (2.0 mg Fe/100 g diet); durum wheat pasta diet (3.0 mg Fe/100 g diet); durum wheat/bean pasta diet (2.0 mg Fe/100 g diet); and durum wheat/bean pasta diet (3.0 mg Fe/100 g diet) (Table 1).

The control diet was supplemented with two levels of $FeSO_4.7H_2O$ to achieve the final iron concentrations of about 2.0 and 3.0 mg/100 g diet. Uncooked semolina and prepared pastas were incorporated into animal diets following the recommendations of the American Institute of Nutrition (Bieri, 1977; Reeves et al., 1993) (Table 1). Procedures used for preparing pastas were the same as in the in vitro studies. Semolina, obtained from the same source as durum wheat flour was used due to the non-availability of a sufficient quantity of the durum wheat flour that was used to make the pastas. All diets were analyzed for iron concentrations before feeding to the animals.

Table 1. Compositions of diets (g/100g) used in animal study[a].

	Control	$FeSO_4$-1	$FeSO_4$-2	Semolina 1	Semolina 2	Wheat Pasta-1	Wheat Pasta-2	Wheat/Bean Pasta-2	Wheat/Bean Pasta-2
Casein	20.0	20.0	20.0	17.2	14.1	18.0	14.0	18.0	14.0
Dyetrose	13.2	13.2	13.2	-	-	-	-	-	-
Cornstarch	39.75	39.75	39.75	26.95	1.25	25.95	0.95	29.65	8.45
Semolina				28.8	57.6				
Wheat Pasta						29.0	58.0		
Wheat/Bean Pasta								25.3	50.5
Fe as $FeSO_4.7H_2O$ (mg/100 g)		1.0	2.0						
Total Fe (analyzed;mg/100g)	1.2	2.3	3.1	2.2	3.1	2.1	3.0	2.2	3.1

[a]The following ingredients (g/100g diet) were added to all diets: sucrose - 10.0; soy oil - 7.0; Solka-floc - 5.0; AIN-76 iron-free mineral mix - 3.5; AIN-76 vitamin mix - 1.0; L-cystine - 0.3; and choline bitartrate - 0.25.

Chemical Analyses

Diet samples were wet-ashed with concentrated HNO_3 and H_2O_2. The ash was dissolved in 0.1N HCl and total iron was determined using atomic absorption spectrophotometry (AAS) (Perkin-Elmer Model 2380). Hemoglobin concentrations were determined using a cyanmethemoglobin method (hemoglobin kit from Sigma Chem. Co., Catalog No. 525-A). Rat livers were freeze-dried and wet-ashed before iron was quantitated using AAS. During all analyses, the accuracy of the ashing procedure and the AAS instrument was checked by analyzing NIST (National Institute of Science and Technology) bovine liver (1577b) and NIST wheat flour (1567a). Iron content (μg/g) of NIST bovine liver was 178.5±3.5 (certified value: 184±15). In NIST wheat flour, total iron (μg/g) was 14.7±0.14 (certified value: 14.5±0.5).

Calculations

Hemoglobin iron (Hb-Fe) gain was calculated for each rat as the difference between Hb-Fe at the end of the repletion period and that at the start of repletion. For the calculation of initial and final Hb-Fe, blood was assumed to be 67 g/kg body weight and Hb was assumed to contain 3.35 mg Fe/g (Miller, 1982). Iron intake for each rat was calculated from food intake and the analyzed iron content of the diet. The hemoglobin regeneration efficiency (HRE) was calculated for each rat as the percentage of iron consumed that was retained in circulating hemoglobin (Miller, 1982; Forbes et al., 1989).

Statistical Analyses

The data reported are mean values with standard error of the mean. For the in vitro data, differences between means were statistically analyzed by Student's t test at 95% confidence level. The in vivo data were analyzed using a 2-factorial ANOVA where the types of diets and iron dosage were taken as the independent variables. When there was no significant interaction between the diet and iron dose ($p>0.1$), diet means of pooled data of both doses were used for statistical comparisons. Final Hb concentration, Hb-Fe gain, HRE, and liver iron concentration were tested using the Bonferroni-Dunn's test for differences between means.

RESULTS AND DISCUSSION

In Vitro Results

Table 2 presents the total iron and dialyzable iron contents of raw and cooked semolina, and cooked durum wheat flour, wheat pasta, and wheat/bean pasta. Iron content of durum wheat flour was similar to that of wheat pasta suggesting that iron contamination during extrusion did not occur. Addition of 15% raw navy bean flour slightly increased the total iron content of the pasta product compared to wheat flour. Total iron contents of all products are consistent with the USDA Agriculture Handbook No. 8 Food Composition Tables (USDA, 1986; USDA, 1989).

Iron dialyzability was significantly lower ($p<0.05$) in cooked semolina (17.0%) than in cooked durum wheat flour (24.6%). Many factors could contribute to the lower dialyzability of iron in semolina compared to wheat flour. One such factor may be a greater particle size of semolina than flour which could influence the extent of protein digestion and iron release, thus altering iron dialyzability. An increase in iron dialyzability was seen after cooking of semolina. Cooking enhances the digestibility of the product and may change the solubility and chemical form of iron. The in vitro results suggest a loss in iron dialyzability after extrusion of durum wheat flour. Dialyzable iron in cooked wheat pasta (7.3%) was significantly lower ($p<0.05$) than in cooked durum wheat flour (24.6%) or cooked semolina (17.0%).

Table 2. Total iron and dialyzable iron (D-Fe) content (μg/g dry weight) of durum wheat flour, semolina, wheat pasta, wheat (85%)/navy bean (15%) pasta and navy bean flour[a].

Product	Total Fe (μg/g)	D-Fe (μg/g)	D-Fe (% total)
Durum wheat flour[b]	32.7±0.55[c]	8.05±0.39[d]	24.6±1.20[d]
Semolina, uncooked	37.7±0.17[d]	2.63±0.12[c]	7.0±0.32[c]
Semolina[b]	35.5±0.50[cd]	6.02±0.13[e]	17.0±0.36[e]
Wheat pasta[b]	34.7±0.17[cd]	2.53±0.12[c]	7.3±0.36[c]
Wheat/bean pasta[b]	37.0±1.59[d]	6.20±0.14[e]	16.8±0.39[e]
Navy bean flour[b]	67.7±1.15[e]	8.70±0.61[d]	12.9±0.91[f]

[a]Each value represents mean ± SEM of triplicates; different letters within a column indicate significant differences using Student's t test at 95% confidence level.
[b]Samples were prepared as described in methods section and freeze-dried before analysis.

However, supplementation of navy bean to wheat flour produced different results. Dialyzable iron in cooked wheat/bean pasta (16.8%) was significantly higher ($p<0.05$) than in cooked wheat pasta (7.3%), significantly lower ($p<0.05$) than in cooked wheat flour, but not significantly different ($p>0.05$) from cooked semolina.

In Vivo Results

There were no significant differences in body weight gain by anemic rats fed either control, ferrous sulfate diets or any of the test diets (Table 3). However, rats fed the highest level of iron tended to have increased body weight gains which may have been due to improved health status resulting from a faster rate of iron repletion.

As expected, final Hb concentration and Hb-Fe gain were significantly higher ($p<0.05$) in rats fed semolina than in rats fed the low iron control diet (Tables 3 & 4). When compared to rats fed ferrous sulfate diets, the rats fed semolina diets had significantly lower ($p<0.05$) final Hb concentrations but no significant differences were found in Hb-Fe gain or HRE. Final Hb concentration, Hb-Fe gain, and HRE were significantly lower ($p<0.05$) in the rats fed wheat pasta compared to rats fed either ferrous sulfate diets or semolina diets, but significantly higher ($p<0.05$) than in rats fed the control diet (Table 4). The final hemoglobin concentration, Hb-Fe gain and HRE in rats fed wheat/bean pasta

Table 3. Iron bioavailability of extruded and non-extruded products in rats using the hemoglobin repletion technique[a].

	Control	$FeSO_4$-1	Semolina 1	Wheat Pasta-1	Wheat/Bean Pasta-1	$FeSO_4$-2	Semolina 2	Wheat Pasta-2	Wheat/Bean Pasta-2
Diet Fe (mg/100g)	1.2	2.3	2.2	2.1	2.2	3.1	3.1	3.0	3.1
Wt.gain (g)	57±5.7	53±1.2	61±1.6	53±2.0	55±4.9	59±2.9	69±3.7	61±3.7	59±1.6
Food intake (g)	237±5.7	237±10.6	244±6.9	224±9.8	248±10.2	243±9.0	261±4.5	253±11.0	230±8.6
Fe intake (mg)	2.7±0.1	5.6±0.3	5.4±0.2	4.8±0.2	5.5±0.2	7.6±0.3	8.0±0.1	7.7±0.3	7.2±0.3
Final Hb (g/dl)	8.5±0.3	11.3±0.3	10.4±0.3	9.4±0.4	10.5±0.3	13.0±0.4	12.3±0.2	11.1±0.4	11.9±0.2
Hb-Fe gain (mg)	1.5±0.2	3.2±0.2	3.1±0.3	1.8±0.3	2.6±0.2	5.0±0.2	4.1±0.4	3.3±0.2	3.8±0.3
HRE[b] (%)	53.3±7.1	57.3±3.7	56.0±4.4	38.3±5.8	47.2±2.8	66.6±4.7	50.8±4.0	43.9±3.3	53.1±4.8
Liver dry wt. (g)	3.8±0.2	3.6±0.2	3.7±0.1	3.4±0.2	3.3±0.2	3.9±0.2	3.9±0.2	3.6±0.1	3.4±0.1
Liver Fe (ug)	204±7.0	237±9.7	276±14.1	260±32.9	244±17.7	349±6.4	328±20.9	250±24.2	298±22.6

[a]Each value represents mean ± standard error of the mean of 6 replicates.
[b]HRE - Hemoglobin Regeneration Efficiency.

