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Nutritional characteristics of hydrothermally cooked soymilk

Makuba Aime Lihono
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**Nutritional characteristics of hydrothermally
cooked soymilk**

by

Makuba Aime Lihono

**A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY**

Major: Nutrition

Major Professor: Robert E. Serfass

Iowa State University

Ames, Iowa

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For the ~~Graduate~~ College

To my son Noah Nlandu

To my mother Walengi Mboyo Emerance

To my late father Lihono Alipi Iphride

*To my family, the Lihono's, Jack, Diane, Didier, Lyly and
Angile*

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ABSTRACT

Hydrothermally cooked (HTC) soymilk (cooked at 154°C for 20-40 seconds) was reported to have more desirable physico-chemical properties than soymilk prepared in the conventional manner (100°C for 60 minutes) and to present acceptable decreases in activities of antinutritional factors.

HTC and conventionally processed soymilks were compared with respect to protein efficiency ratio (PER) and zinc bioavailability in Sprague-Dawley rats fed isocaloric, isonitrogenous diets. When dietary zinc was 50 mg/kg, PER (mean \pm SD, n = 12) was greater for HTC (processed for 20 sec) (2.69 ± 0.34) than for conventional soymilk (2.39 ± 0.17). When dietary zinc was 20 mg/kg, PER (n = 10) was less for HTC processed for 40 sec (1.86 ± 0.17) than for conventional soymilk (2.08 ± 0.19). Processing (HTC versus conventional) did not have a significant effect on zinc bioavailability by the slope ratio bioassay procedure. PER of HTC processed for 20 sec is better than that of reference casein and conventional soymilk if dietary zinc is near recommended levels. Pancreatic hypertrophy was not observed in rats fed spray-dried HTC processed soymilk.

Effects of microbial phytase on the bioavailability of calcium from calcium citrate malate (CCM) added to corn/soy diets were investigated in chickens. Reference diets contained

calcium as the carbonate. No statistically significant effect of phytase on the bioavailability of calcium from CCM was found, as shown by probabilities greater than 0.17 for weight gain, feed intake, tibia/body weight, ash%, and ash Ca% for corn/soybean meal and corn/hydrothermally cooked soymilk diets. For the corn/hydrothermally cooked soymilk diet with calcium at 0.76% of the weight of the diet as the carbonate, statistically significant ($P < 0.05$) decreases in the means for weight gain, feed intake, tibia/body weight, and ash% were observed in the group not treated with phytase. We suggest that CCM is less amenable to the formation of calcium phytate complexes than are other calcium salts and, therefore, should be preferred for fortification of soymilk products.

GENERAL INTRODUCTION

Soybean seed is an important source of high quality vegetable protein. It offers nutritional advantages to humans due to its lack of cholesterol, and the relative abundance of unsaturated fats and fiber. About 80-90% of the fat is unsaturated, mostly polyunsaturated (USDA, 1986; Snyder and Kwon, 1987). Soybeans are ground in water and the mixture heated to produce soymilk. Soymilk, a widely known beverage from soybean, contains as much protein (3.5-4.0%) as cow's milk does (Lee, 1995). Soymilk and other soy products may provide more than enough protein of high quality to the increasing number of vegetarians.

Soymilk presents an amino acid pattern similar to that of human milk and cow's milk (Liener, 1978). The sulfur containing amino acids, methionine and cystine, are considered to be the limiting amino acids in rat assays for soy protein products. Soymilk contains substantial amounts of minerals such as phosphorus, calcium, zinc, iron, copper, and sodium (Waggle and Kolar, 1979). Unprocessed soybeans contain antinutritional factors such as trypsin inhibitors, lectins, isoflavones, stachyose, raffinose, and phytate (Liener and Kakade, 1980). Because appropriate cooking or processing of soybeans eliminates or inactivates most of these unwanted substances, their presence in raw soybeans has less

significance when cooked soybeans or soy products are consumed as part of mixed diets (Harry, 1987).

Phytates are salts of phytic acid. Phytic acid is chemically designated myoinositol 1,2,3,4,5,6- hexakis (dihydrogen phosphate). Phytic acid can be classified as a nutrient source because it contains phosphorus (Chapman et al., 1955; Reddy et al. 1982). It can be considered antinutritional because it binds various essential divalent cations and decreases their availability for absorption from the diet (Cheryan, 1980; Cosgrove, 1966; Maga, 1982; Erdman, and Poneros-Schneider, 1989) and digestive juices (Savage et al., 1964; Davies and Nightingale, 1975).

In vitro studies of binding of phytic acid (with single minerals at pH 7.4 indicated the following decreasing order of stability: Cu (II) > Zn (II) > Co (II) > Mn (II) > Fe (III) > Ca (II) (Vohra et al., 1965; Oberleas, 1983; Wise, 1983). Maddaiah and others (1964) proposed an order of stability where zinc phytate was the most stable complex of all the phytate complexes with metals.

Despite the low relative binding of calcium to phytic acid, the formation of calcium phytate is of great importance since calcium is the dominant divalent mineral in most diets (Wise, 1983). Calcium and magnesium respond similarly to phytate. Calcium phytate coprecipitates with magnesium to form phytin that binds the trace metals cadmium, copper, lead and

zinc (Wise, 1983).

The binding of minerals by the phytic acid may depend on the pH of the food systems or in the GI tract. Researchers have shown that at neutral pH phytic acid is negatively charged resulting in the phosphate groups associating loosely or binding chemically with a variety of food components (De Rham and Jost, 1979). At low pH, half of the protons of the phytic acid are dissociated and since proteins are strongly positively charged, the formation of new phytate-protein complexes is favored (Cheryan, 1980; Reddy et al., 1982). It is assumed in stomach acid that most phytate is either soluble, or, more likely, complexed with protein (Wise, 1983). In the upper duodenum, the acid is neutralized leading to a maximum precipitation of Zn phytate and Zn-Ca-phytates complexes (Oberleas, 1983; Reddy et al., 1982).

Enzymes that catalyze hydrolysis of phytate to inositol and inorganic phosphate are called phytases. Microbial phytases have been shown to be effective in decreasing phytate content in animal feeds (Simmons et al., 1990; Edwards, 1993; Roberson and Edwards, 1994; Biehl et al., 1995). The possible chelating effect of phytate on the bioavailability of minerals in soybean products has led to studies on elimination or reduction of phytates in soybean products for human use. Anion-exchange resin (Smith and Rackis, 1957), acid and salt-disruption of phytate-protein bond (Ford et al., 1978; De Rham

and Jost, 1979) and ultrafiltration (Omosaiye and Cheryan, 1979) have been used to decrease phytates in soybean protein isolates. Enzymatic treatment using microbial phytase in human soy products has not been reported.

The characteristic off-flavor of conventionally prepared soymilk, the low bioavailability of minerals and the existence of antinutritional factors have limited the nutritional importance of soybeans for humans. Several methods have been used to reduce the beany flavor in soymilk: grinding the beans in boiling water (Wilkins et al., 1967), blanching (Nelson et al., 1967), lactic acid fermentation and the addition of fructose (Mital and Steinkraus) or skimmed cow's milk (Chien and Snyder, 1983). But none of these methods has proven to be low in cost and high in yield. A rapid-hydration hydrothermal method was developed (Johnson, 1978) to produce soymilk with improved qualities. This short-time (20-40 seconds) and high-temperature (154°C) cooking produces hydrothermally cooked (HTC) soymilk with increased yields of solids that possess remarkable dispersive properties, as well as with adequate inactivation of trypsin inhibitors and lipoxygenase activity (Johnson, et al. 1981; Kim, 1988). HTC soymilk presents less chemical browning and less off-flavor than the conventional soymilk (Kim, 1988).

Although sensory, functional, physico-chemical, and organoleptic properties of HTC soymilk have been studied, in

vivo nutritional characteristics have not yet been investigated. The purpose of the study reported here was to do additional chemical analyses and to perform *in vivo* characterization of HTC soymilk. Analytical determinations included indigestible oligosaccharides (sucrose, stachyose), phytate, minerals (Ca, Mg, P, Zn, Cu, Fe). Protein quality as protein efficiency ratio (PER), pancreatic hypertrophy, and Zn bioavailability of HTC soymilk at low and recommended Zn concentrations have been determined in a rat feeding study.

Studies (Lonnerdal et al., 1988; Zhou et al., 1992) have shown that the reduction of phytic acid in soybean products improved zinc bioavailability to monkeys and rats. Similarly, the reduction of phytic acid using microbial phytase could increase the bioavailability of added calcium in soymilk. Thus, bioavailability of calcium added to microbial phytase-treated, hydrothermally cooked soymilk was determined by a bioassay procedure in broiler chicks.

Dissertation organization

This dissertation begins with a General Introduction followed by a Literature Review. The main component of the dissertation consists of two papers written in the format required by the Journal of Food Science. The first one titled "Hydrothermal Cooking Affects Protein Efficiency Ratio and

Zinc Bioavailability of Soymilk-Based Diets in Rats" was published in the Journal of Food Science, year 1996, volume 61, number 5, pages 1043 to 1074. The second paper titled "Bioavailability of Calcium Citrate Malate Added to Microbial Phytase-Treated, Hydrothermally Cooked Soymilk" has been submitted to the same journal for publication. The dissertation concludes with general conclusions and acknowledgements. References are listed at the end of the chapter in which they are cited.

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LITERATURE REVIEW

Chemical composition of soybean seed and soymilk

As the number of vegetarians grows, the interest for soybean products increases in the United States. Nutritional attributes of soybean and soy products include their high quality protein, their abundance of unsaturated fats and fiber and their lack of cholesterol. The common way to process soybeans is to grind the beans with water and to extract a beverage called soymilk. Soymilk has been of considerable interest for its use as a substitute for cow's milk to feed infants who are allergic to animal milks. Other processed products from soybeans are soyflour, soy protein concentrate and isolated soy protein containing about 50, 60, and 90% protein, respectively. Applications of soy protein isolate as the sole source of protein include the preparation of infant formulas and other enteral nutrition products.

Proximate composition and vitamins

Proximate chemical composition of soybeans can vary according to the variety and the growing conditions. Average figures indicate 40% protein, 20% lipid, 35% carbohydrates and 5% ash on a dry basis (Snyder and Kwon, 1987; USDA, 1986). Soybeans contain all the essential amino acids. They are low

in sulfur containing amino acids, especially methionine, but high in lysine. About 80% of fatty acids are unsaturated, mostly polyunsaturated (Snyder and Kwon, 1987; USDA, 1987). Linoleic acid is the most abundant fatty acid (about 50% of the total fatty acids). Other predominant fatty acids are palmitic acid, oleic acid, and linoleic acid. The nontriglyceride fraction in crude soy oil is characterized by a high content of phospholipids (1-3%). Soybeans have high amounts of potassium, phosphorus, magnesium, and sodium. Also, they contain substantial amounts of calcium, iron, zinc, and copper. Among vitamins, folacin, vitamin A, and ascorbic acid are the most represented in soybeans.

The composition of soymilk is a function of its water content (90-95%). Soymilk has in average 3.5-4% protein, 2-3% carbohydrates, 2% lipids. The relative amount of amino acids, fatty acids, and minerals in soymilk correspond to their relative abundance in raw soybeans.

"Non-nutritional" constituents

Trypsin inhibitors.

Trypsin inhibitors are protease inhibitors that include two main types: 1) the Kunitz trypsin inhibitors, which possess molecular weights of about 20,000 with 2 disulfide bridges and are specific against trypsin and 2) the Bowman-Birk inhibitors which have molecular weights of only 6,000 to

10,000 with a high proportion of disulfide bonds and the potentiality of inhibiting chymotrypsin and trypsin. There are other types which are variants of these two main ones (Liener, 1994).

Trypsin inhibitors constitute at least 6% of the protein of soybeans (Ryan, 1973) and are known to have antinutritional effects (Gallanger and Schneeman, 1986). These effects include growth depression, and pancreatic hypertrophy (Chernick et al., 1948; Yanatori and Fujita, 1976). Feeding of raw soybeans to rats (Klose et al., 1946), chicks (Ham et al., 1945), mice (Westfall and Hauge, 1948), and young guinea pigs has demonstrated the existence of the effects mentioned above. On the contrary, no enlargement of the pancreas has been found in adult guinea pigs (Hasdai et al. 1989), dogs (Schneeman and Gallaher, 1986), adult pigs (Struthers et al., 1963), calves (Kakade et al., 1976), humans (Gallanger and Schneeman, 1986) and primates. Studies in the latter species lasted 5 years (Harwood et al., 1985).

Pancreatic hypertrophy and the consequent increase in proteolytic enzyme concentrations in the intestinal lumen caused by trypsin inhibitors are hormone-mediated responses. The sequence of events is: 1) Trypsin inhibitor binds trypsin in the gut; 2) the activity of the trypsin is depressed; 3) cholecystinin pancreozymin (CCK) secretion increases; 4) pancreas responds accordingly by producing more enzyme (Green

and Lyman, 1972). Because pancreatic enzymes are rich in sulfur-containing amino acids, it has been hypothesized that growth depression is the result of the pancreas diverting sulfur-containing amino acids from the synthesis of body tissue protein to the synthesis of pancreatic enzymes (Nitsan and Liener, 1976; Lyman and Lepkovsky, 1957; Booth et al., 1960).

Trypsin inhibitors do not present any substantial risk because they are inactivated by moist heat routinely used in the processing of soybean foods (Snyder and Kwon, 1987). Most commercially available edible grade soybean products retain 5 to 20% of the original raw soybean trypsin inhibitor activity (Rackis and Gumbmann, 1982; Dipietro and Liener, 1989; Churella et al., 1976). Manufacturers cannot destroy all the trypsin inhibitor activity because they try to achieve a balance between the amount of heat necessary to destroy the trypsin inhibitors and that which may result in damage to the nutritional or functional properties of the protein (Rackis and Gumbmann, 1982). Soymilk processed by hydrothermal cooking at 154°C for 40 seconds was reported to possess 7.6% residual trypsin activity; equivalent to that of soymilk prepared by conventional cooking at 99°C for 60 minutes (Johnson et al., 1980).

Flatulence-producing factors

Oligosaccharides such as stachyose and raffinose are believed to cause flatulence, namely the production of gas such as carbon dioxide, hydrogen, and to a lesser extent, methane, in the gastrointestinal tract (Liener, 1980; Rackis et al., 1970). The absence in the human intestinal mucosa of enzymes that hydrolyze 1,6- galactosidic linkages (Gitzelman and Auricchio, 1965) results in the accumulation of intact oligosaccharides in the lower intestine. Fermentation of these oligosaccharides by anaerobic bacteria produces gases responsible for flatulence (Rockland et al., 1969; Rackis et al., 1970).

Rackis et al. (1970, 1981) showed in human subjects that whey solids from soyflour had the highest flatus activity of soybean products. Full-fat soyflour and soy protein concentrate gave rise to much less gas, and soy isolate was devoid of flatus activity. Traditional soybean foods such as tofu and tempeh present little flatus activity (Calloway et al., 1971). Ku et al. (1976) reported a significant reduction of flatulence-related oligosaccharides when soybeans were cooked in water.

Lectins

Lectins are carbohydrate-binding proteins or glycoproteins. Kocourek and Horesji (1981) wrote: "They are of

non-immunoglobulin nature and are capable of specific recognition of and reversible binding to carbohydrate moieties of complex glycoconjugates without altering the covalent structure of any of the recognized glycosyl ligands".

Liener (1953) demonstrated the ability of soybean lectins from raw soybeans to inhibit the growth of rats. He showed that this growth inhibition was about 25% of the total growth inhibition caused by feeding of raw soybeans. Some researchers (Greer et al., 1985; De Oliveira et al., 1988; Grant et al., 1988) noticed that soybean lectins caused rapid and progressive increases in the weight and length of the small intestine. This hypertrophy greatly restricts the amount of nutrient available to the animal to maintain and facilitate growth of other body tissues (Liener, 1986). It is believed about 60% of the dietary content of lectins reaches the intestine where it binds to the epithelium causing disruption of the brush border (Pusztai et al., 1990), atrophy of the microvilli (Jindal et al., 1984) and decreased viability of epithelial cells (Ishiguro et al., 1992). Lectins are able to agglutinate the red blood cells from various species of animals because of the interaction of multiple binding sites on the lectin molecule with specific glycoconjugate receptors on the surface of the erythrocyte membrane (Liener, 1994). Other possible effects of lectins are: 1) Inhibition of disaccharidases and proteases in the intestines; 2) pathologic

changes in liver, kidney and pancreas; 3) interference with absorption of non-heme iron and lipids.