Table 4. Statistical analyses of data obtained from rat study[a].

Diet	vs	Final [Hb]	Hb-Fe gain	HRE
Semolina	Control	S	S	NS
	$FeSO_4$	S	NS	NS
Wheat pasta	Control	S	S	S
	$FeSO_4$	S	S	S
	Semolina	S	S	S
Wheat/bean pasta	$FeSO_4$	S	S	S
	Semolina	NS	NS	NS

[a]S - significantly different; NS - not significantly different using 2-factorial ANOVA followed by Bonferroni-Dunn's test at 95% confidence level. Since there was no interaction between diets and dose level ($p>0.1$), diet means over both doses were used for statistical comparisons.

were significantly lower ($p<0.05$) than in rats fed ferrous sulfate diets; however, they were not significantly different ($p>0.05$) from rats fed semolina diets. Liver iron concentrations did not show a consistent pattern. Since they represent iron stores in the animal, the repletion period may need to have been continued for a longer time to observe significant changes.

The results of the in vivo study suggest that the extruded products had lower iron bioavailability than ferrous sulfate; bioavailability of iron decreased in wheat pasta and this effect of extrusion was not seen when pasta was made from durum wheat flour supplemented with navy bean flour.

Contradictory results have been reported on the influence of extrusion on iron bioavailability. An increase in in vitro iron dialyzability was reported in extruded maize-based snack foods (Hazell and Johnson, 1989) and defatted soy flour (Latunde-Dada, 1991). Speculations have been made that during extrusion some of the high molecular weight compounds are degraded, thus releasing iron. Other researchers have reported that extrusion decreased iron dialyzability (Lombardi-Boccia et al., 1991; Kivisto et al., 1986) or produced no changes in iron absorption in humans (Fairweather-Tait et al., 1987; Fairweather-Tait et al., 1989).

In previous experiments, we observed that, in the case of legume flours, percent dialyzable iron varies in the same products extruded under different temperature, pressure and shear conditions thus indicating that extrusion conditions play a major role in determining the effect of extrusion on iron bioavailability (Ummadi et al., 1993). Differences in extrusion conditions used by different researchers may partially account for the disagreement between studies involving extruded products and suggests that further work needs to be done to fully understand the effects of extrusion on iron bioavailability.

The results obtained in the rat study indicate that iron bioavailability is lower in an extruded wheat flour pasta than in non-extruded durum wheat. The extruded products also had lower iron bioavailability compared to the diets containing the standard source of iron, i.e. ferrous sulfate. The relatively higher bioavailability of iron in uncooked semolina observed in the animal study compared to the in vitro study is not clear. The specific effects of extrusion cannot be evaluated on the basis of the results of the animal study because the non-extruded product tested (semolina) was not the same durum wheat flour that was used to make the pastas. However, the results of the in vitro study clearly demonstrate the negative effects of extrusion on iron dialyzability of durum wheat flour.

Both in vitro and rat studies showed that the addition of 15% navy bean flour to durum wheat flour before extrusion compensated for the negative effects of extrusion processing on iron bioavailability in durum wheat products. An explanation for this improvement is not readily apparent. It might be speculated, however, that a slight improvement in protein quality of the wheat/bean products may have an impact on iron bioavailability. Another explanation could be that the introduction of bean protein favorably alters the protein-phytate-iron interactions during extrusion thus increasing iron bioavailability.

At present, the mechanism(s) by which extrusion may alter mineral bioavailability is poorly understood. Many mechanisms have been suggested including amylose-lipid complexes, Maillard reaction products and increase in the lignin fraction (Fairweather-Tait et al., 1989). It may also be proposed that alterations in bioavailability of iron during extrusion may be due to interactions between protein, phytate and iron. It is well documented that high temperature extrusion (used to make our product) results in extensive protein denaturation, decreased protein digestibility and solubility (Jeunink and Cheftel, 1979; Phillips, 1989; Gujska and Khan, 1991). Protein denaturation caused by processing could enhance protein-phytate-mineral complex formation and thus alter mineral bioavailability.

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CHAPTER 4. PROTEIN-PHYTATE-MINERAL INTERACTIONS IN EXTRUDED PRODUCTS

INTRODUCTION

Durum wheat flour and navy bean supplemented durum wheat flour were extruded to make pasta products. The effects of extrusion on iron bioavailability from these products, determined by in vitro and in vivo assays suggest that iron availability is lower in extruded durum wheat pasta compared to non-extruded durum wheat or navy bean supplemented durum wheat pasta (Chapter 3). In order to explain the effects of extrusion and navy bean supplementation on iron dialyzability, the role of protein-phytate-iron associations were investigated in this study using in vitro assays. Since zinc balance is of concern in infants (Hambidge, 1986; Keen and Gershwin, 1990) and extrusion is widely used in the manufacture of weaning foods, it is important to study the effects of extrusion on zinc interactions with other components in a food.

Since the solubility and the molecular weight of a mineral complex are considered to be important factors affecting mineral dialyzability (Clydesdale, 1989), the effects of extrusion, dephytinization and digestion/dialysis with pepsin and pancreatin on the soluble forms of iron and zinc were determined. Regular and dephytinized durum wheat flour, navy bean flour, durum wheat pasta and wheat/navy bean pasta were analyzed for total and soluble protein, phytate, iron and zinc before and after in vitro digestion.

MATERIALS AND METHODS

Extrusion Processing

Procedures used in the manufacture of pastas made from durum wheat flour and durum wheat (85%)/navy bean flour (15%) have been described in Chapter 3.

Cooking

Durum wheat pasta, wheat/navy bean pasta, durum wheat flour and navy bean flour were cooked by mixing the product with hot water (90°C) in a 1:3 ratio (w/v) and allowing the mixture to stand for 10 min. The mixture was then blended to a smooth consistency and freeze-dried.

Dephytinization

Uncooked or cooked/freeze-dried durum wheat pasta, wheat/navy bean pasta, durum wheat flour and navy bean flour were dephytinized using a phytase solution containing 100 mg of phytase (Sigma Chem. Co., P-1259) dissolved in 10 ml of double deionized water (DDW). To each flask containing approximately 1.00 g sample, DDW was added to make a slurry. The pH of the slurry was adjusted to 5.0, 1 ml of phytase solution was added and the mixture was incubated at 55°C for 2 h (Sandberg and Svanberg, 1991).

In Vitro Iron and Zinc Dialyzability

In vitro digestion and dialysis in the presence of enzymes, pepsin and pancreatin was carried out according to the procedure of Miller et al. (1981) as modified by Kane and Miller (1984). The molecular weight cutoff of the dialysis bag was 6000-8000 Da. The dialysate (solution entering the dialysis bag) and retentate (solution remaining in the flask) were centrifuged at 12,000 x G for 20 min and the supernatants were analyzed for soluble protein, phytate, iron and zinc (Fig. 1).

Protein Determination

The total protein contents of the flours and pastas and the protein contents of the supernatants of dialyzed and undialyzed samples were determined by the Lowry method.

Phytate Determination

The total phytate contents of the flours and pastas and the phytate contents of the supernatants of dialyzed and undialyzed samples were determined by ion-exchange chromatography according to Graf and Dintzis (1982). The eluent was collected and wet-ashed with nitric acid and hydrogen peroxide. The ashed solution was analyzed for phosphorus according to Fiske and Subbarow (1925). Phytate content was calculated by using a phosphorus to phytate conversion factor of 3.55.

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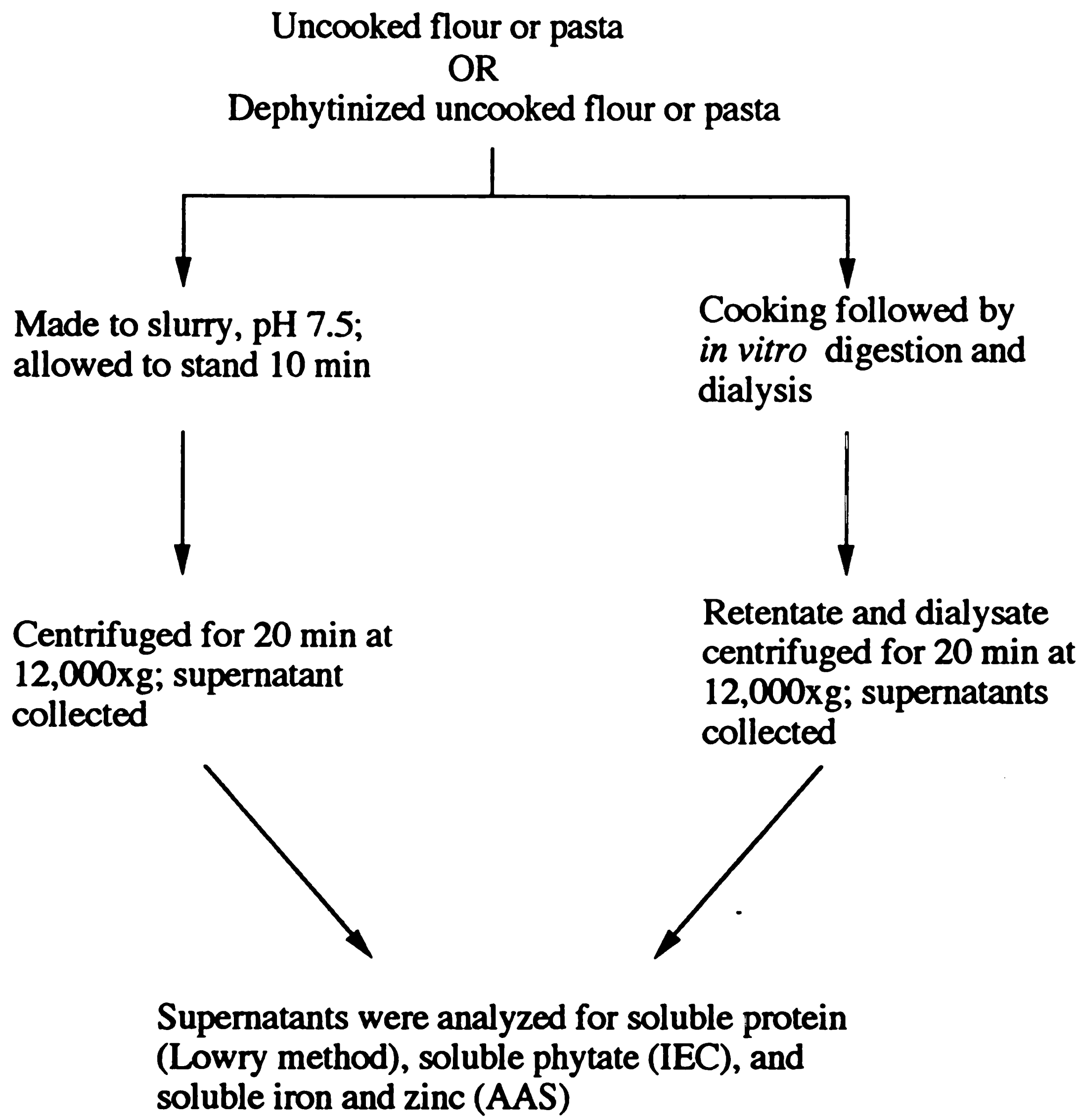

Figure 1. Flow chart for analyses of soluble protein, phytate, iron and zinc in raw and extruded products.

Iron and Zinc Determination

Iron and zinc contents of the supernatants of dialyzed and undialyzed samples were determined using atomic absorption spectrophotometry (Perkin-Elmer Model 2380).

Statistical Analyses

A one-way analysis of variance followed by the test for least significant difference at a 95% confidence interval were used to test means for significant differences.

RESULTS AND DISCUSSION

Extruded Wheat Pasta

Table 1 presents the soluble protein, phytate, iron and zinc in durum wheat flour as affected by dephytinization and extrusion. A hypothetical model of the changes in the chemistry and interactions of protein, phytate, iron and zinc is presented below.

When durum flour is dephytinized:

Gluten proteins are known to have >50% of amino acids as glutamine/glutamic acid and proline (MacRitchie et al., 1990). At pH 7.5, the protein would have a net charge that is slightly positive such that it may bind phytate as a binary complex.

Phytate as a hexaphosphate can bind wheat proteins and decrease the solubility of the protein (Lasztity and Lasztity, 1990). Dephytinizing wheat flour may release protein which may explain the increase in protein solubility from 6.9 to 9.0 mg/g ($p<0.05$). Also existing in durum flour are phytate-mineral complexes which may dissociate when dephytinized releasing the minerals. Hence, an increase in soluble iron (from 20.0 to 26.3 ug/g) and zinc (from 5.7 to 10.4 ug/g) in dephytinized flour ($p<0.05$).

When durum flour is extruded:

Extrusion processing via heat, shear and pressure can

Table 1. Changes in solubilities of protein, phytate, iron and zinc in durum wheat flour caused by extrusion, dephytinization and dialysis with pepsin and pancreatin.

	TOTAL[a]	SOLUBLE (NON-DEPHYTINIZED SAMPLES)			SOLUBLE (DEPHYTINIZED SAMPLES)		
		Undialyzed[b]	Dialysate[c]	Retentate[c]	Undialyzed[b]	Dialysate[c]	Retentate[c]
DURUM FLOUR							
PROTEIN (mg/g)	136.0	6.9	46.3	64.0	9.0	28.6	75.0
PHYTATE (ug/g)	8400	617	119	338	1302	96	344
IRON (ug/g)	32.7	20.0	7.6	27.4	26.3	8.8	30.9
ZINC (ug/g)	17.6	5.7	1.8	3.4	10.4	0.9	4.6
DURUM PASTA							
PROTEIN (mg/g)	120.0	5.6	25.3	64.0	15.8	26.9	76.0
PHYTATE (ug/g)	8400	900	133	476	1555	107	725
IRON (ug/g)	34.7	16.6	5.5	10.7	19.2	10.6	11.3
ZINC (ug/g)	14.9	6.3	0.7	3.3	8.9	1.5	4.1

[a]total amount in the uncooked product expressed as mg or μg per g product.
[b]represents solubilities in uncooked, undigested samples.
[c]represents solubilities in cooked, freeze-dried and _in vitro_ digested samples. Dialysate is the solution that enters the dialysis bag (MWCO - 6000 to 8000) and retentate is the remaining sample.

denature gluten proteins. As heat treatment continues, denaturation of proteins is followed by gelation or aggregation (Nakai and Li-Chan, 1989) leading to a decrease in solubility of proteins.

However, extrusion is also known to degrade phytates to lower inositol phosphate forms (Chapter 2) which may bind to insoluble peptides and form soluble protein-phytate complexes. This may offset the decrease in protein solubility caused by extrusion. Hence, in our experiments, we did not see a significant decrease from 6.9 to 5.6 ($p>0.05$) in soluble protein after extrusion of durum flour. An increase in soluble phytate from 617 to 900 ug/g ($p<0.05$) may have been due to the degradation of inositol hexa-phosphate to its soluble, lower phosphate forms.

When extruded durum pasta is dephytinized:

Our results indicate that dephytinization of extruded products produced a two fold increase in protein solubility from 5.6 to 15.8 mg/g ($p<0.05$). It may be that extrusion processing enhances protein-phytate interactions forming either binary complexes or ternary complexes with iron or zinc.

Speculations on the mechanisms by which these interactions may be promoted in the extruder are: denaturation of proteins which opens up binding sites for phytates and minerals, peptide formation and phytate degradation. When extruded pasta is dephytinized, phytate is degraded and the protein-phytate or protein-mineral-phytate complexes may dissociate; thus explaining the increase in the solubilities of protein, phytate (900 to 1555 ug/g), iron (16.6 to 19.2 ug/g) and zinc (6.3 to 8.9 ug/g) ($p<0.05$).

When durum flour is dialyzed:

Protein is digested by enzymes pepsin and pancreatin increasing the solubility of protein from 6.9 to 100.3 mg/g (dialysate+retentate) ($p<0.05$). The free mineral content in the dialysed fractions of durum flour was negligible (0.54 μg/g iron and 0.22 μg/g zinc); hence, it is assumed that a large percentage of total iron and zinc exist as complexes with phytate, protein or other components of the flour.

When durum flour is dephytinized and dialyzed:

A decrease ($p<0.05$) in protein dialyzability from 46.3 to 28.6 mg/g was seen. However, no change in total soluble protein (dialysate+retentate) was observed following dephytinization. Phytate is degraded to lower inositol phosphate forms by phytase. Iron bound to phytate may be released causing a nonsignificant increase ($p>0.05$) in the dialyzability of iron from 7.6 to 8.8 ug/g. Dialyzability of zinc decreased significantly ($p<0.05$) from 1.8 to 0.9 ug/g but no change ($p>0.05$) in total solubility (1.8+3.4 vs 0.9+4.6) was observed; these changes may have occurred because of the

high molecular weight phytate-protein-zinc complexes.