Lectins are destroyed by moist heat treatment but are resistant to dry heat (Liener, 1994). No active lectins were found in soy protein meat substitutes, low levels of active lectins were present in milk substitutes and bakery products, and relatively high levels of lectins were detected in a cereal-type food and a cookie made from soyflour (Klurfeld and Kritchevsky, 1987). It is highly improbable that lectins present in many processed soy products pose any risk for human health (Liener, 1994).

Goitrogens

Enlargement of the thyroid gland occurs when unheated soybeans are fed in rats (Sharpless et al., 1939) and chicks (Patton et al., 1939; Wilgus et al., 1941). Rare cases of goiter have been found in human infants fed soymilk formula (Van wyk et al., 1959; Hydowitz, 1960; Shepard et al., 1960). Heat treatment of soybeans and the administration of iodine respectively have been shown to partially (Wilgus et al., 1941) and totally eliminate (Halverson et al., 1949; Block et al., 1961) the goitrogenic effect.

Goitrogenic substances in soybeans have not been identified with certainty, although one has been reported to be soyasapogenol (Suwa and Kimura, 1981). A second report

suggests an oligopeptide of low molecular weight (Konijn et al., 1972; Konijn et al., 1973).

Goitrogenic activity has been detected in the curd of tofu (Suwa et al., 1979) and to a lesser extent in toasted soyflours, concentrates, and isolates (Fillisetti and Lajolo, 1980).

Saponins

Saponins are glycosides containing a steroid triterpenoid aglycone (sapogenins). They are characterized by one or more of the properties including hemolytic activity, cholesterol-binding properties, and bitterness (Price et al., 1987; Liener, 1994). Sapogenins (soyasapogenols) identified in soybeans are linked to any of the following sugars: galactose, arabinose, rhamnose, glucose, xylose, and glucuronic acid (Liener, 1994). Because saponins are thermostable (Birk et al., 1963), toasted soybeans contain the same amount of saponins (about 0.5%) as whole soybeans do (Fenwick and Oakenfull, 1981). Using a thin-layer chromatography method, Fenwick and Oakenfull reported saponin content of 0.3 to 2.5% for four commercial soy protein isolates. The method of processing affects the saponin content of processed soybean products (Fenwick and Oakenfull, 1981). Fermentation and the processing of protein concentrate by washing to remove soluble oligosaccharides reduce saponin content. Processed products

with low moisture content contain high level of saponins.

Ishaaya et al. (1969) fed chicks, rats, and mice saponin levels three times greater than those found in soybeans and did not detect any deleterious effects. Also, studies in rats have indicated that soybean saponins lowered the blood and cholesterol levels (Oakenfull et al., 1984; Sidhu et al., 1987). Liener (1994) suggested that saponins in soybeans should not be regarded as antinutritional factors.

Lipoxygenases

Lipoxygenases are enzymes that catalyze the oxidation of lipids containing a *cis, cis* 1,4-pentadiene structure to produce hydroperoxides. Lipid hydroperoxides are decomposed to compounds such as aldehydes, alcohols, and ketones, which may be responsible for off-flavors (Sessa and Rackis, 1977). Lipid hydroperoxides can also lead to loss of nutritive value by destroying some vitamins. Soybeans possess three isoenzymes of lipoxygenase designated L-1, L-2, and L-3 with pH optima respectively of 8.3, 6.5, and 6.5 (Christopher et al., 1970). Linoleic acid and structurally related fatty acids are substrates for both L-1 and L-2. In the dry soybean, enzyme-substrate contact is limited by substrate immobility and for this reason lipoxygenase is inactive. Upon hydration, enzyme and substrate gain mobility and oxidation accelerates.

Isoflavones

Isoflavones are glucosides with estrogenic activity. Soybeans, soybean products, and isolated soybean isoflavones have been shown to cause estrogenic responses in experimental animals (Matrone et al., 1956; Drane et al., 1980; Farmakalidis and Murphy, 1984). Estrogens are capable of stimulating the growth of the vagina, uterus, and mammary gland and the development of female secondary sex characteristics (Liener, 1994).

About 65%, 25%, and 10% of the isoflavone content of soybean products is present as genistin, diadzin, and glycitein-7-o-glucoside, respectively. The amount of coumesterol is negligible. Compared with unprocessed soybeans, high-protein soy ingredients such as soyflour and texturized vegetable protein contain slightly lower isoflavone concentrations (total isoflavones approximately 1.1-1.4 mg/g) (Xia, 1996). In general, there is a reduction of the amount of isoflavones during processing.

In vivo, genistin and daidzin are roughly 10^5 times less potent than estrogens as shown by mice uterine enlargement assays (Bickoff et al., 1962). Isoflavones may be able to reduce biosynthesis of estrogens (Adlercreutz et al., 1992). Other effects of isoflavones are antioxidant activity and anticarcinogenic effects. Naim et al. (1976) reported that isoflavones inhibited lipoxigenase action and prevented

peroxidative hemolysis of sheep erythrocytes *in vitro*.

Anticarcinogenic activities of soy isoflavones are probably due to their profound impact on control of cell growth and proliferation of tumor cells (Xia, 1996).

Estrogenic effects of isoflavones have been demonstrated when isolated forms of isoflavones have been used at high levels in animals. The same estrogenic effects can be seen in humans only if soybeans constitute the sole component of diet. But, Verdeal and Ryan (1979) indicated that the levels of soy isoflavones that humans are exposed to is far lower than that required for a physiological response.

Nutritional characteristics of soymilk and soybean products

Protein quality

Biological quality of soybean proteins is higher than that of cereal proteins and a little less than that of most animal proteins. The pattern of soybean essential amino acids when compared to the pattern for whole egg gives a chemical score of 70%, cysteine and methionine being limiting. It is interesting to note that this method of evaluating protein presents some shortcomings when evaluating soybean protein (Snyder and Kwon, 1987): 1) Whole egg protein is particularly rich in sulfur amino acids with respect to human requirements and ; 2) the emphasis on limiting amino acids obscures the

very positive aspect of the high lysine content of soybean protein.

The *in vivo* quality of proteins in rats/chicks is based on protein efficiency ratio (PER), digestibility, biological value (BV), and net protein utilization (NPU) assessment. As soybean products are processed using heat, PER increases with time until heating results in loss of available lysine and sulfur amino acids through non-enzymatic browning reactions (Snyder and Kwon, 1987). Hackler et al. (1965) reported that cooking soymilk 1-6 hours at 93°C had no adverse effect on PER, growth, or available lysine. But with cooking for 32 minutes at 121°C, there was a definite decline in PER and in available lysine. This decrease in lysine was higher when soymilk was heated for 40 minutes at 121°C.

Studies have indicated that supplementation of soybean products with methionine, sometimes combined with other amino acids, improves PER and NPU greatly. Sarwar et al. (1993) improved the relative protein efficiency ratio (RPER) and the relative net protein ratio (RNPR) values (casein + methionine = 100) of diets containing a soy-based infant formula. Both RPER (initial value 71-81) and RNPR (initial value 78-85) were increased to 100 by supplementing formula diets with lysine (0.2%), methionine (0.2%), threonine (0.1%), and tryptophan (0.05%). Emmert and Baker (1995) found that the PER in chicks of soybean meal was higher than that of soy protein

concentrate and soy protein isolate. Supplementation with methionine and threonine increased the PER of all three. Mori et al. (1993) added methionine at 0.3% to 0.7% in a diet containing 10% soy protein isolate and noticed an improvement in food efficiency and NPU in rats.

Watanabe et al. (1971), after conducting digestibility tests of soybean proteins from several soybean food products, reported digestibility values from 65 to 93 %. Soy curd and soymilk film had the highest digestibilities. Bressani (1981) found that digestibilities in adult humans of soybean protein products were similar to that of milk, beef and casein. Soy protein concentrates and isolates had higher digestibility than soyflours.

Nitrogen balance studies using soybean products have been carried out in humans. Feeding infants, Fomon and Ziegler (1979) reported that methionine-supplemented soy isolate formulas were as efficient as milk-based formulas in promoting retention of nitrogen and growth in infants. Furthermore, infants utilized less efficiently soy protein as indicated by body weight in the absence of methionine supplementation. Torun (1979) found that feeding soy protein isolate at 1 g/kg body weight per day in preschool-age children resulted in normal growth and nitrogen retention. Also, he noticed that the soy protein isolate without methionine was not different from cow's milk when it came to nitrogen retention,

absorption, and balance. In feeding trials of soy protein isolate in young men, Scrimshaw and Young (1979) concluded that methionine supplementation improved nitrogen balance when low levels of soy protein were used and when the subjects were in negative nitrogen balance. Methionine supplementation did not result in any improvement at a level of soy protein that supported positive nitrogen balance. Torun et al. (1981) proposed that methionine supplementation in soybean protein in human diets may be justifiable only when total dietary protein intake is marginal.

Fatty acid profiles

Linoleic acid, an essential fatty acid for humans, accounts for about 50% of the total fatty acids (about 90% of the total polyunsaturated fatty acids of soy lipid (USDA, 1986). Polyunsaturated fatty acids are known to lower plasma cholesterol in humans (Mattson and Grundy, 1985). About 60% of the total soy fatty acids is polyunsaturated which consists mainly of linoleic and linolenic acids (USDA, 1986). Oleic acid has as much hypocholesterolemic property as linoleic acid does (Mattson and Grundy, 1985). Oleic acid accounts for about 25% of the total fatty acids in soy lipid (USDA, 1986).

Mineral bioavailability - zinc, calcium, iron, magnesium*Zinc*

Studies (Forbes et al., 1979; Erdman et al., 1980; Forbes and Parker, 1977) have shown that the relative bioavailability of zinc from soy products in rats is low compared with the availability of added zinc carbonate. Results suggest that bioavailabilities of zinc from isolates and protein concentrates are lower than those from soyflour and soy beverage; the acidic forms of isolates and concentrates presenting a better bioavailability than the neutralized forms. Zinc bioavailabilities of defatted soybean meal and soy protein isolate are moderate and low, respectively, compared with cow's milk protein in chicks (Rackis and Anderson, 1977).

Lönnerdal et al. (1984) studied the effect of phytate on zinc bioavailability from soy formula and cow's milk formula using meals extrinsically labeled with ⁶⁵Zn. Results in human adults suggested that phytate inhibited zinc absorption. The zinc absorption was reduced by half when soyflour formula having two times the phytate content of soy isolate was used. Zinc from cow's milk was absorbed twice as well as zinc from soy isolate formula. In another study (Lönnerdal et al. 1988), absorption of zinc from dephytinized soy formula has been found to be 45% and 47% in rat and monkey, respectively, compared with values of 27% and 16% for conventional soy formula.

Calcium

Harrison and Mellanby (1939) produced rickets in dogs by adding phytic acid to dog diets. They increased dietary calcium and vitamin D and eliminated rickets. That was the first demonstration of the adverse effect of phytic acid on calcium bioavailability. Work by Weingartner (1981) indicated that the bioavailability of exogenous calcium (as calcium carbonate) in soyflour, soy concentrate, and soy beverage was as high as that of the same calcium added to casein. Rats were fed control casein or soy diets *ad libitum* for either 6 weeks (experiment 1) or 4 weeks (experiment 2 and 3). In experiment 1, diets containing 0.0, 0.2, 0.4, or 0.65 calcium were made by addition of calcium carbonate to casein or soy basal diets. All diets contained 0.4% phosphorus; the calcium to phosphorus ratios were between 0 and 1.5. In the second experiment, diets were made to provide 0.08%, 0.18%, 0.28%, or 0.68% total dietary calcium with added calcium carbonate. In the third experiment, whole soybean flour, dehulled soybean flour, or dehulled soybean flour plus added fiber (2% solka floc) basal diet containing 0.08, 0.18, or 0.28% calcium were compared to casein control with the same levels of calcium. Femur or tibia calcium content was related to dietary calcium concentration in experiment 1. Results showed no differences in added calcium carbonate among the different soy diets and the casein control. In experiment 2, femur calcium content of rats fed

soy diets at 0.08% (calcium supplied entirely by soy) was not different from that of rats fed casein diet at the same calcium level (from calcium carbonate). Results of experiment 3 did not show any difference in bioavailability between the casein control and whole soybean flour, dehulled soybean flour, or dehulled soybean flour plus added fiber. It was supposed that phytic acid as well as fiber do not affect negatively the bioavailability of calcium in soy products. However, it is questionable if results of experiment 2 based on the level of calcium at 0.08% (less than 20% of the NRC of rats) could justify this assumption.

Heaney et al. (1991) compared the absorption of calcium from boiled soybeans low or high in phytate with that from labeled 2% milk in 15 healthy premenopausal women. The soybeans were grown hydroponically and intrinsically labeled with ⁴⁵calcium. Test meals were consumed as breakfast and the average test load of calcium for all three sources was 2.45 mmol. Fractional calcium absorption from the high-phytate soybeans averaged 0.310 ± 0.070 ; from the low-phytate soybeans, 0.414 ± 0.074 ; and from milk, 0.377 ± 0.056 . Results showed that phytate content significantly affected calcium absorption from soybeans.

Iron

Bioavailability of iron from soy products is about the same as that of iron from other plant sources (Erdman and Forbes, 1981). Experiments in rats (Steinke and Hopkins, 1978) showed soy isolates having a relative iron bioavailability of 61% for endogenous iron when compared to ferrous sulfate. Endogenous soybean iron from full fat soyflour and neutralized soy concentrate restored the level of hemoglobin in anemic rats as much as did ferrous sulfate (Erdman and Forbes, 1981). Studies in adult humans (Lynch et al., 1994; Hurrell et al., 1992) using extrinsic radioiron labeled in liquid formula meals indicated that the reduction of phytate in soy protein isolates improved significantly the absorption of iron. It should be noted that phytic acid had to be reduced to less than 0.3 mg/g of isolate before a meaningful increase in iron absorption was observed (Hurrell, 1992). A negative effect of phytic acid on iron metabolism was seen in a study with infants (Davidsson et al., 1994). Soy formula with the native content of phytic acid was compared with a dephytinized formula when it comes to the incorporation of iron into the red blood cells, and the mean fractional iron incorporation improved significantly from 3.9% to 8.7% with dephytinization.

Welch and Van Campen (1975) found that intrinsic labeled iron in mature soybeans was more available than iron in immature soybeans even though mature soybeans contained three

times more phytic acid. Hurrell et al. (1992) discovered that, after removal of almost all the phytic acid, extrinsic iron absorption from the soy-protein isolate meal was only half that from the egg white control. This finding led the authors to think that there are factors other than phytic acid that affect iron bioavailability (Hurrell et al., 1992). Lynch et al. (1994) found that phytic acid and a protein-related moiety contained in the conglycinin (7s) fraction are the two main inhibitors of extrinsic iron absorption in soybean-protein isolates.

Magnesium

Forbes et al. (1979) and Lo et al. (1980) demonstrated that endogenous and exogenous magnesium ($MgCO_3$) from soy products is highly bioavailable (not significantly different from that of casein or lyophilized beef); soy concentrates showing poorer utilization of magnesium than soyflour, soy beverage, and soy isolates. Brink et al. (1991), using $MgSO_4 \cdot 7H_2O$, found that added magnesium at the same level used by Lo et al. (0.82 mmol/100g) decreased femur zinc content of rats fed soybean protein isolates (dietary magnesium from soy isolate was negligible) compared with those fed casein protein. This effect disappeared when the level of added magnesium increased to 1.64 mmol/100g (dietary magnesium requirement for rats). Research with chicks (Guenter and Sell, 1974) showed that

magnesium bioavailability in soybean meal is as high as in skim milk. The high bioavailability of magnesium coupled with the high magnesium content of most soy products makes them significant sources of this mineral.