When extruded pasta is dialyzed:

Protein solubility increased ($p<0.05$) from 5.6 to 89.3 mg/g (dialysate+retentate) because of protein digestion by enzymes, pepsin and pancreatin. However, the soluble protein in dialyzed wheat pasta (25.3 ug/g) is significantly lower ($p<0.05$) than in dialyzed wheat flour (46.3 ug/g) indicating the formation of insoluble protein complexes as a consequence of extrusion.

Total soluble (dialysate+retentate) iron and zinc contents are significantly lower ($p<0.05$) in dialyzed wheat pasta compared to dialyzed wheat flour suggesting that insoluble protein-phytate-zinc/iron complexes formed during extrusion have not dissociated.

When the extruded pasta is dephytinized and dialyzed:

Total soluble (dialysate+retentate) protein, phytate, iron and zinc and dialyzable iron and zinc contents increased ($p<0.05$) compared to non-dephytinized dialyzed pasta. The phytate from the insoluble protein-phytate-zinc/iron complex may have been released making the protein-zinc/iron complex soluble (and somehow more susceptible to proteases?).

Extruded Wheat/Bean Pasta

In the case of wheat bean pasta (Table 2), similar patterns as

Table 2. Changes in solubilities of protein, phytate, iron and zinc in wheat bean pasta and navy bean flour caused by dephytinization and dialysis with pepsin and pancreatin.

	TOTAL[a]	SOLUBLE (NON-DEPHYTINIZED SAMPLES)			SOLUBLE (DEPHYTINIZED SAMPLES)		
		Undialyzed[b]	Dialysate[c]	Retentate[c]	Undialyzed[b]	Dialysate[c]	Retentate[c]
WHEAT BEAN PASTA							
PROTEIN (mg/g)	132.0	8.1	37.0	83.0	16.1	37.0	85.0
PHYTATE (ug/g)	8500	2073	101	419	3218	94	594
IRON (ug/g)	38.1	18.9	7.0	15.6	21.5	10.5	17.3
ZINC (ug/g)	16.3	7.2	2.1	4.2	12.1	2.9	10.6
NAVY BEAN FLOUR							
PROTEIN (mg/g)	223.0	20.5	45.0	134.0	20.3	44.0	136.0
PHYTATE (ug/g)	9200	1707	148	997	2586	209	1283
IRON (ug/g)	67.7	34.1	7.9	13.5	49.9	8.8	16.0
ZINC (ug/g)	30.3	16.5	5.4	8.1	19.5	5.2	9.0

[a]total amount in the uncooked product expressed as mg or μg per g product.
[b]represents solubilities in uncooked, undigested samples.
[c]represents solubilities in cooked, freeze-dried and in vitro digested samples. Dialysate is the solution that enters the dialysis bag (MWCO - 6000 to 8000) and retentate is the remaining sample.

in wheat pasta were observed. However, since a new type of protein (navy bean protein) was introduced along with wheat protein, the results are different. Soluble protein and phytate in undialyzed wheat bean pasta were higher ($p<0.05$) than in undialyzed wheat pasta. Total soluble (dialysate+retentate) protein, iron and zinc contents were also higher ($p<0.05$) in non-dephytinized wheat bean pasta compared to non-dephytinized wheat pasta. Addition of navy bean to durum wheat pasta seems to have decreased the negative effects of extrusion processing on iron and zinc dialyzability and solubility. Dephytinization of wheat bean pasta increased ($p<0.05$) total soluble iron and zinc in the undialyzed and dialyzed samples.

In conclusion, extrusion of durum wheat flour decreased soluble protein and iron but increased soluble phytate. Extrusion combined with dephytinization caused a significant increase in protein, phytate, iron and zinc solubility. Extruded durum wheat pasta had lower dialyzable protein, iron and zinc compared to durum wheat flour. However, dephytinization of extruded durum wheat pasta increased dialyzable iron and zinc compared to non-dephytinized extruded durum wheat pasta. It is speculated that high molecular weight insoluble protein-phytate-iron/zinc complexes may form during extrusion and release the minerals upon phytate degradation.

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CHAPTER 5. EXTRUSION PROCESSING OF SEMOLINA. I. CHANGES IN THE SOLUBILITY AND DISTRIBUTION OF PROTEINS

ABSTRACT

The effects of extrusion processing on protein solubility and molecular weight distribution were investigated. Enriched semolina was extruded using a twin-screw extruder at two different temperatures, 50°C and 96°C. A modified Osborne fractionation showed that the amount of protein extracted as albumin, globulin, gliadin and glutenin fractions decreased with an approximate three-fold increase in the insoluble residue following extrusion. The effect on protein solubility was greater with the higher extrusion temperature. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) patterns of reduced and unreduced protein fractions suggest that disulfide linkages formed during extrusion may be responsible for the increase in insoluble residue fraction.

INTRODUCTION

Wheat is the largest U.S. cereal crop used for human food (Potter, 1986). Extrusion processing of wheat is widely used in the manufacture of breakfast cereals, infant foods, crispbread, snacks and sweets and pasta products. Extrusion processing has the potential to alter protein structure, solubility and digestibility due to heat, pressure and shear (Phillips, 1989). The effects of extrusion processing on protein solubility in soybean (Cumming et al., 1973), field bean (Jeunink and Cheftel, 1979) and navy and pinto beans (Gujska and Khan, 1991) have been reported. However, little is known about the changes in solubility and distribution of wheat proteins during extrusion. Although proteins are known to undergo changes due to heat treatment, depending on the temperature conditions, information on how the physiochemical properties of wheat proteins are affected by extrusion is not available.

The purpose of this study was to determine the effects of extrusion processing at two different temperatures on the solubility and distribution of proteins in semolina. The role of disulfide linkages in the effects of extrusion on protein solubility and distribution also was investigated.

MATERIALS AND METHODS

Extrusion Processing

Semolina (30 mesh), milled from durum wheat with approximately 65% extraction and enriched with niacin, iron, thiamin and riboflavin was obtained from the North Dakota Mill & Elevator, Grand Forks, ND. Enriched semolina is known to have added amounts of 3.13 mg iron, 0.53 mg thiamin, 0.49 mg riboflavin and 2.68 mg niacin per 100 g (USDA, 1989). Semolina was extruded to make a product in the shape of small "O's". A corotating twin-screw extruder (Creusot-Loire, Model 45) was used at two different temperatures (47-50°C and 92-96°C). Water was injected into the feed at the rate of 0.12 L/min. The screw was operated at 900 rpm and feeder was set to deliver 2.06 kg semolina/min. The final product was dried in a vat dryer/blower to a moisture content of 8 to 9%. The extruded semolina cereals were milled in a coffee mill to a coarse powder (able to pass completely through a 30 mesh sieve) prior to analyses.

Protein Fractionation and Determination

Albumins, globulins, gliadins, glutenins and the insoluble residue were fractionated from raw semolina and extruded semolina cereals according to the Osborne procedure as modified by Chen and Bushuk (1970). A dialysis bag with a molecular weight cutoff of 6000-8000 Da was used in the fractionation of albumins and globulins. The protein fractions were freeze-dried and stored in a dessicator at room temperature prior to all analyses. The protein content of raw and extruded semolina and each of the protein fractions was determined by the micro-Kjeldahl method (AOAC, 1980) using a nitrogen to protein conversion factor of 5.83.

Electrophoresis

The protein fractions from raw semolina and extruded semolina were electrophoresed with or without a reducing agent (5% 2-mercaptoethanol) on 17.5% (w/v) gels in the presence of sodium dodecyl sulfate (SDS-PAGE) according to Ng and Bushuk (1987). The weights of protein fractions were adjusted so that the same amount of protein was loaded into each column. Gels were stained for protein using Brilliant Blue-R. Protein extract from the flour of cultivar Neepawa was used as a reference for molecular weights.

Statistics

Means of the percentage of total protein in each fraction were compared for statistical significance using 2-way analysis of variance followed by the test for least significant difference at 95% confidence level.

RESULTS AND DISCUSSION

Protein Distribution

Results of the protein fractionation of raw semolina, semolina cereal extruded at 50°C (SC I) and 96°C (SC II) are presented in Table 1. The recovery of samples is reported on the basis of weight and protein content. Recovery by weight was 93 to 95% and protein recovery ranged from 93 to 98%. The variability in recovery may be attributed to the loss of low molecular weight proteins during dialysis and loss of sample during analysis.

In raw semolina, protein was present in the highest amount in the alcohol-extractable protein fraction (gliadin) (41.8% of total protein) followed by the insoluble glutenin fraction (27.7%), soluble glutenin (14.2%), albumin (11.7%) and globulin (3.6%). These results are in general agreement with the findings of Chen and Bushuk (1970).