Summary

When it comes to mineral bioavailability of soy products, it is important to make a difference between various soy products. Evidence suggest that minerals from soy isolates are poorly utilized compared with the other forms. Phytic acid accounts for most of the decreased bioavailability of minerals. Factors such as dietary fiber, oxalates, phenolic compounds, and protein-related moieties in the conglycinin fractions may affect mineral utilization. Also, there are indications that manufacturing chemical conditions and physiological factors influence the action of phytic acid. The phytate-protein complexes formed during processing are probably responsible for the variability of zinc utilization in diets containing isolates manufactured by different processes (Rackis and Anderson, 1977). The reduced bioavailability of zinc in neutralized as compared to acid-precipitated products may be a result of the formation of stable protein-phytic acid zinc complexes in the dried neutral product (Erdman and Forbes, 1981). As protein-phytic acid-mineral complexes are formed in solution at neutral pH,

tightly-bound complexes are favored during drying of soy protein; the lack of water creating conditions thermodynamically advantageous. Since calcium is the most prevalent cation in most diets, the formation of calcium-magnesium complexes with phytic acid (phytin) in the gastrointestinal tract is of great importance. Zinc in soybean is apparently not associated with phytate but, once, in the intestine, phytin binds it and decreases its availability (Ellis and Morris, 1981). Indications from *in vitro* studies (Oberleas, 1966) are that calcium enhanced the formation of phytate-zinc complexes at pH comparable to that of the small intestine. Forbes et al. (1983) concluded that the inhibitory effect of calcium in tofu on zinc bioavailability in high phytate diets is the result of the formation of insoluble complexes in the gastrointestinal tract and not the result of processing during tofu preparation. Another factor that may act on the phytic acid effectiveness is the nature of the amino acids or peptides originated by the hydrolysis of protein. Likuski and Forbes (1984) showed in chicks fed a mixture of amino acids as the sole source of nitrogen that addition of phytic acid at 1.8% decreased the availability of zinc to as great extent as when casein was fed. Hence, it appears that either protein or specific amino acids/peptides may act with phytic acid to chelate zinc or other minerals.

Soy milk vs. cow's milk-soy milk as milk substitute?

Because of its health benefits and nutritious profile, soy milk can be used as a basis for a substitute for infants who are allergic and intolerant to other protein sources. Important factors influencing the choice of nutritional substitute include nutritional composition, evidence of successful use with particular reference to satisfactory growth and nutritional status, palatability, ease of preparation, cost, availability and allergenicity (MacDonald, 1995).

Soy milk presents nutritional similarities and differences compared with cow's milk. Soy milk has about 3.5-4% protein, about the same amount as cow's milk. Soy milk protein is of high quality with an amino acid pattern similar to that of human and cow's milk, except methionine. Because of its vegetable origin, soy milk is without cholesterol. The high content of polyunsaturated fats and the presence of isoflavones in soy milk are desirable because they are related to hypocholesterolemic and anticarcinogenic activities, respectively. Soy milk has high content of phosphorus, magnesium, potassium, and sodium. If consumed often, soy milk may provide enough zinc, iron, and copper.

Although soy milk provides calcium, its calcium content is insignificant compared with that of cow's milk. Soybeans contain approximately 0.20% calcium, and a beverage consisting

of 6% soy solids would contain 3mM calcium. On the other hand, cow's milk contains about 25mM calcium and 2/3 of the total calcium is in colloidal complexes with phosphate, citrate, and protein components (Weingartner et al., 1983). One cup of soymilk contains about 50 mg calcium soymilk, 6 times lower than the 300 mg of calcium per cup of 2% cow's milk.

Fortification of soymilk to a calcium level comparable to that of cow's milk will certainly be of nutritional benefit. Weingartner et al. (1983) were able to fortify pasteurized or thermally processed soy beverage (6%) with 25 mM (or 30 mM) mixtures of calcium citrate and tricalcium phosphate. Addition of these calcium sources did not adversely affect protein stability after 10 days and 6 months for the pasteurized and the thermally processed soy beverage, respectively. Since tricalcium phosphate is virtually insoluble in cold water, none of the tricalcium phosphate remained in solution. Approximately 10 mM of calcium citrate remained in the top of beverage. Calcium citrate malate was found 10 times more soluble than calcium citrate in a solubility test in water at neutral pH (Heaney et al., 1990). In the same study, the bioavailability of calcium citrate malate expressed as fractional absorption in adult women was higher than that of calcium citrate and tricalcium phosphate.

There are commercially available palatable nutritionally complete soya protein isolate formulas. These formulas are

supplemented with L-methionine, taurine and carnitine. They are cheaper and more palatable than hydrolysate formulas from whey, casein, and beef (MacDonald, 1995). Still, there are concerns with respect to mineral bioavailability and allergenicity (MacDonald, 1995), mineral bioavailability being affected by the phytate content of soy formulas (Lönnerdal et al., 1984). Lönnerdal et al. (1988) proved that reduction of phytate content of soy formula improved zinc absorption. Phytate removal may enhance calcium bioavailability but calcium fortification may be more beneficial because of the low calcium content of soy protein.

Hydrothermally cooked soymilk vs. conventionally processed soymilk

The easiest and most common way to process soybeans to a high protein food is to grind the beans with water. The resulting beverage is referred to as soymilk. Traditional soymilk flavor has been characterized as beany, painty, bitter and rancid (Kim, 1988). Nutritional studies of soymilk have included the improvement of both the nutritional characteristics and the organoleptic properties of soymilk. Researchers have looked for ways to decrease the antinutritional factors, to increase the availability of the minerals especially calcium, and to reduce the beany flavor of

the soymilk.

One approach is to heat the soybeans before or during processing to destroy lipoxygenase. Wilkens et al. (1967) ground unsoaked and dehulled soybeans with hot water (80 - 100°C) to inactivate the lipoxygenase and to produce an acceptable bland soymilk.

A second approach is to use blanching to minimise development of undesirable flavors due to degradation and oxidation of lipids. Nelson et al. (1967) produced soymilk by blanching in 0.5% NaHCO₃ prior to the grinding of soaked beans. They found that this processing gave soymilk a mouth feel and colloidal stability. Moreover, this processing resulted in a bland flavored product having reduced off-flavors, lipoxygenase and trypsin inhibitor activities.

A third approach is the use of fermentation to modify and improve flavor. Wang et al. (1974) reported that the beany flavor was masked by fermentation with *Lactobacillus acidophilus*. Mital and Steinkraus (1976) using defatted soyflour to which they added 2% sucrose and 2.5% refined soy oil, produced soymilk with flavor acceptability inferior compared with that of cow's milk. Soymilk from the defatted flour fermented with *Streptococcus thermophilus* has a satisfactory gelatinous curd but its flavor was still inferior to cow's milk fermented with the same organism.

None of these approaches has produced soymilk at low cost

and with efficient recoveries of solids and protein. A rapid-hydration hydrothermal method was developed (Johnson, 1978) to improve the quality of the soymilk. At 154°C for 34 seconds, this steam-infusion processing produced soymilk with yields of approximately 90% protein and 86% solids from whole soybean flour (Johnson et al., 1980). Hydrothermal cooking involves: 1) Dry grinding dehulled soybeans to form a flour; 2) forming a slurry of soyflour in water; 3) heating the slurry by injecting steam under pressure; 4) cooling the slurry (Kim, 1988).

Kim (1988) studied the physico-chemical and flavor properties of hydrothermally cooked (HTC) soymilk processed at 154°C and compared them with those of conventionally cooked soymilk processed at 97-100°C for 60 minutes. Following findings were reported:

- HTC soymilk was stable and had higher yields of solids and protein, and higher viscosity
- At equivalent residual trypsin inhibitor activities, HTC soymilk presented more available lysine, less chemical browning and higher protein digestibility
- HTC soymilk had lower level of oxidative degradation as measured by thiobarbituric acid (TBA) and hexanal contents.

Zinc bioavailability studies of soy products in rats

Zinc bioavailability of soy products

Momcilovic et al. (1975a, b), by demonstrating that the logarithm of total femur zinc is a linear function of dietary zinc for periods of two to three weeks, proved that bone zinc is a reliable measure of zinc bioavailability in slope ratio-type assays in weanling rats. Forbes et al. (1979) stated that bone zinc response is a more sensitive measure of zinc bioavailability than is weight gain because it is the sum of two effects of absorbed zinc: the promotion of increased tissue mass and the promotion of increased zinc concentration in the tissue. Moreover, bone zinc response is less susceptible to daily variation than is body weight.

Some researchers have speculated that bone zinc may serve in rats as a reserve for periods of low dietary zinc intake (Hurley and Swenerton, 1971; Brown et al., 1978). King (1996), while not supporting the idea of zinc stores, remarked that high zinc intakes may cause modest increases in bone, liver, and intestinal zinc concentrations (Emmert and Baker, 1995). In summary, zinc accumulates in bone during periods of high zinc intake and is redistributed from bones to other growing tissues during periods of marginal zinc intake.

It has been reported from rat experiments that bioavailability of zinc is poor from most soy protein products (Rackis and Anderson, 1977; Forbes et al. 1979; Momcilovic et

al. 1976; Forbes and Parker, 1977; Erdman et al., 1980; Erdman and Forbes, 1981). Momcilovic et al. (1976) used a slope ratio method with an egg-white basal diet (0.8 μg zinc/g) where zinc sulfate, milk and soy-protein based infant formulas (containing zinc sulfate) were added to provide diets with levels of 3, 6, 9, and 12 μg zinc/g. They found that the relative biological availabilities of endogenous plus added zinc sulphate in soy-based formulas were about 20% less than zinc in milk-based formula. Forbes and Parker (1977) used a 20% protein egg-white diet and reported that zinc added to the diet in the form of full-fat soyflour was utilized 34% efficiently as zinc carbonate. However, femur zinc responses to increasing levels of zinc carbonate were not affected by the presence of soyflour in the diet. Forbes et al. (1979) confirmed in 20% protein, basal egg-white diets that zinc was poorly available from soy products. Zinc added to the diet as soy beverage and soy concentrate was 40% and 20% utilized respectively compared with the egg white control. Exogenous zinc added to the soy concentrate was not fully available. In another experiment, where 30% protein egg white was employed, Erdman et al. (1980) compared the bioavailability of zinc to weanling rats from soy concentrates and isolates prepared under neutral or acid conditions. At about similar phytate-to-zinc molar ratios, the acidic products (concentrate and isolate) supported growth better than the neutral products. No

significant differences were found in the log of total zinc per tibia.

Erdman and Forbes (1981) summarized studies of the effects of soya protein and phytic acid on the bioavailability of zinc (Forbes et al., 1979; Erdman et al., 1980; Forbes and Parker, 1977). They wrote that the relative bioavailability of zinc from soy products was highly variable and often low compared with the bioavailability of zinc from zinc carbonate. Relative bioavailability from full-fat soyflour and soy beverage is higher than that from soy isolates and concentrates, acid forms of isolates and concentrates exhibiting bioavailabilities higher than those of neutral forms. Results from studies mentioned above (Forbes et al., 1979; Erdman et al., 1980; Forbes and Parker, 1977) also demonstrated that the presence of soybean products did not appear to affect the bioavailability of zinc as zinc carbonate added into rat diets, except for soy concentrates. Erdman and Forbes (1981) suggested that the reduced bioavailability of zinc in neutralized as compared with acid forms of soy products is likely to be the result of the formation of stable protein-phytic acid-zinc complexes at neutral pH in solution (De Rham and Jost, 1979), with subsequent tight binding of the complexes during drying of the soy protein.

Phytate-to-mineral molar ratio

Earlier, experiments by O'Dell and Savage (1960) supported the interpretation that phytic acid was involved in making zinc less available. They found, in contrast to a casein-gelatin diet containing the same amount of zinc, that growth of chickens fed soy protein basal diet was improved by addition of supplemental zinc. Addition of phytic acid to the casein-gelatin diet reduced growth to a level similar to that of soy basal diet. Chicks on casein-phytic acid overcame symptoms of zinc deficiency with supplementary zinc. Later, Oberleas et al. (1966) suggested that phytate played a physiological role in decreasing the bioavailability of zinc in rats. They also proved that high calcium acts synergistically with phytate in reducing zinc. In this study, there was no effect of calcium among groups fed 0.8% or 1.6% calcium without phytate. The addition of 1% phytate resulted in rats fed 0.8% calcium having body weight gain significantly higher compared with those fed 1.6% calcium; the body weight gain of the rats with low and high calcium being about 60% and 20% of the control group, respectively. Analysis of variance showed a highly significant interaction between calcium and phytate.

Several rat experiments have shown that phytate and calcium exert a synergistic inhibitory effect on zinc bioavailability. Forbes et al. (1983) compared the

bioavailability of zinc in tofus coagulated with CaSO_4 and MgCl_2 , to bioavailability of ZnCO_3 in an egg-white diet and found similar responses from both tofus. They explained these responses by the fact that these diets were designed in a way that phytate/zinc molar ratio and calcium and magnesium were to be equal (26-27, 0.7%, 0.1%), thus the adverse effect of the high calcium concentration in the Ca-tofu on zinc utilization was suppressed. In another experiment (Forbes et al., 1983), rats were fed diets at 9 mg zinc/kg from the two tofus and the egg-white. Three levels of calcium content at 0.4, 0.7, and 1.2% were fed. Rats fed tofus had similar tibia zinc values, values which were lower than those of egg-white. But within each tofu-diet, 0.7 and 1.2% calcium levels decreased the zinc content of the tibias. These results illustrated the negative effect of high calcium level in the utilization of zinc in phytate-containing diets with low zinc content. The authors concluded that the calcium/zinc ratio of the diet must be considered as well as the phytate/zinc ratio in evaluating dietary effects of zinc bioavailability.

The roles of calcium and phytic acid on the utilization of dietary zinc was recognized early in studies with semi-purified diets. Likuski and Forbes (1965) demonstrated that the addition of 2.0% phytic acid in semi-purified diets reduced weight gain, zinc absorption, and zinc in the femur ash when zinc supply was at the requirement level but not when

excess zinc was present. Also, these reductions were more prominent when level of calcium was increased to 0.8% and 1.2%. Morris and Ellis (1980), while investigating the bioavailability of dietary zinc at different phytate/zinc molar ratios in semi-purified diets in rats confirmed the synergistic action of calcium on the phytate-zinc interaction. They concluded that at dietary zinc concentration near requirement and at calcium levels of 0.75% (1.5 times the requirement level), dietary phytate/zinc molar ratios of about 12 might be tolerated if the dietary zinc concentration exceeded the minimum requirement (2.5-5 times). They also noticed that high calcium levels (1.75%) reduced zinc bioavailability at a phytate/zinc ratio of 10 as compared with diets containing 0.75% calcium. Results from semi-purified diets may be applied to soy-based diets because it was found that phytate in semi-purified diets (the authors used the term synthetic) may act similarly to that present in legumes in reducing zinc bioavailability to rats (Kumar and Kapoor, 1983).

At phytate \times calcium/zinc molar ratios > 5.88 , Fordyce et al. (1987) showed that bone zinc (tibia or femur) in studies using 18-20% protein from egg white reached a minimum while body weight did not. The authors suggest a "plateau theory" explaining that bones contain a small amount of zinc necessary for bone metabolism which is not altered at phytate \times

calcium/zinc above the breakpoint in the diet. As the phytate \times calcium /zinc ratio is reduced (zinc becomes more available), zinc is deposited in bone matrix and total bone zinc increases.

Fordyce et al. (1987) validated that phytate \times calcium/zinc molar ratios are indicative of zinc bioavailability from processed soybean foods. From previous studies (Forbes and Parker, 1977; Weingartner et al., 1984; Erdman et al., 1980; Forbes et al., 1980; Forbes et al., 1983, 1984; Ketelsen et al., 1984), bioavailability of zinc was predicted by fitting the calculated molar ratios to absolute weight gain and bone zinc accumulation. Phytate \times calcium/zinc molar ratios appeared to predict bone zinc accumulation and weight gain better than did phytate/zinc molar ratios. However, the authors cautioned that molar ratios of phytate \times calcium/zinc were less predictive of zinc bioavailability from processed soy foods where binding of phytic acid to minerals was altered (i.e. neutral soy concentrates).