The distribution of proteins differed in the extruded semolina cereals compared to raw semolina (Table 1). Extrusion processing at both experimental temperatures caused a marked decrease in the percentage of total protein present as albumin, globulin, gliadin and glutenin fractions with a corresponding increase in the insoluble residue. The insoluble residue in raw semolina is considered to represent insoluble glutenins (Wrigley and Bietz, 1988); however, after extrusion other proteins of wheat were also extracted in this fraction. Extrusion, at a higher temperature of 96°C caused

Table 1. Protein fractionation of raw and extruded semolina.

	Product		
	Raw Semolina	SC I[1]	SC II[2]
Protein (%)	14.2	14.4	14.9
ALBUMIN FRACTION			
Weight (g/10g prod.)	0.52	1.07	0.92
Protein content (%)[3]	31.3	14.8	2.9
% total protein[4]	11.7[a]	10.8[b]	2.0[c]
GLOBULIN FRACTION			
Weight (g/10g prod.)	0.09	0.03	0.24
Protein content (%)	54.6	49.9	9.6
% total protein	3.6[a]	1.4[b]	0.7[c]
GLIADIN FRACTION			
Weight (g/10g prod.)	0.95	0.47	0.54
Protein content (%)	62.1	22.5	9.4
% total protein	41.8[a]	7.3[b]	3.4[c]
GLUTENIN FRACTION			
Weight (g/10g prod.)	0.33	0.19	0.18
Protein content (%)	60.1	20.7	32.4
% total protein	14.2[a]	2.8[b]	4.0[b]
INSOLUBLE FRACTION			
Weight (g/10g prod.)	7.38	7.59	7.73
Protein content (%)	5.3	14.1	16.1
% total protein	27.7[a]	74.0[b]	83.2[c]
RECOVERY			
Weight (%)	92.8	95.1	94.7
Protein (%)[5]	98.6	96.2	93.3

[1]Semolina cereal prepared by extrusion processing at 47-50°C.
[2]Semolina cereal prepared by extrusion processing at 92-96°C.
[3]Protein content (N X 5.83).
[4]% total protein = Protein (%) x fraction wt. (g) x 10 ÷ total protein. Means (of 2 replicates) with different superscript letters in each row are significantly different ($p<0.05$).
[5]Sum of % total protein in each fraction.

a greater increase in insoluble residue compared to the lower temperature of 50°C. Previously, Gujska and Khan (1991) reported that extrusion temperatures of 110°C to 135°C decreased the solubility of protein constituents in navy and pinto beans. A decrease in solubility of field bean proteins after extrusion was observed by Jeunink and Cheftel (1979). They concluded that non-covalent interactions between the polypeptide chains and the formation of extended unfolded protein networks may be responsible for the decrease in solubility. Cumming et al. (1973) reported that extrusion of soy meal resulted in a four-fold loss in the solubility of water-extractable proteins and a breakdown of the remaining protein into subunits. The marked decrease in the solubility of semolina proteins at an extrusion temperature as low as 50°C (Table 1) may have potential applications in the food industry.

Electrophoresis

The molecular weight (MW) distribution profiles obtained from SDS-PAGE under reduced or unreduced conditions for albumins of raw and extruded semolina are presented in Figure 1 (lanes 2-4, 11-13). After extrusion of raw semolina, the reduced SDS-PAGE pattern of albumins showed a disappearance of the high molecular weight polypeptides (estimated MW: 61,500) and an appearance of a low MW (LMW) polypeptide in the range of 49,800 Da. Although the patterns were similar for both

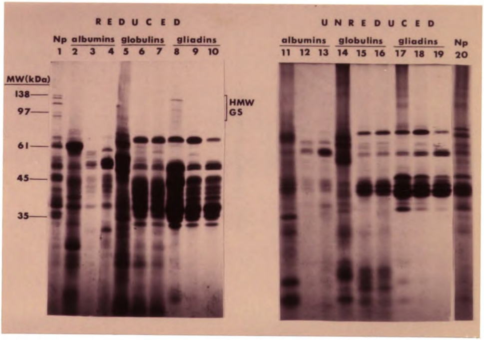

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns of unreduced and reduced (with 2-mercaptoethanol) albumin, globulin and gliadin fractions from raw and extruded semolina; Np-protein extract from flour of cultivar Neepawa; 2,5,8,11,14,17 represent fractions from raw semolina; 3,6,9,12,15,18 represent fractions from semolina extruded at 50°C; 4,7,10,13,16,19 represent fractions from semolina extruded at 96°C; HMW-GS - high molecular weight glutenin subunits.

extruded semolina cereals, extrusion at 96°C resulted in bands of greater intensity than from extrusion at 50°C. The high MW (HMW) polypeptides of albumins were most likely depolymerized after extrusion at these temperatures. There were no obvious changes between the SDS-PAGE patterns of reduced and unreduced raw (lane 2 vs 11) or extruded (lane 3 vs 12, 4 vs 13) semolina.

The globulin fraction of raw semolina contained five major regions of polypeptides with estimated molecular weights of 34,600, 37,000, 45,200, 49,800, and 61,500, under reduced conditions (Fig. 1; lane 5). The presence of several fainter bands in the MW range of 34,600 to 67,500 was also observed. Extrusion temperature of 50°C and 96°C affected protein patterns similarly causing a decrease in intensity of some bands in the HMW region (49,800 to 63,300) and an increase in intensity of some bands in the LMW region (below 45,200) (lanes 5 vs 6 and 7). There were no apparent differences between the SDS-PAGE patterns of reduced and unreduced globulin fractions from raw (lane 5 vs 14) or extruded (lane 6 vs 15, 7 vs 16) semolina, although the reduced fractions (in the MW region of 35,000-45,000 Da) diffused more than the unreduced fractions.

The SDS-PAGE patterns of the reduced gliadin fraction of semolina, shown in Figure 1 (lanes 8-10), were similar before and after extrusion at 50°C or 96°C. However, a band representing a polypeptide with estimated MW of 67,500 appeared fainter after extrusion at 96°C compared to raw semolina or extrusion at 50°C. A similar pattern was observed with an unreduced gliadin fraction from raw and extruded semolina (lanes 17-19). In addition, a few HMW glutenin subunits appeared in the raw semolina (lane 8) but not in either of the extruded semolina cereals. These subunits most likely were depolymerized after extrusion at the temperatures used. As in the case of globulins, there were no apparent differences between the SDS-PAGE patterns of reduced and unreduced gliadin fractions from raw (lane 8 vs 17) or extruded (lane 9 vs 18, 10 vs 19) semolina, although the reduced fractions (in the MW region of 35,000-45,000 Da) diffused more than the unreduced fractions.

The reduced glutenin fraction of raw semolina appeared to have six major regions of polypeptides in the MW ranges of 37,000, 45,000-50,000, 65,000, 92,400, 100,000 and 120,000 (Fig. 2; lane 2). After extrusion, disappearance of bands in the HMW region (above 65,000), an increase in intensity of the band at 65,000, and appearance of some bands in the LMW region (below 45,200) were observed (lane 2 vs 3 and 4). Extrusion at 50°C and 96°C showed similar patterns (lane 3 vs 4); however, the 65,000 band was more intense after extrusion at the higher temperature (96°C) compared to the lower temperature (50°). There were no obvious differences in the SDS-PAGE patterns of reduced versus unreduced extruded semolina cereals (lane 3 vs 9, 4 vs 10). In raw semolina,

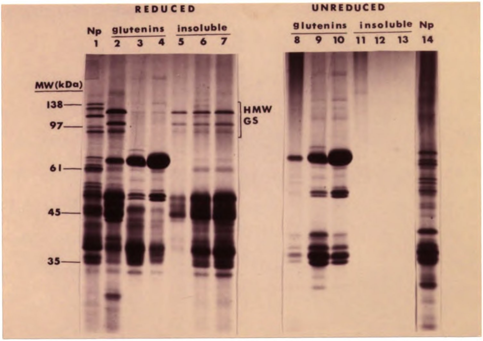

Figure 2. Sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns of unreduced and reduced (with 2-mercaptoethanol) glutenin and insoluble fractions from raw and extruded semolina; Np-protein extract from flour of cultivar Neepawa; 2,5,8,11 represent fractions from raw semolina; 3,6,9,12 represent fractions from semolina extruded at 50°C; 4,7,10,13 represent fractions from semolina extruded at 96°C; HMW-GS - high molecular weight glutenin subunits.

however, the unreduced pattern (lane 8) did not show the HMW polypeptides (above 65,000) and some LMW polypeptides (45,000 to 50,000) that were seen in the reduced fraction (lane 2).

The reduced insoluble residue of raw semolina produced an SDS-PAGE pattern with bands in the MW ranges of 45,000-50,000 and 92,000-128,000 (Fig. 2; lane 5). After extrusion, the intensity of all bands was greater and there was appearance of bands in the MW range of 61,000-67,500 (lane 5 vs 6 and 7). Extrusion at 96°C (lane 7) produced slightly more intense bands compared to the extrusion temperature of 50°C (lane 6), although both showed similar SDS-PAGE patterns. In the unreduced SDS-PAGE pattern of the insoluble residue of raw semolina (lane 11), no bands were visible. The unreduced insoluble fraction of extruded semolina cereals showed only two sets of very faint bands in the MW regions of 34,600 and 65,000 Da (lane 12, 13).