Recently, Zhou et al. (1992) and Lönnerdal et al. (1988) reported that reduction of phytate content of soybean flour, soy isolate, and soy infant formula improved zinc bioavailability in rats.

Is the rat model suitable for humans?

Zinc metabolism in the rat may not reflect that in the human. One of the reasons for this thinking is that intestinal phytase activity is more pronounced in rats than in humans. Intestinal phytase might act on phytate to render zinc more available and more absorbable in the intestines. Another reason is rats coprophagize. This means they feed from their feces. In doing so, the calculation of their feed consumption may be wrongly estimated. Considering that phytate \times calcium/zinc molar ratios in rats are predictive of zinc bioavailability from processed soybean foods, there is considerably less calcium in human diets than in rat diets. In fact, a molar ratio of calcium to zinc in excess of 660:1 is recommended for weanling rat diets, while for man the ratio is between 80:1 and 160:1 (Forbes et al., 1983). Thus, the phytate \times calcium/zinc molar ratios studied in rats do not reflect those in human diets.

However, studies in rats may serve as basis for zinc bioavailability hypotheses in humans. These hypotheses may be of practical use when confirmed in well-planned human studies. As an example, researchers have indicated that the calcium levels commonly found in mixed human diets appear less likely to promote a phytate-inhibitory effect on zinc availability because the high calcium levels used in rat studies are unlikely to be found in omnivorous human diets (Hogarth, 1981;

Forbes et al., 1984). Yet, Bindra et al. (1986) thinks that lacto-ovo vegetarians may be at risk for sub-optimal zinc status because they consume diets that are relatively high in both phytate and calcium but low in readily available zinc. Davies et al. (1985) went even further and assumed that phytate \times calcium/zinc molar ratios exceeding 0.5 may negatively affect zinc bioavailability in humans.

Fordyce et al. (1987) investigated the hypothesis that phytate \times calcium/zinc molar ratios may predict zinc bioavailability in humans. They recalculated data from a study by Bindra et al. (1986) in which diet composites from Punjabi Sikh, predominantly lacto-ovo vegetarian, were compared with those of Canadian omnivorous. From an initial sample of 112 Punjabi Sikh, 15 composite records were selected from the males, and 15 records were selected from the females. For both sexes, five dietary records for each of day one, two and three were randomly selected to give a total of 30 records. Diets comparable to those recorded for each of the thirty selected diet records were prepared and 50 ml aliquots analyzed for energy, calcium, zinc, phytate, and neutral detergent fiber. The same chemical analyses were performed on foods made from 30 diet composites collected from a previous study of Canadian pre-menopausal omnivorous women. Analysis of diets indicated: calcium 568 mg/1000 vs. 422 mg/1000 kcal , zinc 4.5 mg/1000 kcal vs. 5.1 mg/1000 kcal, phytate 788 mg/1000 kcal vs. 423

mg/1000 kcal, for Punjabi and omnivores respectively. Recalculated phytate × calcium/zinc molar ratios by Fordyce et al. (1987) are about 250 mM/1000 kcal and about 90 mM/1000 kcal for the Punjabi and omnivorous groups, respectively. Expression of phytate × calcium/zinc molar ratios in terms of moles/kg of diet results in values about 0.9 and 0.3/kg in Punjabi and omnivorous diets, respectively. Results showed that 32% Punjabi had low serum zinc values (<70 µg/dl) compared with 4.5 and 8.4%, respectively, of the Caucasian adult subjects in the Canada Health survey and the American NHANES II survey. The high phytic acid in Punjabi diets is due to the regular consumption of chapattis prepared from 100% extraction wholewheat flour by the Punjabi subjects. Cereals provided the major source of zinc for the Punjabi while meat was the primary source of zinc for the omnivorous diets. Punjabi consume milk and yogurt on a regular basis, resulting with their diets being high in calcium. The practical use of phytate × calcium/zinc molar ratios may reside in that groups with high phytate intake (Middle Easterners, vegans substituting soybean based synthetic meats for animal protein) exercise control in their calcium intake in order to prevent any substantial reduced zinc bioavailability. In fact, studies (Prasad, 1961; Reinhold, 1971) have shown that middle easterners present clinical signs of zinc deficiency because of their high phytate intake. Fermented products with reduced

phytate content might be preferable.

Phytic acid in soy and interaction with minerals

Phytates are salts of phytic acid. They constitute about 1 to 2% by weight of many cereals and oilseeds (Cheryan, 1980). Lolas and Markakis (1975) analyzed more than 20 varieties of soybeans and reported phytic acid percentage by weight of 0.72 to 1.8 % from defatted meal and 1 to 1.47 % from soybeans seeds. About 60 % of total phosphorus in soybean meal is phytate phosphorus (Kornegay, 1996). The location of phytate varies from one seed to another. In oilseeds, phytate accumulates in the storage sites of globoids (Cheryan, 1980). Soybean protein bodies appear not to possess such globoids and phytate lies throughout the kernel (Snyder and Kwon, 1987). Other researchers (Lott and Buttrose, 1978; Bair, 1979) think that soybean protein bodies have globoid inclusions. These inclusions have been shown to contain considerable phosphorus and for that reason they are believed to contain phytic acid.

Phytic acid is an essential component of all seeds. The phytic acid molecule has a high phosphorus content (28.2 %). Possible roles of phytic acid can be a phosphorus store, a reserve of reactive phosphoryl groups, an energy store, or a source of cations. The binding of minerals, and /or protein by phytic acid makes them unavailable. That is of concern

especially in diets high in phytate. The mechanism for this binding can be explained by the structure of phytic acid (Cheryan, 1980). There is a consensus that the structure proposed by Anderson ($C_6H_{18}P_6O_{24}$) is the correct structure of phytic acid. Based on that structure, phytic acid is chemically identified as myoinositol-1, 2, 3, 4, 5, 6 - hexakis (dihydrogen phosphate). Research has shown that at neutral pH, phytic acid is negatively charged and binds a variety of food components (De Rham and Jost, 1979). This explains the chelating of proteins and metals by phytic acid in common foods. An example is the processing of soya isolates (Jaffe, 1981) where various phytate-protein complexes are formed. At acid pH, phytic acid reacts with proteins, and upon neutralization insoluble complexes are precipitated. These complexes will eventually bind metal ions. Chelating reactions occur within a phosphate group, between 2 phosphate groups of a molecule, or between phosphate groups of different phytic acid molecules (Gosselin and Coghlan, 1953). A molecule of phytic acid which formed a complex with mixed cations of calcium and magnesium used to be referred as phytin. In an *in vitro* study (Oberleas et al., 1966) showed that the formation of phytate-Zn complexes that were least soluble at pH 5-7 (about the pH range in the upper small intestine) was enhanced by calcium (Byrd and Matrone, 1965). At pH 3-5, about that of the stomach, the zinc phytate complexes were more soluble

(Oberleas et al., 1966).

Researchers have used the "pH drop" method to study the solubility and stability of complex formation between various metal ions and phytic acid. When a mole of phytic acid is completely neutralized by NaOH, phytic acid is dissociated and releases 12 titratable hydrogens. This presence of 12 dissociable hydrogens alters the shape of the titration curve for phytic acid in presence of various metal ions. When a metal is added in the solution of sodium phytate, the hydrogen ions are replaced by metal ions subsequently dropping the pH (Maddaiah et al., 1964). The more stable the metal-phytate complex, the greater the drop of the pH is (Vohra et al., 1965). Maddaiah and al. (1964) concluded that the stability of the complexes formed between phytic acid and the metals at pH 7.4 was in the order $Zn^{++} > Cu^{++} > Co^{++} > Mn^{++} > Ca^{++}$. Vohra et al. (1965) found the Zn^{++} -phytate complex to be less stable than the Cu^{++} -phytate complex. However, Vohra and et al. prepared sodium phytate which had the formula of $C_6H_6O_{24}P_6Na_{12} \cdot 3H_2O$ (the accepted formula is not hydrated).

Studies in the reduction of phytic acid

Procedures for the removal of phytic acid are mechanical, water extraction and differential solubility, autoclaving, and enzymatic methods.

Mechanical processes

Since in corn 90% of the phytate is concentrated in the germ, and in wheat and rice most of the phytate is in the outer layers, milling and grinding operations should be able to separate phytic acid-containing fractions from the rest of the seed (Cheryan, 1980).

Water extraction and differential solubility

Cheryan (1980) summarized different techniques used in this processing. Phytic acid is almost totally water-soluble, and because the solubility profiles of protein and phytic acid differ, a careful adjustment of the pH of a water extract of soybeans will create a solubility differential between protein and phytic acid. The insoluble component may be removed by filtration or centrifugation. Three different pH regions of water extraction have been determined: high (pH > 11), moderate (pH 6-10), and low (pH < 5.5).

The high pH method may result in the production of lysinoalanine. When the moderate pH method is used, there is the formation of a ternary protein-cation-phytic acid complex. Thus, the addition of competitive chelators such as EDTA to the system is required to bind preferentially some cations and prevent them from being complexed to phytic acid. At this stage, both phytic acid and protein are in solution and additional steps such as dialysis, ultrafiltration or the

addition of excess NaCl must be used to separate them. At lower pH (< 5.5), both phytic acid and protein are insoluble. Dissociation of the protein-phytate complex is done by the presence of excess calcium combined with dialysis and diafiltration. From a product point of view, this method is undesirable because soybean globulins are known to be denatured at this pH.

Dialysis and ultrafiltration

In cases where phytate is in solution without complexing with protein, methods based on molecular size differences can be used. Dialysis can be used as long as pH and cation concentrations are controlled. It is not of practical use on a large commercial scale. Ultrafiltration, on the other hand, is a pressure-activated process that results in greater removal of solution compared with dialysis. However, it may be used only after phytate has been dissociated from protein.

Autoclaving

O'Dell (1969) reported that autoclaving of soy isolates at 115°C for 4 hours appears to destroy phytic acid by hydrolysis. This excessive heat treatment probably destroys essential amino acids and lowers nutritive value (Rackis, 1974).

Enzyme treatment

Phytase is an enzyme that is present in many plant seeds like cereals and soybeans, and is formed by microorganisms, such as fungi, yeasts, bacteria and rumen microbes (Heinzl, 1996). Soaking of seeds reduces phytic acid content through the enzymatic action of native seed phytase (Jaffe, 1981). The activity of phytase in soybean seeds was reviewed by Goldman (1995). The activity of phytase is typically very low or undetectable in dry or dormant seed. This activity increases gradually during sprouting and peaks at 8 to 12 days. Soybean phytase has optimal activity at pH 4.8-4.9 and 60°C, and pH 4.5-4.8 and 55°C. Activity decreases at temperatures above 60°C.

Phytases from different microorganisms have been used to decrease phytic acid content. Phytic acid from a soybean slurry was completely removed after 4 hours incubation at 37°C with *Aspergillus oryzae* phytase (Anonymous, 1976). Reduction of 1/3 phytate content of tempeh has been obtained using preparations from *Rhizopus oligosporus* (Sudarmadji and Markakis 1977). During breadmaking, phytic acid is destroyed by enzymatic activity of the yeast. Ranhotra et al. (1974) noted that, in contrast to wheat where fermentation caused sizable hydrolysis of phytic acid, there was little phytase activity from soy preparations. When soy-fortified bread was made without yeast, less than half of the phytate was

hydrolysed. As yeast was added, there was a progressive increase in the hydrolysis; and at levels used in bread formulation, phytate hydrolysis was maximum. Microbial phytase commonly used nowadays is made from *Aspergillus ficuum* (Heinzl, 1996).

Microbial phytase and calcium bioavailability studies in chicks

Recent reports have recommended the use of microbial phytase in decreasing phytate content and improving the bioavailability of phosphorus in animal feeds (Nelson et al., 1971; Simons et al., 1990; Edwards, 1993; Biehl et al., 1995; Mitchell et al., 1996; Perney et al., 1993; Yi et al., 1996). This enzyme catalyzes the cleavage of the phosphoric acid esters of inositol freeing ortho-phosphates, and hence, renders ions of P, Ca, Mg, and Zn more available and more absorbable. Phytase is an acid phosphatase. It can be found in plant seeds as well as in microorganisms. Since phytase is a protein, it is normally degraded in the digestive tract of mammals (Heinzl, 1996). Calcium experiments with chickens have the advantage of using large number of animals, and thus, to reduce variability.

Earlier studies of the potential of microbial phytase used crude phytase extract because of lack of commercial microbial phytase. Nelson et al. (1968) fed white leghorn

cockerels either untreated or phytase-treated soybean meal. The phytase used was from a culture of *Aspergillus ficuum* NRRL 3135. An *in vitro* experiment showed that the active culture hydrolyzed 92% of the phytate phosphorus while the autoclaved culture filtrate hydrolyzed only 8%. Birds fed soybean meal treated diets at 0.28%, and 0.31% phosphorus at graded hydrolysis of soybean meal phytate showed responses in the phosphorus equivalent equal or higher than the nonphytate phosphorus (nP). Responses in bone ash and phosphorus equivalent were related to the concentration of the culture filtrate. Also, the authors observed that excess calcium depressed the utilization of the available phosphorus. The negative effect of calcium was not evident in diets low in phytate where a large percentage of total phosphorus (tP) was non-phytate even though the calcium/tP ratio exceeded 2/1 and the calcium/nP ratio approached 3/1.

Nelson et al. (1971) discovered that chicks utilized the hydrolyzed phytate as well as supplemental inorganic phosphorus. They fed chicks diets containing 0.18% natural phytate phosphorus from corn and soybean meal either treated or untreated with a mold phytase from *Aspergillus ficuum* NRRL 3135. At 0.47% tP, chicks fed diets containing 3g of phytase supplement/kg had bone ash similar to that of the group of chicks fed the diet supplemented with inorganic phosphorus and containing 0.67% tP and 0.46% nP; 0.47% nP would be the nP

content of the phytate treated group in case of total hydrolysis of phytate phosphorus. No difference in phytate phosphorus was noticed between a diet containing 4 g of phytase supplement/kg and a diet without any enzyme supplement at the end of the experiment. This experiment demonstrated that the phytase activity occurred in the alimentary tract of the chick and not in the feed prior to ingestion.

Recent developments in biotechnology have made possible the production of large quantities of enzyme. Microbial phytase (Natuphos, BASF, Germany) is commonly used in poultry. It is obtained by fermentation from a genetically modified *Aspergillus niger* strain. *Aspergillus ficuum* provides the genetic information which is transferred to *Aspergillus niger*. Different from plant phytases, microbial phytase has 2 pH optima at pH 2.5 and pH 5.5. This can explain the biological activity of microbial phytase in the fundus of the stomach and its biological superiority to plant phytases.

The manufacturer of the microbial phytase (Natuphos, BASF, Germany) suggests a phytase dose of 600 units/kg diet (Natuphos Safety Sheet). One unit of phytase activity (= FTU) is defined as the quantity of enzyme which liberates 1 μ mole of inorganic P per minute from .00051 moles sodium phytate/L at pH 5.5 and 37°C. Likewise, Mitchell and Edwards (1996) found that 600 units of a commercial phytase (BASF corp., Mt. Olive, NJ)/kg diet would effectively replace up to 0.1% of

inorganic phosphorus in corn-soybean meal diets of young broilers without negatively affecting chickens performance.

The primary use of phytase in chicken studies is to increase the bioavailability of phytate phosphorus to reduce the environment pollution caused by phosphorus in the chicken wastes and to decrease the inorganic phosphorus required as supplement to poultry diets. Researches have focused on the cost-effectiveness of using phytase and the influence of dietary calcium and calcium/phosphorus ratio on the effectiveness of microbial phytase. It is known that excess dietary calcium interferes with the ability of the animal to absorb phosphorus as well as Mg, Mn, and Zn (Kornegay, 1996). Higher than appropriate calcium/phytate ratio may cause the formation of insoluble phytate-calcium complexes which will probably bind various metal ions. Edwards et al. (1992) found that a high level of dietary calcium adversely affected the availability of phytate phosphorus.

Studies using corn-soybean meal diets and multiple levels of phytase and phosphorus have demonstrated that the effectiveness of microbial phytase depends on: the available or non phytate phosphorus (nP), total phosphorus (tP), and the phytase activity. Similar to their results with turkeys (Qian et al. 1996a), Qian et al.(1996b) reported with broilers that the calcium/tP ratio higher than 1.4/1 negatively affected the effectiveness of microbial phytase at levels of microbial

phytase of 300, 600, and 900 units/kg of diet. The dietary phosphorus level in the corn-soybean meal diet at 23% crude protein was formulated at .27% nP (and .51% tP). Responses of body weight gain, feed intake and toe ash were improved by the addition of vitamin D₃. In agreement with Kornegay (1996), Edwards (1993) and Biehl et al. (1995) reported positive effects of the addition of 1, 25-(OH)₂ D₃ in the utilization of phosphorus in chick diets low in phosphorus.

When Denbow et al. (1995) fed broilers for 21 days corn-soybean diets supplemented with phytase (Natuphos) at a calcium/tP ratio of 2/1, they noticed that the amount of phosphorus released per 100 units of phytase decreased as the total amount of phytase was increased. Responses in body weight gain, ash percentage, and tibia shear force showed the same trend; the magnitude of each of the responses for all nP levels was greatest at lower rates of phytase addition for the lower nP levels. Levels of nP and phytase used were respectively .20%, .27%, .34% (.38, .45, .52% tP) and 0, 200, 400, 600, 800, 1,000, and 1,200 units/kg diet. In this experiment, 821 units of phytase would be equivalent to 1g of phosphorus.

Qian et al. (1996b) proved in broilers that addition of phytase to corn-soybean diets improved histological and tibia characteristics in the same way as supplementation with inorganic phosphorus did. Microbial phytase (Natuphos) was

used, and soybean meal provided the only organic phosphorus source (0.11% nP). Inorganic phosphorus was added to diets to reach nP levels of .20, 0.27, 0.34, 0.45 and 0.54% and the calcium/tP was 2/1. A high level of cholecalciferol (26 times the NRC) was used. Based on the evidence that 1g phosphorus from defluorinated phosphate is equivalent to 800 units phytase (Yi et al., 1996; Denbow et al., 1995), different levels of phytase were added to diets at different levels of nP (800 units at 0.20% nP, 600 units at 0.27%, 400 units at 0.34 nP in experiment 1; 350 and 1,050 units at 0.27% nP, 1,050 units at 0.54% nP in experiment 2). Results showed that body weight gain and feed intake linearly increased when phytase was added and as nP content of the diet increased; the increase reached a plateau at 0.45% nP. Gain/feed ratios were not significantly affected by either nP or phytase supplementation of the basal diet. Graded phytase supplements and inorganic phosphorus additions significantly narrowed the hypertrophic zone in the tibias; these tibias were longer and wider than those of broilers fed the phosphorus deficient diets. Tibia breaking strength was improved in broilers fed the phosphorus deficient diet supplemented with phytase. Although there was an increase in ash and Mg content in phytase treated group, no increase was noticed in the zinc content of tibia.

Studies in poultry have pointed out the benefit of using

microbial phytase on the bioavailability of minerals other than phosphorus. Roberson and Edwards (1994) have suggested that phytase increased zinc utilization. In their experiment, 600 units phytase/kg of diet with 35 ppm zinc, adequate phosphorus, and low calcium improved body weight gain. Yi et al. (1996) demonstrated that adding zinc and phytase in a corn-soybean isolate diet with 20 ppm zinc linearly increased the concentration and amount of zinc in toe and tibia. In the same experiment, the addition of zinc and phytase to the low zinc basal diet linearly increased body weight gain and feed intake. Phytase levels used were 150, 300, 450, and 600 units/kg of diet and calcium/nP ratio was 2.22/1 in all diets.

Calcium would be expected to become more available as phytate phosphorus becomes more available. A study mentioned above found that 600 units of phytase/kg diet enhanced calcium retention at 5 and 10 days (Roberson and Edwards, 1994). But, Edwards (1993) and Nahashon et al. (1994) did not show the positive effect of phytase on calcium bioavailability. Nahashon et al. (1994) fed Leghorn layers .25 and .45% available phosphorus in corn-soybean diets supplemented with cane molasses solubles (CCMS)-*Lactobacillus* and CCMS at 2% of diets. No differences in calcium retention were observed with CCMS-*Lactobacillus* supplementation even though higher phosphorus retention were noted in the .25% available phosphorus diet.

A three week feeding trial using a low-phosphorus diet fed to male and female broilers showed that the effect of phytase supplement is prominent in males and particularly in the head portion of the tibia (Sebastian et al., 1996). Experimental diets included a control corn-soybean meal diet, a low phosphorus corn-soybean meal without phytase, and a low-phosphorus corn-soybean diet plus phytase. Phytase treatment had a positive effect on phosphorus (males and females) and calcium utilizations (females only) as shown by significant increases in phosphorus and calcium in the tibia head dry matter content. Neither low-phosphorus nor phytase supplementation to a low-phosphorus diet had an effect on any mineral content in dry matter of the tibia shaft, except that the low-phosphorus diet caused a significant reduction in phosphorus content in the tibia shaft of male chickens. Phytase treatment caused an increase in ash content of the tibia compared with chicks fed the low-phosphorus diet; this increase was more pronounced in the head portion than in the shaft portion. No change in phosphorus or calcium in the ash caused by low-phosphorus or phytase supplementation was seen. No difference in feed to gain ratio was observed. However, phytase treatment increased body weight in male and female chickens by 13.2% and 5.8%, respectively. Also, phytase supplementation increased the relative retention of total phosphorus, calcium, copper, and zinc.

The potential exists for using microbial phytase in human nutrition to increase the bioavailability of calcium and trace minerals such as zinc and iron in soy products. Once microbial phytase is accepted as a food-grade ingredient, it can be sold as a mineral-releasing factor for vegetarians. Also, microbial phytase can be used directly in the processing of phytate-containing foods. In this way, hydrolysis of phytate will be carried on during processing in contrast to the application of microbial phytase in poultry where the action of phytase occurs in the digestive tract. The challenge for human use of phytase will be to come out with forms of phytase that can be added directly to foods, and to develop processing conditions favorable to the activity of microbial phytase.

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**HYDROTHERMAL COOKING AFFECTS PROTEIN EFFICIENCY
RATIO AND ZINC BIOAVAILABILITY OF SOYMILK-BASED
DIETS IN RATS**

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Abstract

Hydrothermally cooked (HTC) and conventionally processed soymilks were compared with respect to protein efficiency ratio (PER) and zinc bioavailability in Sprague-Dawley rats fed isocaloric, isonitrogenous diets. When dietary zinc was 50 mg/kg, PER (mean \pm SD, n = 12) was greater for HTC (processed for 20 sec) (2.69 ± 0.34) than for conventional soymilk (2.39 ± 0.17). When dietary zinc was 20 mg/kg, PER (n = 10) was less for HTC processed for 40 sec (1.86 ± 0.17) than for conventional soymilk (2.08 ± 0.19). Processing (HTC vs. conventional) did not have a significant effect on zinc bioavailability by the slope ratio bioassay procedure. PER of HTC processed for 20 sec is better than that of reference

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casein and conventional soymilk if dietary zinc is near recommended levels, pancreatic hypertrophy was not observed.

Introduction

Soybean seeds contain substantial quantities of high-quality protein and unsaturated fats, without cholesterol. However, raw soybean seeds must be processed to decrease various antinutritional factors to acceptable levels before human consumption. Depending upon conditions of processing, nutritional quality may be adversely affected; protein quality may be decreased, lipids may be autooxidized, and mineral bioavailability may be decreased.

Hydrothermal cooking entails continuous dispersion of soy in water from a hot slurry of ground seeds (Johnson et al., 1980, 1981; Kim, 1988). Processing temperature is higher and processing time shorter than for conventional methods. Nearly all the solids of the soybean are dispersed in a manner that precludes subsequent separation by sedimentation or centrifugation.

Characteristics with advantages over conventionally processed soymilk have been demonstrated by physical and chemical analyses of hydrothermally cooked soymilk (Johnson et al., 1980, 1981; Kim, 1988; Wang, 1993). Hydrothermal cooking results in lower levels of lysinoalanine and browning

products, as well as acceptable decreases in activities of trypsin inhibitor and lipoxygenase. However, previous reports have not documented the nutritional performance of hydrothermally cooked soymilk by bioassay.

Protein efficiency ratios (PER) for adequately processed soy products have been reported to be 62-92% that of casein (Erdman and Forbes, 1981; Rackis et al., 1975; Torun et al., 1981). Dependence of protein utilization from phytate-containing plant seeds on dietary zinc content has been documented (Oberleas and Prasad, 1969). It was concluded that mineral mixtures should contain zinc when quality of vegetable protein concentrates is being evaluated (McLaughlan et al., 1977).

The bioavailability of zinc from various soy-based products has been reported to be, in general, low (Forbes et al., 1979; Fordyce et al., 1987; Lönnerdal et al., 1988; Zhou et al., 1992). This can be of concern when soy-based products predominate in the diet, such as in infants and children fed only soy-based formulas (Erdman and Fordyce, 1989). Dietary phytic acid content adjusted for calcium and zinc content can be used to predict zinc bioavailability to rats (Fordyce et al., 1987; Lönnerdal et al., 1988; Likuski and Forbes, 1965; Zhou et al., 1992). Processing methods that reduce the phytic acid content of soy products can improve zinc bioavailability (Ketelsen et al., 1984; Prattley et al., 1982; Zhou et al.,

1992).

The objective of this study was to assess protein utilization of and zinc bioavailability from hydrothermally cooked soymilk as compared with conventionally processed soymilk when fed to rats. Several experiments at various levels of dietary zinc concentration were performed. The potential for pancreatic hypertrophy that could result from ingestion of inadequately processed soy was considered, and the nutritional composition of the products was extensively analyzed.

Materials & methods

Animal care

Male weanling Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) with initial weight 45 to 55 g were housed individually in stainless-steel wire-mesh cages throughout the experiments. The laboratory room had controlled temperature (23°C), humidity (70%), and lighting (12-hr light, 0600 to 1800 hr). All animals were allowed free access to a pelleted, commercial diet (Teklad rodent diet, Harlan Teklad, Madison, WI) for 3 days during adjustment to the laboratory environment. All procedures used were approved by the Iowa State University Committee on Animal Care.

Experimental design

After environmental adjustment, each animal was assigned randomly to an experimental group (weight range in each experiment was ≤ 10 g) and had ad libitum access to an experimental diet and to drinking water, the zinc concentration of which was below the detection limit of atomic absorption spectrophotometry ($< 2 \mu\text{g/L}$). Feed intakes were measured daily, and body weights were recorded weekly. At the end of the feeding period for each experiment, each rat was euthanized by exposure to an atmosphere of 100% carbon dioxide, and liver, pancreas, and femurs were excised and weighed. Femurs were dried in an oven at 100°C to constant weight. Livers and pancreas were stored at -20°C until further analyses.

Protein efficiency ratio

Three groups of 12 rats each were fed for 28 days. A standardized protocol (AOAC, 1990a) was used to compare the PER of ground dehulled soy that was hydrothermally cooked (HTC) with that of soy prepared by a conventional process for the production of soymilk (Takai Automatic Soymilk Plant, Nichii Corporation, Jefferson, IA). The third group was fed a diet that contained casein as the sole source of protein. The amount of AIN-76 mineral premix used with each soy source was calculated according to the protocol for PER (AOAC, 1990a).

Processing comparisons and zinc effects

A 3-wk zinc study and a 4-wk protein utilization study were performed simultaneously. Three groups of 10 animals each were used for the protein utilization study, and 3 groups of 8 animals each for the zinc study. The three dietary treatments were similar to those of PER experiment except that: 1) The zinc concentration of the soy-based diets was decreased to ≈ 20 mg/kg diet by deleting some zinc sulfate during preparation of the AIN-93G mineral mix used for these soy-based diets; 2) Eight animals for each dietary group were euthanized after 3 wk to provide organs for comparison with published studies of pancreatic hypertrophy (Liener and Kakade, 1980); and 3) The remaining 10 animals per group were euthanized after 4 wk to permit comparison of the protein utilization measurements with those of PER experiment.

Zinc bioavailability

The objective was to compare zinc bioavailability from the HTC and the conventional soy-based diets by the slope ratio method (Forbes et al., 1983). PER was not measured, and casein-fed groups were not included. Four analyzed dietary zinc concentrations (10, 17, 24, 31 mg/kg diet) were studied for each of the two soy processing treatments by using 5-6 animals in each of the 8 subgroups. Calcium concentration of the AIN-93G mineral mix was adjusted to bring the overall

calcium concentration of both soy-based treatments to ≈ 0.5 g calcium/kg diet, based on calcium analyses.

Soybean flour preparation

A pilot-plant scale steam-infusion cooking system was assembled for the production of hydrothermally cooked soymilk (Kim, 1988). Dehulled beans were ground in a Fitzmill comminuting machine (Hammermill, model D, W.J. Fitzpatrick Co., Chicago, IL) through a 20-mesh screen to make a full-fat soy flour. The flour was slurried with tap water to give a soy extract of 12% solids. The slurry was poured into a surge tank from which it was pumped with a variable-speed Moyno pump (2MI type SSQ, Robin and Myers, Inc., Springfield, OH) to a hydroheater valve (size 300, type B, Hydrothermal Corp., Milwaukee, WI) where it was infused with steam. The slurry enveloped the steam flowing into the combining tube. Thereafter, the cooked slurry flowed through an insulated stainless flash chamber into a stainless-steel, tubular heat exchanger where it was cooled to 49°C. Soymilk (≈ 10 L) was obtained from 1.5 kg of beans. The cultivar of soybean used was Vinton 81. The batch of HTC soymilk was processed for 20 sec in experiment PER and for 40-45 sec in experiments of the processing comparisons and zinc effects, and zinc bioavailability. The temperature used was 152-154°C. All batches of conventional soymilk were cooked (Takai Automatic

Soy milk Plant, Nichii Corporation, Jefferson, IA) at 95°C for 7 min, followed by heating to 100°C for 40 sec. The conventional soymilks had 11% solids.

Conventional and HTC soymilks were bulk spray-dried in a pilot plant tower spray dryer (Food Processing Center, University of Nebraska at Lincoln, Lincoln, NE). The flours were analyzed for nitrogen, lipid, moisture, ash, zinc, calcium, magnesium, phosphorus, iron, copper, phytate, sucrose, stachyose, and raffinose. Composition was determined (Table 1) on conventional- and HTC spray-dried soymilks used in the processing comparisons and study of zinc effects, and zinc bioavailability.

Diet preparation

Diets were formulated to contain by weight: protein 10%, lipid 8%, minerals 5%, vitamin mix 1%, fiber 1%, water 5% according to the standard methods of the AOAC (AOAC, 1990a) for PER measurements. Except for casein diets, salt mix was < 5% to compensate for mineral in soy. Corn starch was added to make 100%. A reference diet was prepared by using casein (Casein Vitafree, United States Biochemical, Cleveland, OH) and by assuming the product composition to be 100% protein by weight. AIN-76 mineral premix (United States Biochemical, Cleveland, OH) was used in the preparation of the casein-based diet.

Protein efficiency ratio .

The amount of spray-dried soy (conventional or HTC-processed) included in each diet as source of protein at 10% by weight, was calculated. This was based upon macrokjeldahl nitrogen analyses of each batch of soy product. A protein factor of 5.71 was used (USDA, 1986) according to the formula: amount of soy (g/100 g diet) = $10000 / [(mg\ N / g\ soy) \times 5.71]$ The amount of corn oil, salt mixture (AIN-76), and cellulose, respectively, were calculated (AOAC, 1990a) based upon the reported lipid (21.86%), ash (5.86%), and crude fiber (2.23%) composition of full-fat soyflour (USDA, 1986), as:

corn oil (g/100 g diet) = $8 - [0.2186 \times (g\ soy/100\ g\ diet)]$

salt mix (g/100 g diet) = $5 - [0.0586 \times (g\ soy/100\ g\ diet)]$

cellulose (g/100 g diet) = $1 - [0.0223 \times (g\ soy/100\ g\ diet)]$

The water added to each diet was calculated (AOAC, 1990a) based on moisture determination of soy products according to the formula:

water (g/100 g diet) = $5 - [(\%moisture/100) \times (g\ soy/100\ g\ diet)]$

The protein and moisture content of the soy products used in PER experiment as analyzed in triplicate were (mean \pm SD): HTC, $41.97 \pm 0.21\%$ protein, 3.07% moisture; conventional, $47.34 \pm 0.27\%$ protein, 2.24% moisture (Table 2).