Results indicate that high MW polypeptides of albumins, globulins and glutenins of semolina were most likely depolymerized after extrusion at 50°C or 96°C. In contrast, the MW distribution of gliadin proteins seemed to be unaffected by extrusion at both experimental temperatures. In the case of albumin and globulin fractions, the similarity of electrophoretic patterns of reduced versus unreduced fractions demonstrate that disulfide linkages were not likely to have played a major role in the structural changes that occurred during extrusion.

In the glutenin fraction, under reduced conditions, the HMW polypeptides (above 65,000) seen in raw semolina are not visible in the extruded cereals. In contrast, under reduced conditions, the insoluble fractions from extruded cereals show a greater intensity of bands in the same region (above 65,000) when compared to the insoluble fraction from raw semolina. Thus, after extrusion, there was disappearance of bands in the glutenin fractions and an increased intensity of the same bands in the insoluble fractions suggesting that extrusion caused the soluble glutenin polypeptides to move into the insoluble glutenin fraction (insoluble residue) of semolina.

The insoluble fraction from raw and extruded semolina produced different electrophoretic patterns under unreduced versus reduced conditions. In case of both raw and extruded semolina, there was an apparent lack of bands under unreduced conditions and an appearance of bands under reduced conditions suggesting the presence of disulfide bonds between polypeptides of the insoluble fraction. The results of this study also suggest that extrusion may have promoted the formation of disulfide bonds which, when broken, release the polypeptides; hence there is an increase in intensity of bands for the insoluble fraction of extruded semolina compared to raw semolina. These findings may have some implications in the dough rheological properties of extruded wheat.

The role of disulfide bonds in thermal extrusion effects on protein solubility has been studied previously. However, most of these studies were conducted using soy proteins. It was shown by Hager (1984) that disulfide bonds contribute to the new, extended protein networks produced by extrusion of soy concentrate. Rhee et al. (1981) reported that extrusion at 138°C reduced protein solubility; an increase in protein solubility was achieved by the use of 2-mercaptoethanol and SDS, suggesting that disulfide linkages may be responsible for the decrease in protein solubility after extrusion.

In conclusion, extrusion processing at both 50°C and 96°C caused a marked increase in the percentage of total protein present in the insoluble residue fraction. Results of this study also indicate the presence of disulfide linkages between polypeptides of the insoluble fraction which may have been promoted by extrusion.

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CHAPTER 6. EXTRUSION PROCESSING OF SEMOLINA. II. DISTRIBUTION OF IRON AND PHYTATE IN PROTEIN FRACTIONS

ABSTRACT

Proteins from semolina, raw and extruded (at 50°C and 96°C), were fractionated to study the distribution of iron and phytate and to identify the iron binding polypeptides. The amount of iron and phytate in the insoluble protein fraction increased after extrusion at 50°C or 96°C. Almost all of the soluble iron and phytate were seen in the albumin and globulin fractions in both raw and extruded semolina. There were negligible amounts of iron and phytate present in the gliadin and glutenin fractions of raw and extruded semolina. Extrusion at both temperatures increased the amount of iron and phytate associated with the albumin and globulin fractions. SDS-PAGE patterns indicate that albumins, globulins and the insoluble fraction (high MW glutenins) possess iron binding polypeptides (estimated MW: 16,000 Da). No iron binding components were apparent in the gliadin and low MW glutenin fractions. There was no apparent effect of extrusion temperature in this study. An increase in the amount of iron bound to the soluble, relatively low MW proteins of wheat, albumins and globulins, may explain the increased dialyzability of iron after extrusion at both experimental temperatures.

INTRODUCTION

Cereals and legumes are important sources of minerals, including iron, in the diets of many populations. The effects of extrusion processing on iron bioavailability of cereals and legumes has been studied previously. An increase in in vitro iron dialyzability was reported in extruded maize based snack foods (Hazell and Johnson, 1989) and defatted soy flour (Latunde-Dada, 1991). An increase in iron solubility and dialyzability in low impact extruded legume products when compared to non-extruded or high impact extruded legume flours was seen in our laboratory (Chapter 2). In contrast, Lombardi-Boccia et al. (1991) found that iron dialyzability was decreased, although not significantly, after extrusion of some legumes.

The mechanism(s) by which extrusion processing alters iron dialyzability is not known. Chemical changes during extrusion that have been suggested to influence iron dialyzability are formation of Maillard products, increase in lignin fraction, formation of amylose-lipid complexes (Fairweather-Tait et al., 1989), promotion of minerals-tannins interactions (Lombardi-Boccia et al., 1991) and the deactivation of endogenous phytase in cereals and legumes (Kivistö et al., 1986).

The interaction between protein, phytate and minerals as a factor affecting mineral solubility or dialyzability in extruded foods has not been investigated. It is known that phytates exist naturally in the proteinaceous matrix of cereals (Lasztity and Lasztity, 1990) and, from a theoretical point of view, neutral or high pH and the presence of divalent cations may favor the formation of protein-mineral-phytate complexes. It is also well-documented that extrusion processing results in changes in protein digestibility and solubility (Cumming et al., 1973; Jeunink and Cheftel, 1979; Gujska and Khan, 1991) and the nature of protein-phytic acid interactions may be affected depending on the extent of heat treatment (Lasztity and Lasztity, 1990). However, the effect of extrusion processing of cereals on the solubility and distribution of phytate and minerals is not known.

In an earlier study (Chapter 5), the effect of extrusion temperatures on protein solubility and distribution in semolina was determined. Extrusion processing at 50°C and 96°C caused a marked decrease in the albumin, globulin, gliadin and glutenin fractions with an approximate three-fold increase in the insoluble residue. The effect on protein solubility was greater with the higher extrusion temperature. Because changes in protein solubility and distribution can affect the interactions between proteins, phytate and minerals in cereals, the present study was based on the hypothesis that extrusion processing would result in a redistribution of iron and phytate. Iron and phytate contents were determined in individual protein fractions from raw and extruded semolina.

Polypeptides that bind iron in raw and extruded semolina were also identified and their molecular weights determined.

MATERIALS AND METHODS

Extrusion Processing and Protein Fractionation

These procedures are described in Chapter 5.

Iron Determination

Known amounts of raw and extruded semolina cereals and the isolated protein fractions were wet ashed with concentrated nitric acid and 30% hydrogen peroxide. The ash was dissolved in 0.1 N hydrochloric acid (trace metal grade) and analyzed for total iron using atomic absorption spectrophotometry (AAS) (Perkin Elmer Model 2380). National Institute of Science and Technology standards, bovine liver (1577b) and wheat flour (1567a), were used to monitor the accuracy of the ashing and AAS analyses. The analyzed values of the NIST standards were within the range of the certified values.

Phytate Determination

Total phytate in raw and extruded semolina cereals and the isolated protein fractions was determined using the ion-exchange procedure of Graf and Dintzis (1982) followed by wet ashing and phosphorus analysis.

Ion-Exchange Procedure - Samples of 0.5 g were extracted under mechanical agitation with 20 ml 0.5 M HCl for 2 hours at 20°C. The extract was centrifuged and supernatant decanted, frozen overnight and filtered (o.45 μm) under pressure. The filtrate was diluted with 10 ml distilled deionized water (DDW) and passed through an ion-exchange column containing 0.65 ml resin (AG 1-X8, 200-400 mesh) at 0.4 ml/min followed by 10 ml of 0.025 M HCl. Inositol phosphates were removed from the resin with ten 1 ml portions of 2 M HCl.

Phosphorus Analysis - The solution was wet ashed with concentrated nitric acid and 30% hydrogen peroxide. The ash was dissolved in de-ionized distilled water and phosphorus was determined according to Fiske and Subbarow (1925). Total phosphorus in the solution was converted to total phytate using a factor of 3.55.

Electrophoresis

The electrophoresis procedure has been described in Chapter 5. Gels were stained for protein using Brilliant Blue-R. Duplicate gels were stained for iron using a ferene-thioglycolic acid reagent according to Chung (1985). Protein extract from the flour of cultivar Neepawa was used as a reference for molecular weights; human hemoglobin (Sigma Chem. Co., H7379) was used as a standard protein containing iron.

In Vitro Iron Dialyzability

Estimation of dialyzable iron in six replicates of raw and extruded semolina cereals was carried out according to Miller et al. (1981). Iron content of the dialysate was determined using a ferrozine color reagent.

Statistics

Means of the percentages of total iron (or phytate) were compared for statistical significance using 2-way analysis of variance followed by the test for least significant difference at 95% confidence level.