Processing comparisons, zinc effects, and zinc bioavailability

The ingredients of diets in these experiments are shown in Tables 3 and 4. The analyzed composition of spray-dried soymilk (Table 1) was the basis for the design of diets (Tables 3,4). The formulation of diets for soyflour, corn oil, salt mix, cellulose, and water was based upon analyzed values for protein, lipids, ash, crude fiber, and moisture of the spray-dried soymilks. However, when processing techniques were compared at marginal zinc intakes, the amount of corn oil was based on the USDA lipid value for full-fat soyflour (21.86%). Mineral mixes were designed to complement the mineral in spray dried soy and were prepared to provide the minimal nutrient composition specified by AIN-93G (Reeves et al., 1993) except for zinc. When processing techniques were compared at marginal zinc intakes, mineral mixes were designed to contain 20 mg zinc/kg.

Diets were analyzed by atomic absorption spectrophotometry (Video 12, Thermo Jarrell Ash, Franklin, MA) in our laboratory for zinc, calcium, magnesium and by a commercial laboratory (AGP Limited, Courtland, MN) for phosphorus. Mineral mixes in the zinc bioavailability experiment were adjusted based on differences between analyzed and expected values for calcium, magnesium, and phosphorus of the diets that had been used when processing techniques were compared at low zinc intakes. In the zinc bioavailability

experiment, mineral mixes contained no added zinc. The soyflour basal diets contained about 10 mg zinc/kg solely supplied by the soy. Zinc sulfate heptahydrate was added (30.79 mg, 61.57 mg, and 92.40 mg/kg of diet) with the added water during diet mixing to provide diets with 17, 24, or 31 mg zinc/kg.

Analytical methods

Right femurs were cleaned of all adhering tissues and dried in an oven at 100°C to constant weight. Femurs were digested in a microwave digestion apparatus (Milestone, model mls 1200 mega, Buck Scientific, Norwalk, CT) using concentrated HNO₃ and 30% H₂O₂. The residues were dissolved in 0.7% HNO₃ and transferred to volumetric flasks for zinc and calcium analysis by atomic absorption spectrophotometry (Video 12, Thermo Jarrell Ash, Franklin, MA). Absorbance was measured at 213.9 nm for zinc and at 422.7 nm for calcium and compared with certified reference standards (National Institute of Standards and Technology, Gaithersburg, MD).

Soyflours and diets were also wet-digested before atomic absorption analysis for zinc and calcium. In addition, soyflours were analyzed for magnesium, iron, copper, and phytate. Magnesium, iron, and copper were determined as for zinc and calcium. Certified reference standard was used for

iron (National Institute of Standards and Technology, Gaithersburg, MD), and reference materials for magnesium and copper were prepared from magnesium ribbon and metallic copper.

Phytic acid was separated by the anion-exchange method of Thompson and Erdman (1982) and determined by comparison with a standard curve prepared as described (AOAC, 1990b). Phosphorus was determined by AGP Limited (Courtland, MN). Lipid content of soyflours was determined by the Goldfisch (American Association of Cereal Chemists, 1983a) method (Goldfisch fat extraction apparatus #35001, Labconco, Kansas City, MO) using petroleum ether as solvent, and the results were confirmed by the acid extraction method (American Association of Cereal Chemists, 1983b). Protein determinations in both diets and soyflours were based upon macrokjeldahl nitrogen analyses (Kjeltec Digestion/Distillation System #1003, Tecator, Inc, Boulder, CO).

Statistical analyses.

PER was calculated based upon nitrogen analyses of formulated diets. In all experiments, the statistical significance of differences between group means was determined with Student's t-test that used the pooled experimental error

taken from the ANOVA (SAS Institute Inc., Cary, NC) of the completely randomized design. In zinc bioavailability experiment, linear regression analysis was performed of the logarithm of total femur zinc content vs. dietary zinc intake, and slopes and intercepts were compared by ANOVA and t-test of group means. Also, results for feed intake, body weight gain, and PER were subjected to multiple analysis of variance. Statistical significance was established at $p \leq 0.05$. Values in the text are means \pm SD.

Results

The nutrient composition of spray-dried HTC soymilk (Table 1) was similar to that of conventional soymilk with regard to calcium, magnesium, phosphorus, and sugars (sucrose, stachyose, and raffinose). Phytate and zinc concentration of spray-dried HTC soymilk were less than for spray-dried conventional soymilk, but phytate/zinc molar ratio was greater for the HTC product (27.70 compared with 24.44). Protein and lipid concentrations of HTC spray-dried soymilk were slightly lower than those of spray-dried conventional soymilk.

Protein efficiency ratio

Results for PER, pancreatic weight, and log total femur zinc were compared (Table 5). The PER for the HTC-fed group

was significantly higher than for the conventional and the casein-fed groups. Mean pancreatic weight for the conventionally-fed group was below the range of 0.5-0.6% body weight and was different from that of the HTC-fed group. Mean pancreatic weights for the HTC- and the casein-fed groups were within normal range and did not differ. There were no differences in log of mean total femur zinc and log of mean femur zinc/g femur among the three groups.

Processing comparisons and zinc effects

When the dietary zinc concentration was 20 mg/kg, the PER of the HTC-fed group was significantly less (Table 5) than the PER of the conventional group after 4 wk of feeding. Mean pancreatic weights were not different after 3 wk. Mean zinc intakes after 3 wk were different, but means for zinc concentration/g femur at 3 wk were not different. Means for log total femur zinc were less for the HTC- than for the conventional-fed group at 3 wk. Means for femur calcium did not differ between conventional and HTC-fed groups at 3 wk. Values at 4 wk for the casein-fed group, where zinc was at 50 mg/kg, were comparable to those of the casein-fed group in PER experiment.

Zinc bioavailability

Regression equations when log total femur zinc (Table 6) was plotted against analyzed dietary zinc intake after 21 days of feeding were:

- for HTC fed group: $y = 1.136 + 0.0514x$ $r = 0.98$

- for conventional-fed group: $y = 1.156 + 0.0518x$ $r = 0.95$

where y is the log total femur Zn in μg and x is the dietary zinc intake in mg. The slopes and intercepts for log mean total femur zinc were not different between the two soy processing methods ($P = 0.98$ for slopes and $P = 0.82$ for intercepts). Fig. 1. ANOVA showed a significant effect of type of soy-processing (conventional vs. HTC) on body weight gain, feed intake, efficiency of protein utilization, and log total femur zinc ($P < 0.05$). Similarly, ANOVA showed an effect of dietary zinc on weight gain, feed intake, efficiency of protein utilization, and log total femur zinc ($P < 0.05$). Interactions between dietary zinc and soy-processing on body weight, feed intake, efficiency of protein utilization, and log total femur zinc were not noted ($P > 0.05$).

Discussion

In the PER and processing comparisons the efficiency of protein utilization for the HTC-fed group was higher than that

of the conventionally-fed group when dietary zinc concentration was 50 mg/kg. However, it was lower than for the conventionally-fed group when dietary zinc was \approx 20 mg/kg. In both studies, the casein control with zinc at 50 mg/kg was in the normal PER range (2.39 and 2.48). This may suggest that the protein quality for rats of the HTC soymilk diet was more related to its zinc content than was that of the conventional soymilk diet. McLaughlan et al. (1977), evaluated protein quality of vegetable protein concentrates high in phytate. They concluded that conventional salt mixtures should contain at least 50 mg/kg zinc when the source of protein was rapeseed or similar protein of plant origin. Hence, the PER where zinc was at 50 mg/kg (PER experiment) may be the more appropriate measure of the protein quality of the soy products we evaluated. The higher PER of HTC vs. conventional soymilk in PER experiment could be attributed to the short processing time (20 sec) that might result in higher available lysine and methionine. This was first observed by Hackler et al. (1965), who demonstrated that available lysine seemed to be a better indicator of PER in spray-dried soymilk than in freeze-dried soymilk. Their soymilk was cooked at 121°C before spray-drying or freeze-drying.

Mean pancreatic weight can be considered as an indicator of trypsin inhibitor activity in rats (Liener and Kakade, 1980). In the PER and the processing comparisons, hydrothermal

cooking resulted in mean pancreatic weights in the normal range (0.5-0.6%) (Liener and Kakade, 1980). Perhaps the 20-sec processing time for hydrothermal cooking used in the PER experiment provided sufficient inactivation of trypsin inhibitor activity. Johnson et al. (1980) reported that hydrothermal cooking (154°C, 40 sec) produced trypsin inhibitor inactivation equivalent to that of conventionally processed soymilk (99°C, 60 min). The optimal duration of hydrothermal cooking to maximize protein efficiency ratio without elicitation of pancreatic hypertrophy remains to be identified.

The data for zinc bioavailability or total femur zinc met the criteria (linearity of responses with common y-intercepts) of Momcilovic et al. (1975a, b). They established that comparison of slopes of log total femur zinc vs. zinc intake was effective for determination of zinc bioavailabilities from different dietary treatments to growing rats fed for 2-3 wk. These were more reliable than body weight, weight gain, and femur zinc/g fresh or dry weight. By using this slope-ratio bioassay procedure, we have shown that bioavailabilities of zinc from HTC and conventionally processed soymilk-based diets were not different.

Phytic acid x Ca : Zn molar ratio is considered to be a reliable predictor of zinc bioavailability in processed products (Fordyce et al., 1987; Lönnerdal et al., 1988;

Likuski and Forbes, 1965; Zhou et al., 1992). Also, the type of processing of soy products affects zinc bioavailability (Erdman et al., 1980; Erdman and Forbes, 1981; Erdman and Fordyce, 1989). In our experiments, dietary calcium and zinc concentrations were nearly the same for both soy treatment groups, so processing and zinc bioavailability differences would be the only potential factors differentiating the two groups.

In the zinc bioavailability experiment, soy processing method and dietary zinc concentration each separately affected body weight gain, feed intake, efficiency of protein utilization, and log total femur zinc. No interaction between processing method and dietary zinc concentration was found. Hence, we did not consider the observed effect of type of processing, when hydrothermal cooking duration was 40 sec, to be attributable to differences in zinc bioavailability from the two types of products.

As observed by Zhou et al. (1992), when experiments are designed in this manner phytate becomes the predominant factor influencing zinc bioavailability. The phytate content of spray-dried HTC soymilk might seem (Table 1) to be slightly less than that of the conventional product. However, the phytate content of the HTC soymilk-containing diet was slightly greater than that of the diet based on conventional soymilk when the diets are formulated for equal protein,

calcium, and zinc content. This is reflected by the phytate/zinc molar ratios for the two products. Because zinc bioavailabilities from HTC and conventional soymilk were not different by the slope-ratio bioassay, the small difference between the phytate/zinc molar ratios of the HTC and conventional soy-based diets probably is not important when dietary calcium content is 0.5%. Probably the lower feed intake, efficiency of protein utilization, body weight gain and log total femur zinc as for HTC soymilk where dietary zinc was suboptimal (< 50 mg/kg) are attributable to the 40-sec processing time that might result in greater lysinoalanine formation and/or decreased digestibility(not assessed in these experiments).

Our results confirmed that fortification with inorganic zinc of soy protein foods improves the zinc bioavailability from the soy products to rats (Erdman and Fordyce, 1989; Forbes and Parker, 1977). Also, fortification of HTC soymilk with inorganic zinc should improve the PER of the final product. Furthermore, suitably processed HTC soymilk has a PER at least equal to that of casein when dietary zinc concentration is at the recommended value of 50 mg/kg.

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Table 1

Analyzed composition of spray-dried soymilk^a

	HTC ^b	Conventional
	g/100 g	
Protein	40.94 ± .98	46.71 ± .16
Lipid	15.95 ± 1.51	19.11 ± .71
Moisture	1.39 ± .22	0.60 ± .05
Ash	5.55 ± .04	5.85 ± .53
Ca	0.21 ± .00	0.22 ± .01
Mg	0.26 ± .01	0.28 ± .01
P	0.71 ± .01	0.75 ± .00
Phytate	1.17 ± .03	1.21 ± .03
Sucrose ^c	6.73	6.71
Stachyose ^c	4.49	4.78
Raffinose ^c	0.69	0.61
	mg/kg	
Zn	42.94 ± .65	48.93 ± 1.27
Fe	55.89 ± 1.18	60.21 ± .53
Cu	12.96 ± .42	15.66 ± .28
	mol:mol	
Phytate/Zn	27.70	24.40

^a Results expressed as mean ± SD, n = 3

^b HTC soymilk processed for 40-45 sec before spray-drying.

^c n = 1

Table 2

Dietary ingredients (Protein efficiency ratio)^a

Ingredient	Casein	HTC	Conventional
		g/100 g	
Protein source ^b	10.00	23.83	21.12
Corn oil	8.00	2.75	3.31
Salt mix ^c	5.00	3.59	3.74
Vitamin mix ^d	1.00	1.00	1.00
Cellulose ^e	1.00	0.46	0.52
Water	5.00	4.27	4.52
Corn starch ^f	69.90	64.00	65.69
Choline ^g	0.10	0.10	0.10

^a According to AOAC, 1990

^b Protein source was either spray-dried soymilk (HTC or conventional) or casein.

^c AIN-76 mineral mixture (United States Biochemical, Cleveland, OH) contains in g/kg mixture: CaHPO₄, 500.0; NaCl, 74.0; K₃C₆H₅O₇·H₂O, 220.0; K₂SO₄, 52.0; MgO, 24.0; MnCO₃, 3.5; Ferric citrate (16-17% Fe), 6.0; ZnCO₃ (70% ZnO), 1.60; CuCO₃, 0.3; KIO₃, 0.01; Na₂SO₃·5H₂O, 0.01; CrK(SO₄)₂·12H₂O, 0.55; sucrose, finely powdered to make 1,000.00.

^d AIN-76A vitamin mixture (United States Biochemical, Cleveland, OH) contains in each kg: thiamine hydrochloride 600.0 mg; riboflavin 600.0 mg; pyridoxine hydrochloride 700.0 mg; nicotinic acid 3.0 mg; D-calcium pantothenate 1.6 mg; folic acid 200.0 mg; biotin 20.0 mg; cyanocobalamin 1.0 mg; retinyl palmitate 800.0 mg; DL-alpha-tocopheryl acetate 20.0 g; cholecalciferol 2.5 mg; menaquinone 50.0 mg; sucrose 972.9 g.

^e Celufil (United States Biochemical, Cleveland, OH)

^f Argo corn starch (CPC International Inc, Englewood Cliffs, NJ)

^g Choline chloride (Sigma Chemical, St. Louis, MO)

Table 3

Dietary ingredients (Processing comparisons and zinc effects)

Ingredient	Casein	HTC	Conventional
		g/100 g	
Protein source ^a	10.00	24.43	21.40
Corn oil	8.00	2.53	3.16
Salt mix	5.00 ^c	3.53 ^b	3.70 ^b
Vitamin mix ^d	1.00	1.00	1.00
Cellulose ^e	1.00	0.46	0.52
Water	5.00	4.66	4.87
Corn starch ^f	69.90	63.43	65.26
Choline ^g	0.10	0.10	0.10

^aProtein source was either spray-dried soymilk (HTC or conventional) or casein.

^b Self-mixed minerals based on AIN-93G, adjusted to the soyflour composition, in g/kg of mixture: CaCO₃, 395.00; KH₂PO₄, 196.00; NaCl, 74.00; K₂SO₄, 46.60; ferric ammonium citrate 6.06; ZnSO₄.7H₂O, 0.76; MnSO₄.H₂O, 0.93; CuSO₄, 0.36; KIO₃, 0.01; Na₂SeO₃, 0.01; (NH₄)₆Mo₇O₂₄.4H₂O, 0.006; Na₂SiO₃.9H₂O, 1.45; CrK(SO₄)₂.12H₂O, 0.275; LiCl, 0.013; H₃BO₃, 0.061; NaF, 0.063; nickel carbonate 0.03; ammonium metavanadate 0.007; finely powdered sucrose 207.94 g to make 1,000.00.

^c AIN-76 mineral mix (United States Biochemical, Cleveland, OH) (Please refer to table 2)

^d AIN-76A vitamin mixture (United States Biochemical, Cleveland, OH) (Please refer to table 2)

^e Celufil (United States Biochemical, Cleveland, OH)

^f Argo corn starch (CPC International Inc, Englewood Cliffs, NJ)

^g Choline chloride (Sigma Chemical, St. Louis, MO)

Table 4

Dietary ingredients (zinc bioavailability)

Ingredient	HTC	Conventional
g/100 g		
Soyflour	24.43	21.40
Corn oil	4.09	3.93
Salt mix ^a	3.64	3.75
Vitamin mix ^b	1.00	1.00
Cellulose ^c	0.44	0.51
Water	4.66	4.87
Corn starch ^d	61.49	64.29
Choline ^e	0.25	0.25

^a Self-mixed Zn-free mineral mixture based on AIN-93G adjusted to the conventional and HTC soyflour composition. Contains in g/kg mixture: CaCO₃, 296.24 for conventional, 367.35 for HTC; KH₂PO₄, 196.00; NaCl, 74.00; K₂SO₄, 46.60; ferric ammonium citrate 6.06; MnSO₄·H₂O, 0.93; CuSO₄, 0.36; KIO₃, 0.01; Na₂SeO₃, 0.01; (NH₄)₆Mo₇O₂₄·4H₂O, 0.006; Na₂SiO₃·9H₂O, 1.45; CrK(SO₄)₂·12H₂O, 0.275; LiCl, 0.013; H₃BO₃, 0.061; NaF, 0.063; nickel carbonate 0.03; ammonium vanadate 0.007; finely powdered sucrose to make 1,000.00.

^b AIN-76A vitamin mixture (United States Biochemical, Cleveland, OH) (Please refer to table 1).

^c Celufil (United States Biochemical, Cleveland, OH).

^d Argo corn starch (CPC International Inc, Englewood Cliffs, NJ).

^e Choline bitartrate (41.1% choline) (Harlan Teklad, Madison, WI).

Table 5

Protein efficiency^a, Body weight, and zinc Relationships^b

	Protein intake (g)	Final body weight (g)	PER	Pancreas wt./body weight (g/100 g)	Log total femur Zinc (Log µg)
Protein efficiency ratio ^c					
HTC ^d	36.2 ± 3.4 ^a	162 ± 10 ^a	2.69 ± .34 ^a	0.57 ± .12 ^a	1.75 ± .03 ^{ab}
Casein	34.9 ± 3.9 ^a	149 ± 23 ^b	2.39 ± .42 ^b	0.56 ± .08 ^{ab}	1.68 ± .05 ^a
Conv ^e	44.2 ± 4.0 ^b	172 ± 15 ^c	2.39 ± .17 ^b	0.48 ± .11 ^b	1.77 ± .05 ^b
Processing comparisons and Zinc effects ^f					
HTC	35.8 ± 2.7 ^{ab}	113 ± 4 ^a	1.86 ± .17 ^a	0.56 ± .09 ^a	1.45 ± .06 ^a
Casein	38.0 ± 3.8 ^a	136 ± 11 ^b	2.48 ± .25 ^b	0.59 ± .08 ^a	1.56 ± .06 ^b
Conv ^e	34.9 ± 2.9 ^b	122 ± 8 ^c	2.08 ± .19 ^c	0.56 ± .12 ^a	1.52 ± .06 ^b

^a Protein efficiency ratio, four week-values for weight gain/protein intake.

^b Results are expressed as mean ± SD. Means within a column within each experiment not sharing a common superscript are significantly different (P < 0.05).

^c n = 12, rats fed for 28 days, all diets contained zinc at 50 mg/kg.

^d Hydrothermally cooked soymilk diet.

^e Conventional soymilk diet.

^f n = 10 for protein intake and PER values, rats fed for 28 days; n = 8 for pancreatic weight /body weight and log total femur zinc, rats fed for 21 days, casein diet contained zinc at 50 mg/kg, zinc in soy diets at 20 mg/kg.

Table 6

Effect of dietary zinc and method of processing on intake, weight and PER Relationships (zinc bioavailability^a)

Diet	Zinc ^b	Feed intake	Wt. gain	Wt. gain/ protein	log total femur zinc
	mg/kg	g	g		µg
HTC ^c	10	236.48 ± 11.95	45.32 ± 5.15	1.90	1.23 ± .06
	17	258.40 ± 23.46	58.25 ± 7.17	2.25	1.39 ± .04
	24	251.93 ± 13.29	56.47 ± 6.31	2.24	1.47 ± .04
	31	279.56 ± 21.30	64.50 ± 4.00	2.31	1.56 ± .05
Conv ^d	10	264.97 ± 16.12	56.62 ± 6.50	2.13	1.25 ± .04
	17	275.95 ± 14.74	67.13 ± 5.95	2.42	1.42 ± .07
	24	278.12 ± 17.72	65.15 ± 9.86	2.33	1.53 ± .03
	31	302.14 ± 13.87	74.64 ± 7.05	2.48	1.59 ± .04

Analysis of Variance - P values

Diet	0.0001	0.0001	0.0031	0.0032
Zinc ^b	0.0001	0.0001	0.0001	0.0001
Diet*Zinc	0.8700	0.9611	0.8317	0.6505

^a Values are means ± SD; n = 6 for dietary zinc of 10, 17, 24 mg/kg; n = 5 for dietary zinc of 31 mg/kg; rats fed for 21 days.

^b Analyzed diet zinc concentration (i.e., 10, 17, 24, 31 mg/kg).

^c Hydrothermally cooked soymilk.

^d Conventional soymilk.

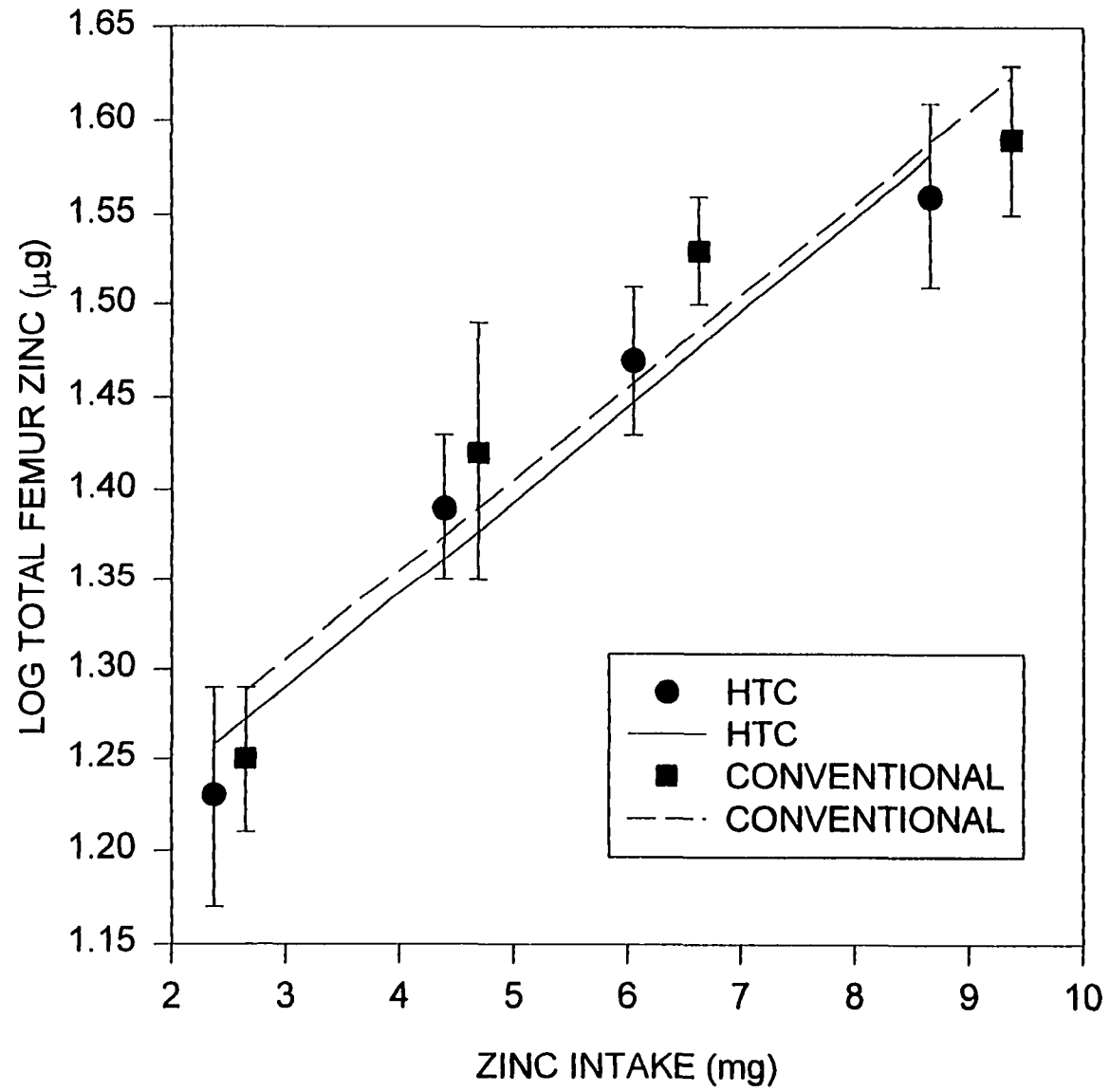
Fig. 1. Zinc bioavailability

Effect of zinc intake on femur zinc in rats fed hydrothermally cooked or conventionally processed, spray dried soymilk-based diets for 21 days. Basal dietary zinc was 10 mg/kg. Zinc sulfate was added in increments to produce additional dietary zinc levels of 17, 24, and 31 mg/kg. Each point represents the mean \pm SD of 6 observations (5 observations at the level of 31 mg/kg of zinc). The linear regression equations are:

HTC-fed group: $y = 1.136 + 0.0514x$ ($r = 0.98$);

conventionally-fed group: $y = 1.156 + 0.0518x$ ($r = 0.95$);

P values between slopes and between intercepts are not significant, ($P > 0.05$).



**BIOAVAILABILITY OF CALCIUM CITRATE MALATE ADDED TO
MICROBIAL PHYTASE-TREATED, HYDROTHERMALLY COOKED
SOYMILK**

A paper submitted to The Journal of Food Science
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Abstract

Effects of microbial phytase on the bioavailability of calcium from calcium citrate malate (CCM) added to corn/soy diets were investigated in chickens. Reference diets contained calcium as the carbonate. No statistically significant effect of phytase on the bioavailability of calcium from CCM was found, as shown by probabilities greater than 0.17 for weight gain, feed intake, tibia/body weight, ash%, and ash Ca% for corn/soybean meal and corn/hydrothermally cooked soymilk diets. For the corn/hydrothermally cooked soymilk diet with calcium at 0.76% of the weight of the diet as the carbonate, statistically significant ($P < 0.05$) decreases in the means

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for weight gain, feed intake, tibia/body weight, and ash% were observed in the group not treated with phytase. We suggest that CCM is less amenable to the formation of calcium phytate complexes than are other calcium salts and ,therefore, should be preferred for fortification of soymilk products.

Introduction

Hydrothermally cooked (HTC) soymilk possesses better physicochemical properties than soymilk cooked in the conventional manner (Johnson et al., 1980, 1981; Kim, 1988). We have reported that the protein efficiency ratio of HTC soymilk processed for 20 seconds is superior to that of conventionally processed soymilk if dietary zinc is at recommended levels (Lihono and Serfass, 1996).

The presence of phytate in soymilk impairs the bioavailability of minerals, especially zinc and calcium (Lönnerdal et al., 1984; Heaney et al., 1991; Zhou et al., 1992). We investigated the zinc bioavailability from HTC soymilk and found it similar to that of conventional soymilk in rat bioassays (Lihono and Serfass, 1996).

Another concern about soymilk is its intrinsically low calcium content. Soymilk containing 6% solids has about 3 mmol/L calcium compared with 25 mmol/L in cow's milk (Weingartner et al., 1983). Fortification of soymilk with

calcium has been difficult because most added soluble calcium salts tend to coagulate protein in soy beverages (Weingartner et al., 1983). The high tolerance of HTC soymilk for calcium salts might be utilized to improve the dispersion stability of calcium-fortified, soy-based dairy analogs (Wang, 1993).

The citrate, malate, carbonate, and tribasic phosphate salts of calcium are commonly used in the fortification of fruit juices and various drinks (FAO and WHO, 1990). However, there are other calcium sources with advantageous properties. Heaney et al. (1990) documented that solubility of calcium citrate malate (CCM) in water at neutral pH is about 80 mmol/L. This is 10, 100 and 500 times higher than that of calcium citrate, tricalcium phosphate, and calcium carbonate, respectively. Moreover, the fractional absorption value in humans for calcium from CCM is higher than from the other forms of calcium cited previously (Heaney et al. 1990).

Heaney et al. (1991) showed that phytate significantly affected intrinsic calcium absorbability from soybeans eaten by humans. This result does not agree with results from studies on rats (Poneros and Erdman, 1988; Churella and Vivian, 1989). Unlike humans, rats have intestinal phytase activity and, for this reason, may not be a good model for assessing calcium absorption from phytate-containing foods (Williams and Taylor, 1985; Heaney et al., 1991).

Chickens constitute a more suitable animal model for

studying calcium bioavailability in humans. Incubation of feedstuffs with microbial phytase has been effective in decreasing phytate phosphorus and in improving phosphorus bioavailability (Nelson et al., 1968; Denbow et al., 1995). In this paper, we report our initial investigations of the effects that microbial phytase treatment of soy has on the bioavailability of calcium from CCM in male broiler chickens. Calcium as the carbonate is used for purposes of comparison.

Materials & methods

Determination of activity of phytase

Phytase activity was measured by incubating 20 mg of the enzyme preparation with 5.1 mmol/L sodium phytate (Sigma Chemical Co., St. Louis, MO) at pH 5.5 and 37°C in a total volume of 250 mL; one unit of phytase activity is defined as the quantity of enzyme that liberates 1 μ mole of inorganic phosphorus per minute. The concentration of disodium citrate (buffer) was 50 mmol/L. After 30 and 60 min incubation, three 2-mL aliquots were taken from each incubation. The reaction was terminated by transferring each aliquot to a 100 mL volumetric flask and adding 20 mL molybdovanadate reagent to form a yellow complex. After dilution to volume, the concentration of liberated inorganic phosphate was determined by spectrophotometry at 400 nm (Method 965.17 of AOAC, 1995).

Incubation of suspended soyflakes with phytase

Dehulled soyflakes (Nichii Co., Jefferson, IA) were suspended at 10% by weight in 1 L tap water and incubated with 0.1 g (\approx 500 units) microbial phytase (Natuphos, BASF, Parsippany, NJ) at pH 5.6 and 37°C. The temperature was kept constant by using a water bath. Citric acid was used as a buffer at 1 g/L. Samples were taken 5, 10, 15, 20, 25, and 30 minutes after the incubation and directly transferred to an Erlenmeyer flask that contained 2.2 ml of 1 M NaOH to terminate the reaction. Samples were boiled to denature the enzyme. An amount equivalent to 2 g of dry sample (before boiling) was used for analysis of phytate phosphorus. Phytate phosphorus was liberated as in the Thompson and Erdman method (1982). Phosphorus was determined according to the AOAC method (Method 986.11, 1995).

Broiler-chicken feeding experiments*General procedures*

One-day-old, male broiler chicks were used in all experiments. The birds were housed in electrically heated battery brooders with wire mesh floors. The temperature was maintained at 22°C for the first week, then reduced to 20°C for the remainder of the experiment. Pens were randomly assigned to diets, and chicks were randomly allotted to pens. Chicks were allowed to consume feed and water *ad libitum*.

Weight gain and feed intake were determined weekly. Trials were conducted for 17 days each. At the termination of each trial, 2 chickens per pen were killed by cervical dislocation. Left and right tibias from the 2 chickens were removed for analysis of dry bone weight, ash, and ash calcium and phosphorus. In addition, ash zinc and liver iron and zinc were determined in one of the trials.