RESULTS AND DISCUSSION

Distribution of Iron

Iron contents of raw and extruded semolina products and their protein fractions are presented in Table 1. Total iron in raw semolina and semolina extruded at 50°C (SCI) was 37.4 and 35.0 μg/g, respectively. Total iron content of semolina extruded at 96°C (SCII) was significantly higher (46.3 μg/g) than in raw semolina or SCI. The reason for the increase in total iron content of semolina after extrusion at the higher temperature is not clear. A similar observation was made by Fairweather-Tait et al. (1987) who reported that extrusion increased the iron content of maize and potato products.

The distribution of iron varied in raw and extruded semolina products. In raw semolina, the highest amount of iron (29.8%) was seen in the insoluble fraction, followed by globulin (16.2%), albumin (11.9%) and very small amounts (1 to 2%) in the gliadin and glutenin fractions. After extrusion at 50°C, the percentage of total iron increased in the albumin and insoluble fractions and decreased in the gliadin and glutenin fractions; however, no effect was seen on the iron associated with the globulin fraction. Semolina extruded at 96°C showed a slightly different pattern of iron distribution. Compared to raw semolina, the percentage of iron increased in the globulin and insoluble fractions, decreased in the gliadin and glutenin fractions but no effect was seen in the albumin fraction.

Table 1. Iron in protein fractions from raw and extruded semolina.

	Product		
	Raw Semolina	SC I[1]	SC II[2]
Total iron (μg/g product)	37.4	35.0	46.3
ALBUMIN FRACTION			
Iron (μg/g fraction)	86.1	94.8	63.7
Iron (μg/10g product)	44.6	101.9	58.4
% total iron[3]	11.9[a]	29.2[b]	12.6[a]
GLOBULIN FRACTION			
Iron (μg/g fraction)	656.3	1627.9	1568.8
Iron (μg/10g product)	60.4	56.3	187.0
% total iron	16.2[a]	16.1[a]	40.4[b]
GLIADIN FRACTION			
Iron (μg/g fraction)	8.7	4.7	5.3
Iron (μg/10g product)	8.3	2.2	2.9
% total iron	2.2[a]	0.6[b]	0.6[b]
GLUTENIN FRACTION			
Iron (μg/g fraction)	12.7	ND[4]	6.2
Iron (μg/10g product)	4.2	ND	1.1
% total iron	1.2[a]	ND	0.2[b]
INSOLUBLE FRACTION			
Iron (μg/g fraction)	15.1	19.2	21.4
Iron (μg/10g product)	114.5	145.7	165.4
% total iron	29.8[a]	41.6[b]	35.7[c]
IRON RECOVERY (%)	61.2	87.4	89.6

[1]Semolina cereal prepared by extrusion processing at 47-50°C.
[2]Semolina cereal prepared by extrusion processing at 92-96°C.
[3]% total iron = iron (μg/10g product) x 10 ÷ total iron (μg/g). Means (of 2 replicates) with different superscript letters in each row are significantly different ($p<0.05$).
[4]Non-detectable.

It is evident that extrusion at both temperatures increased the amount of iron associated with the insoluble fraction. The decrease in protein solubility associated with extrusion (Chapter 5) may be responsible for the increase in the amount of iron seen in the insoluble fraction. Nelson and Potter (1979) reported that the amount of ferrous iron (from ferrous sulfate) bound to the insoluble fraction of purified wheat gluten increased with an increase in temperature. The authors also observed that a ferrous-protein complex was markedly less soluble than the protein alone. Similarly, in our experiments, the binding of iron to proteins during extrusion may have contributed, in part, to the increase in insoluble residue fraction of semolina.

Almost all of the soluble iron was seen in the albumin and globulin fractions in all three products. The presence of high amounts of polar and charged amino acids such as lysine, aspartate, cysteine and arginine in albumins and globulins (Wrigley and Bietz, 1988) may explain the high affinity of these proteins for iron. In contrast, gliadin and glutenin proteins possess high amounts of uncharged amino acids such as proline, glutamine, leucine and serine and thus have less tendency to form ionic interactions with iron (Wrigley and Bietz, 1988).

Extrusion at the higher temperature seemed to cause a shift in iron distribution from the albumin fraction (in SCI) to the globulin fraction (in SCII). Overall, the amount of iron associated with the soluble proteins of semolina, i.e. albumins and globulins was markedly increased after extrusion. Extrusion results in depolymerization of proteins (Cumming et al., 1973; Jeunink and Cheftel, 1979) and owing to the ionic nature of albumin and globulin proteins, extrusion may have caused increased iron binding to these proteins.

Distribution of Phytate

Total phytate contents of raw and extruded semolina products and their protein fractions are presented in Table 2. Extrusion processing did not seem to influence the total phytate content of semolina. However, the distribution of phytate in semolina was greatly affected by extrusion.

The distribution of phytate followed the same pattern as iron. In raw semolina, the amount of phytate associated with the albumin and globulin fractions was very low. After extrusion, the amount of phytate increased markedly in the globulin and especially, in the albumin fractions. As in the case of iron, the ability of albumins and globulins to form ionic interactions may result in high amounts of phytate seen in these fractions. Extrusion processing at both temperatures also caused a marked increase in the amount of phytate seen in the insoluble fraction when compared to raw semolina. Negligible amounts of phytate were seen in the gliadin and glutenin fractions of both raw and extruded semolina.

Table 2. Phytate in protein fractions from raw and extruded semolina.

	Product		
	Raw Semolina	SC I[1]	SC II[2]
Total phytate (μg/g product)	609.1	590.7	578.1
ALBUMIN FRACTION			
Phytate (μg/g fract.)	109.9	3853.2	4105.5
Phytate (μg/10g prod.)	55.2	4216.9	3814.4
% total phytate[3]	0.9[a]	71.4[b]	66.0[c]
GLOBULIN FRACTION			
Phytate (μg/g fract.)	590.6	4949.2	3874.4
Phytate (μg/10g prod.)	55.0	177.2	469.6
% total phytate	0.9[a]	3.0[b]	8.1[c]
GLIADIN FRACTION			
Phytate (μg/g fract.)	57.1	109.2	347.0
Phytate (μg/10g prod.)	54.1	45.4	178.7
% total phytate	0.9[a]	0.8[a]	3.1[b]
GLUTENIN FRACTION			
Phytate (μg/g fract.)	145.1	112.1	121.2
Phytate (μg/10g prod.)	46.5	23.0	22.3
% total phytate	0.8[a]	0.4[a]	0.4[a]
INSOLUBLE FRACTION			
Phytate (μg/g fract.)	11.7	73.2	71.4
Phytate (μg/10g prod.)	87.0	549.7	554.6
% total phytate	1.4[a]	9.3[b]	9.6[b]
PHYTATE RECOVERY (%)	4.9	84.9	87.2

[1]Semolina cereal prepared by extrusion processing at 47-50°C.
[2]Semolina cereal prepared by extrusion processing at 92-96°C.
[3]% total phytate = phytate (μg/10g product) x 10 ÷ total phytate (μg/g). Means (of 2 replicates) with different superscript letters in each row are significantly different ($p<0.05$).

Recovery of phytate was very low in raw semolina (5%) compared to extruded semolina cereals (approx. 85%). Recovery of iron in raw semolina was only 61% compared to about 87% in extruded semolina cereals. In an attempt to determine the reason for the low recoveries of iron and phytate, the fractionation procedure was carried out without the dialysis step which is part of the standard assay; thus, albumins and globulins remained as one fraction. Using this procedure, recoveries of iron and phytate were considerably higher, especially for the raw semolina. Recoveries for iron in raw semolina, SCI and SCII were 97.9%, 99.4% and 95.4%, respectively. Phytate recoveries in the same products were 91.4%, 98.3% and 99.5%, respectively. These results suggest that iron and phytate losses occurred during dialysis. Based on the recoveries of iron and phytate, it may be deduced that approximately 38% of iron and 95% of phytate in raw semolina is present in a low MW (<6000-8000 Da) form since the MW cutoff of the dialysis bag was 6000-8000 Da. After extrusion, only about 13% of iron and 15% of phytate remained in the low MW form. High MW complexes of protein, iron and/or phytate may have been formed during extrusion causing an increase in insolubility of all three components.

It is evident that in raw semolina the iron lost during dialysis was not bound to protein because protein recovery after fractionation of raw semolina was 98.6% (Chapter 5). Thus, iron lost during dialysis was either in a free form or bound to components other than protein, such as phytate. Previously, May et al. (1980) demonstrated that over 60% of iron in wheat bran exists as monoferric phytate. Losses of iron and phytate in raw semolina during dialysis may have occurred, in part, in the form of monoferric phytate.

It is interesting to note that phytate in raw semolina is not associated with the proteins. Unlike commercial soy protein isolates which are known to contain 1.62% to 1.87% phytate (Honig and Wolf, 1987), commercial wheat gluten (obtained from Dyets, Inc. and analyzed in our laboratory) contained only 0.25% phytate bringing into focus the differences in the interactions between proteins and phytate as dependent on the type of protein and/or the procedures used in the isolation of the protein. The finding that after extrusion about 70% of the phytate was associated with the albumin fraction, which comprises only about 12% of the total wheat protein, further emphasizes the importance of the type of protein. The major fractions of wheat protein, i.e. gliadin and glutenin, do not seem to bind phytate in raw or extruded semolina.

Iron Binding Proteins

The SDS-PAGE patterns for albumin, globulin and insoluble (Fig. 1) fractions from raw and extruded semolina show that iron is mostly concentrated in the albumin and globulin polypeptides with an estimated MW of 16,000 Da. Extrusion

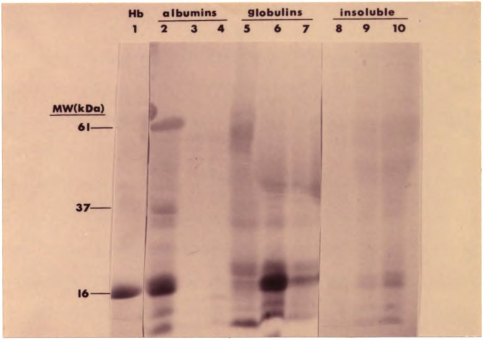

Figure 1. Iron staining of reduced (with 2-mercaptoethanol) albumin, globulin and insoluble fractions from raw and extruded semolina; Hb-human hemoglobin (reduced with 2-mercaptoethanol); 2,5,8 represent fractions from raw semolina; 3,6,9 represent fractions from semolina extruded at 50°C; 4,7,10 represent fractions from semolina extruded at 96°C.

temperature did not appear to influence the pattern of MW distribution, although the intensity of bands varied. The gliadin and glutenin fractions from raw and extruded semolina did not show any iron bound protein subunits. These fractions are known to contain negligible amounts of iron (Table 1). In the case of the insoluble fraction from raw semolina, no bands were apparent. However, in the insoluble fraction from extruded semolina, bands appeared in the low MW range (approx. 16,000); intensity of bands was greater in semolina extruded at the higher temperature of 96°C compared to the lower temperature of 50°C.

The reasons for some differences between the distribution of iron and its electrophoretic patterns are not readily apparent. For example, the percentage of total iron present in the albumin fraction increased after extrusion of semolina at 50°C (Table I). However, the SDS-PAGE pattern does not show any iron staining (Fig. 1, lane 3). Because this same fraction also did not show any protein staining, the polypeptide may have been too large to pass through the resolving gel resulting in the apparent lack of iron bound proteins in this fraction.

Iron Dialyzability

Dialyzable iron in raw semolina, semolina extruded at 50°C and semolina extruded at 96°C were 2.8, 7.3 and 6.0 $\mu g/g$ product, respectively. Previously, an increase in the dialyzability of iron after extrusion processing of maize-based snack foods (Hazell and Johnson, 1989) and defatted soy flour (Latunde-Dada, 1991) was reported.

In conclusion, our results indicate that extrusion causes a redistribution of iron and phytate in semolina. After extrusion, there is more iron and phytate associated with the albumin and globulin fractions. There is also a shift in the distribution of iron from the soluble form in raw semolina to the insoluble form after extrusion. It is not clear how these changes relate to the increase in iron dialyzability after extrusion; however, it may be speculated that the albumins and globulins being soluble and relatively low MW proteins, can potentially increase the dialyzability of iron bound to them. Previously, Nelson and Potter (1980) reported that a wheat gluten-ferrous complex released 94% of iron following HCl-pepsin-pancreatin digestion indicating that protein-bound iron would be readily available for absorption. The protein-bound ferrous iron was also seen to be as biologically available as the standard, ferrous sulfate. Although our experiments do not demonstrate the exact mechanism by which extrusion exerts its effects on iron dialyzability, these results provide an insight into the nature, forms and interactions of protein, iron and phytate and indicate the need for further investigation.

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CHAPTER 7. SUMMARY AND CONCLUSIONS

The extruded products used in this study were extruded under different conditions to produce various products. Legume flours were extruded under low or high impact extrusion conditions which differed in temperature, pressure and shear applied during the extrusion process. In the production of the pastas, durum wheat flour and navy bean flour were extruded at extremely high temperatures with the purpose of producing microwaveable pasta. Durum wheat semolina based breakfast cereals were prepared by extrusion at two different temperatures while keeping all other extrusion conditions constant.

Since the products were extruded under different conditions, it is difficult to compare the bioavailability of iron from the different products. However, it seems apparent that the temperatures used in the extrusion processing of cereals and legumes may play a role in determining the effects of extrusion on iron dialyzability. The dialyzable iron in legume flours extruded at a lower temperature 110°C (230°F) was higher than those extruded at a higher temperature 135°C (275°F) or the non-extruded legume flours. In comparison to raw durum wheat, durum wheat cereals extruded at 50°C (122°F) and 96° (205°F) had higher amounts of dialyzable iron. In contrast, durum wheat pasta extruded at 205°C (401°F) had lower dialyzable iron than raw or boiled durum wheat flour.

The negative effects of high temperature extrusion processing of durum wheat on iron bioavailability were also apparent in an in vivo (rat model) assay. Rats fed a diet containing durum wheat pasta extruded at 205°C had lower hemoglobin regeneration efficiency than rats fed diets containing non-extruded durum wheat or a standard source of iron, ferrous sulfate.

Both in vitro and in vivo assays indicate that navy bean supplementation had a beneficial effect on iron availability of extruded durum wheat. Pasta made from durum wheat flour (85%) and navy bean flour (15%) had higher dialyzable iron than durum wheat (100%) pasta. Hemoglobin regeneration efficiency in rats fed the wheat/bean pasta diet was higher than rats fed the wheat pasta diet.

The mechanisms involved in the effects of extrusion processing on iron availability were studied. The role of phytate and tannins, two known inhibitors of iron availability, was investigated. Although extrusion resulted in extensive degradation of phytic acid and a two-fold decrease in tannin content of legume flours, these factors could not explain the changes in iron dialyzability seen in the extruded products. A more complex system involving the interactions between protein, phytate and iron was hypothesized as a mechanism by which extrusion exerts its effects on iron dialyzability/bioavailability.

The effects of extrusion processing on protein-phytate-iron interactions was studied in durum wheat semolina extruded at 50°C and 96°C. Extrusion caused an increase in the insoluble residue fraction of durum wheat protein at both experimental temperatures. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns indicate an increased occurrence of disulfide bonds between polypeptides of the insoluble fraction of extruded compared to non-extruded durum wheat. The formation of disulfide linkages during extrusion processing may be responsible for the increased insolubility of extruded durum wheat protein.

Extrusion of durum wheat at mild temperatures (50°C and 96°C) increased the amount of iron and phytate associated with the soluble proteins of wheat, i.e. albumins and globulins. Extrusion at these temperatures also seemed to promote the formation of high molecular weight protein-iron-phytate and protein-iron complexes. Using an iron specific stain, an iron binding polypeptide with an estimated molecular weight of 16,000 Da was identified in the albumin, globulin, and insoluble fractions of durum wheat protein. It is expected that the iron bound to the albumin and globulin fractions of durum wheat is likely to be bioavailable because of its soluble, low molecular weight form. Hence, there was an increase in iron dialyzability of low temperature extruded durum wheat products.

The effects of extrusion on iron bioavailability may be small and of minor importance to populations consuming extruded foods only as a small part of a healthy, balanced diet. However, these effects may be significant to populations consuming extruded products alone as their diet, eg. infant foods and weaning foods. In the production of extruded foods such as breakfast cereals which are used widely to meet the daily requirements of iron, it is imperative that the extrusion conditions used be targeted not just towards the production of a high quality product but also be conducive for iron availability.

Although other components of foods, e.g. carbohydrates, fiber, lignins etc., may also influence iron bioavailability, the associations between protein, phytate and iron seem to be a potential mechanism affecting iron bioavailability in extruded cereal and legume foods. The literature offers numerous studies dealing with the protein, phytate and iron interactions in soy protein based products (Abdul-Kadir, 1980; Reddy et al., 1982; Erdman, 1981; Rodriguez et al., 1985; Lynch et al., 1994). Unlike soybean protein, the interactions between protein, phytate and iron in other legumes or cereals or their isolated protein fractions have not been studied and need consideration.

Due to the significance of dietary fiber and complex carbohydrates in nutrition and health, an increase in the consumption of legumes is being promoted (Morrow, 1991). It would prove beneficial to expand the uses of extrusion processing to include cereal foods supplemented with various legumes.

RECOMMENDATIONS FOR FUTURE RESEARCH

1. Study protein-iron-phytate interactions in legumes extruded under a wide range of temperatures.
2. Determine the reason for the protective effect of legume supplementation of durum wheat flour on the negative effects of high temperature extrusion processing on iron availability.
3. Evaluate the feasibility and consumer acceptance of an extruded legume supplemented durum wheat product.
4. Identify and develop a procedure to quantify protein-iron and protein-iron-phytate complexes in foods.
5. Study the effects of extrusion processing on zinc bioavailability in infant foods.

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