Experimental design

Bioavailability of calcium from CCM added to corn/soybean meal diets

Equal numbers of chicks were fed soybean-meal-based diets with or without 600 units of phytase per kg of diet. Each treatment had 5 dietary subgroups that contained four pens of four chickens each, for a total of 160 birds. Chickens in the five subgroups were fed diets with CCM added to provide calcium at nominal levels of 0.45%, 0.55%, 0.65%, or 0.75%, or with calcium carbonate added to provide 1% Ca. Chicks in the CCM subgroups were fed diets formulated to provide 0.56% total phosphorus so that the ratio of calcium to total phosphorus (Ca/tP) would be less than 1.4 even at the highest CCM concentration. Chicks in the calcium carbonate subgroups were fed diets designed to provide 0.45% non phytate phosphorus and 1% Ca, which are equivalent to recommendations of the National Research Council (1994). The calcium carbonate subgroups,

therefore, represent chicks under standardized dietary conditions to which the other subgroups can be compared.

Bioavailability of CCM added to phytase-treated HTC soymilk

Chicks were fed diets based on spray-dried soymilk that had been incubated with microbial phytase before hydrothermal cooking. They were compared with chicks fed diets based on HTC soymilk not treated with phytase. Full-fat soyflour from dehulled beans was suspended at 10% by weight in water and was incubated with ≈ 750 units/L of phytase at 35°C for 25-30 minutes before being processed in the HTC system. Equal amounts of phosphorus from $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ were added to the feed of the nonphytase and phytase-treated groups to provide experimental diets with a Ca/tP ratio less than 1.4 and a calcium/available phosphorus (Ca/nP) ratio less than 2.0 in the phytase-treated diets. Three levels of Ca (0.31%, 0.46%, 0.61%) at one level of P (0.54%) were used in the CCM diets, and one level of Ca (0.76%) was used in the diets of the calcium carbonate control groups. There were 4 chickens/pen and 5 pens per group.

Preparation of phytase-treated HTC soymilk

Dehulled beans were ground in a Fitzmill comminuting machine (Hammermill, model D, W.J. Fitzpatrick Co., Chicago, IL) through a 20-mesh screen to make full-fat soyflour.

Soyflour was slurried with tap water to give a soy extract of about 10% solids. Fifteen L of slurry were poured into a 30 L electrical kettle (Groen, model TDC/CT/40, Elk Grove Village, IL). The kettle was heated to 35°C and the microbial phytase (2.475 g) was added to make a concentration of ≈ 750 units/L. The tube containing the enzyme was rinsed 3 times and the rinse mixed with the slurry. The temperature in the kettle was maintained at 35°C for 30 min. Thereafter, the slurry was taken out and hydrothermally cooked via the steam-infusion system as previously described (Lihono and Serfass, 1996). The soybeans were a 50:50 mixture of two cultivars: Vinton and Lyxoygenasenol. The processing time was 20 s. The control HTC soymilk was prepared in the same manner as the phytase-treated soymilk, except phytase was not added and the incubation step was omitted. Soymilk was bulk spray-dried in an Anhydro Compact Spray Dryer (APV Crepaco Inc., model 93-4601, Attleboro Falls, MA). Flour was analyzed for nitrogen, phytate phosphorus, total phosphorus, fat, and moisture content.

Experimental diets

Bioavailability of calcium from CCM added to corn/soybean meal diets

Basal diet (Table 1) consisting of corn and dehulled soybean meal was formulated to meet or exceed the standards of the National Research Council (1994) except for Ca and P. Corn

and soybean meal were analyzed for protein, Ca, and P. The basal diet was formulated to contain 0.45 and 0.56% Ca and P (total), respectively. Chickens in the CCM dietary subgroups were fed the basal diet with 0.0, 0.1, 0.2, 0.3% Ca added as CCM. The calcium carbonate subgroup diets were formulated like the basal diet except CaCO_3 was the only calcium salt added to provide Ca at 1% of the diet. The calcium salts and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ replaced an equal weight of corn to achieve the desired Ca and P levels. The CCM was reduced to powder and screened through 2 mm mesh. For the phytase-treated diets, phytase was added at a level of 0.12% (≈ 600 units/kg diet). The liquid phytase for a 20 kg batch of diet was mixed with ≈ 35 ml of tap water and premixed with corn before being added to the diet. Mineral premix, vitamin premix, and soybean oil were added (Table 1), and the entire diet was mixed (Hobart mixer, model H600, Hobart Manufacturing Co., Troy, OH) for about 20 min.

Bioavailability of CCM added to phytase-treated HTC soymilk

A computer program (The Brill Corp., Norcross, GA) was used to formulate diets of corn/spray-dried soymilk to meet or exceed NRC standards (National Research Council, 1994) except for Ca and P. Diets were isocaloric and isonitrogenous. The basal diet is shown in Table 1. CCM (or CaCO_3 in control diets) and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ were used to attain the desired Ca and P levels. Solka floc fiber was added at different levels to bring the

energy content of diets to \approx 3200 kilocalories/kg. Also, vitamin premix, D-L methionine, salt, and mineral premix were added.

Sample preparation & analysis

After dilution, calcium from CCM was measured using atomic absorption spectrophotometry (Video 12, Thermo Jarrell Ash, Franklin, MA). Weighed portions of corn, soybean meal, and the diets were digested in fluoropolymer vessels in a microwave digestion apparatus (Milestone, model mls 1200 mega, Buck Scientific, Norwalk, CT) using concentrated HNO_3 and 30% H_2O_2 . The residues were dissolved in 0.7% HNO_3 and transferred to volumetric flasks for Ca and Zn analysis by atomic absorption spectrophotometry. La_2O_3 at 1% was added at the last dilution for Ca determination. For P determination, the samples were dry ashed, dissolved in HCl and HNO_3 , and filtered before analysis according to AOAC (1995). Phytate P was determined the same way as for incubated suspended soyflakes (Thompson and Erdman, 1982; AOAC, 1995).

Tibias were boiled for 3 minutes, and adhering tissues were removed (Waldroup et al., 1975) with cheesecloth. The tibias were dried in an oven at 100°C . When they had reached constant weight, tibias were ashed in the muffle furnace at $575\text{-}600^\circ\text{C}$ for 24 h. Ash portions that weighed ≈ 0.5 g were dissolved in 5 ml of concentrated HCl before dilution and

analysis for Ca and Zn.

Liver (≈ 5 g) from the large lobe was suspended in four volumes of distilled water and homogenized with a Potter-Elvehjem apparatus. About 5 g of each homogenate was digested in the microwave apparatus as described previously for diets, and Zn and Fe were determined by atomic absorption spectrophotometry.

Statistical analysis

In all trials, treatment means of body weight gain, feed consumption, dry bone weight, ash weight, and mineral content were compared by ANOVA (SAS Institute Inc., Cary, NC). Significant differences among treatment means were determined by using Duncan's new multiple range test with a 5% level of probability.

Results

Enzymatic activity of the microbial phytase was confirmed. Residual phytate P of suspended soyflakes was 56, 37, 22, 9, 4, and 3% of control (no phytase added) values after 5, 10, 15, 20, 25, and 30 minutes of incubation (Figure 1).

Analysis of CCM gave a calcium content (mean \pm SD, $n = 3$)

of $21.96 \pm 0.44\%$. Analyzed compositions of calcium, total phosphorus, and available phosphorus of each of the diets is presented in Table 2.

Table 3 presents the effects of supplemental microbial phytase on the bioavailability of calcium from CCM added to corn/soybean meal diets. There were no statistically significant effects of phytase treatment ($P > 0.05$). Calcium, however, affected tibia/body weight values ($P = 0.0009$). No statistically significant differences in any of the measured responses were found when means for the phytase-treated CaCO_3 subgroup at 1% calcium were compared with corresponding means for the nontreated subgroup. P values were 0.18 for weight gain, 0.15 for feed intake, 0.86 for tibia/body weight, 0.78 for ash%, and 0.34 for Ca% in ash.

There were no statistically significant effects ($P > 0.05$) of phytase treatment on the bioavailability of calcium from CCM added to HTC soymilk diets (Table 4). Calcium had a significant effect ($P < 0.05$) on weight gain, feed intake, ash%, Ca% in ash, and Zn content in ash. Means for the phytase-treated CaCO_3 subgroup were significantly different from corresponding means for the nontreated CaCO_3 subgroup when it came to weight gain ($P = 0.002$), feed intake ($P = 0.005$), tibia/body weight ($P = 0.011$), and ash% ($P = 0.001$). There was a significant interaction between phytase and calcium in the effect on liver iron. Mean ash-zinc concentration was

significantly less in the phytase-treated than in the non treated CaCO₃ subgroups.

Discussion

Phytase had no detectable effect on the bioavailability of CCM added to corn-soybean diets. Chicks fed phytase-treated or nontreated CaCO₃ at the optimal calcium level of 1% did not differ in their weight gain, feed intake, and tibia mineral content.

In the corn/soybean meal diets fed to the CCM subgroups, levels of calcium were below the Current National Research Council (1994) recommendation but were in accordance with levels of calcium used in other calcium bioavailability studies in broilers (Reid and Weber, 1976; Burnell et al., 1990; Guinotte et al., 1991). The lowest level of calcium used (0.45%) did not result in any signs of calcium deficiency in birds. Chicks fed CCM to provide calcium at 0.45% had weight gain and tibia ash percentage similar to those of chicks fed diets with a higher content of calcium. In contrast, previous studies by other investigators found that broilers fed other calcium salts at 0.45% exhibited impaired performance, showing that the Ca in CCM has higher bioavailability than in other calcium salts. Since phytase and calcium had no detectable effect on almost all the measured responses, a decision was

made to incorporate a 0.31% calcium level in our next study.

When calcium levels of 0.31, 0.46, and 0.61% were used and when broilers were fed phytase-treated or nontreated HTC soymilk diets where CCM was added, no effect of microbial phytase on the bioavailability of calcium was observed. In contrast, phytase treatment improved body weight gain, feed intake, tibia/body weight, and ash percentage in the broilers fed 0.76% calcium as the carbonate.

Kornegay (1996) and Denbow et al. (1995) have shown that a Ca/tP ratio less than 1.4/1 and a Ca/nP ratio less than 2/1 have a favorable effect on the release of phosphorus from phytate and on the magnitude of measured responses (body weight gain, toe ash percentage). For the trials we have conducted, calculated Ca/tP ratios for formulated calcium levels from 0.45 to 0.65% are lower than 1.4/1 in the phytase-treated CCM subgroups (Table 2). Still, the measured responses for this trial do not indicate any difference between the CCM phytase-treated and nontreated subgroups. Calculated values for Ca/tP in CCM subgroups of chicks fed phytase-treated HTC soymilk are lower than 1.4/1 except for the CCM phytase-treated subgroup at 0.61% calcium (Ca/tP of 1.46).

According to our results, CCM seems to have eliminated the effect of phytase on calcium bioavailability, although that effect can still be observed when calcium is provided as CaCO₃. Chicks fed diets with 0.61% calcium provided as CCM and

with or without phytase treatment had weight gain, tibia ash, and mineral content similar to those of chicks fed phytase-treated diets with 0.76% calcium as the carbonate.

CCM has advantageous solubility and fractional absorption properties (Heaney et al., 1990). Perhaps calcium in CCM is hindered from forming a complex with phytic acid, preventing any adverse effects of phytic acid on calcium in nonphytase treated groups (Cheryan, 1980; Heaney et al., 1990).

More experiments will be needed to confirm the observation that the bioavailability of calcium from CCM is high for broilers. The Ca/nP ratios higher than 2/1 in some of our phytase-treated CCM subgroups may have contributed to the inhibition of the effect of microbial phytase observed in our studies.

The hydrolysis of phytate before processing of HTC soymilk was not as complete as desired. Analysis of the spray-dried soymilk indicated phytic acid content of 0.31% and 1.01% for the phytase and nonphytase treated samples, respectively. We attribute the incomplete hydrolysis to deterioration of the microbial phytase during prolonged refrigerated storage. Calculated values for available phosphorus were 0.37% for the basal phytase-treated experimental diet and 0.28% for the nontreated one. If hydrolysis had been complete, the level of available phosphorus would be about 0.44% in the basal phytase-treated experimental diet. Perhaps an effect of

phytase on bioavailability of calcium from CCM would be observed in such a circumstance, but we consider it unlikely that the effect would be as marked as when calcium is provided as CaCO_3 .

The high dispersion stability of HTC soymilk in the presence of soluble calcium salts such as CCM may help in the preparation of shelf-stable, calcium-fortified, fluid soymilk products.

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Table 1. Composition of the basal diets

Item	Corn/soybean meal	Hydrothermally cooked
	diets ¹	soymilk diets ²
	%	
Corn, crude		
protein 7.01%	52.31	50.85
Soybean meal, crude		
protein 45.95%	41.06	-----
Soymilk ³	-----	42.56
Soybean oil	4.00	-----
CCM ⁴	1.48	0.86
NaH ₂ PO ₄ ·H ₂ O	0.60	0.60
Mineral premix ⁵	0.05	0.05
Vitamin premix ⁶	0.30	0.30
Dl-methionine ⁷	0.20	0.16
Solka floc ⁸	-----	4.52
Salt ⁹	-----	0.11
As formulated		
Protein (%)	22.6	23.0
Total Phosphorus (%)	0.56	0.54
Metabolizable energy (kcal/kg)	3106	3200

¹Calcium carbonate control subgroups contained 2.26% CaCO₃ (38% Ca) as the sole calcium salt, 1.42% NaH₂PO₄·H₂O, and 50.71% corn.

²Calcium carbonate control subgroups contained 1.73% CaCO₃ (38% Ca) as the sole calcium salt, and 3.45% of Solka Floc.

³Phytase-treated or non-treated spray-dried soymilk, 45.94% crude protein.

⁴Calcium citrate malate; Procter & Gamble, Cincinnati, OH.

⁵Supplied per kg of diet: manganese, 11.67 mg; zinc, 6.67 mg; iron, 6.17 mg; copper, 1 mg; selenium, 0.025 mg; sodium chloride, 0.43 g.

⁶Supplied per kg of diet: vitamin A (retinyl acetate), 5,000 IU; cholecalciferol, 1,500 IU; dl- α -tocopheryl acetate, 12 IU; vitamin B₁₂, 11 μ g; vitamin K (menadione sodium bisulfite), 1.8 mg; riboflavin, 2.7 mg; pantothenic acid, 7 mg; niacin, 75 mg; choline, 509 mg; folic acid, 0.55 mg; biotin, 75 μ g.

⁷Degussa Corp., Ridgefield Park, NJ.

⁸Brown Co., Berlin, NH.

⁹Morton Co., Chicago, IL.

Table 2. Analyzed calcium, total phosphorus, and available phosphorus in diets. Calcium/total phosphorus and calcium/available phosphorus ratios of CCM subgroups

Formulated Ca (%)	Ca (n=6) (%)	Total P (n=3) (%)	Phytate P (n=2) (%)	Available P (%)	Ca/total P ratio	Ca/available P ratio
Bioavailability of calcium from CCM added to corn/soybean meal diets						
CCM nontreated						
0.45	0.48 ± 0.06	0.54 ± 0.08	0.25	0.29	0.88	1.66
0.55	0.59 ± 0.06	0.50 ± 0.01	0.26	0.24	1.18	2.45
0.65	0.69 ± 0.07	0.48 ± 0.04	0.27	0.21	1.44	3.29
0.75	0.81 ± 0.06	0.56 ± 0.09	0.24	0.32	1.45	2.53
treated						
0.45	0.49 ± 0.02	0.51 ± 0.03	0.28	0.23	0.96	2.13
0.55	0.57 ± 0.02	0.56 ± 0.1	0.24	0.32	1.02	1.78
0.65	0.68 ± 0.02	0.49 ± 0.06	0.25	0.24	1.39	2.83
0.75	0.82 ± 0.05	0.47 ± 0.04	0.25	0.22	1.74	3.73
CaCO₃ nontreated						
1.00	1.05 ± 0.08	0.66 ± 0.02	0.23	0.43		
treated						
1.00		0.68 ± 0.06	0.25	0.43		
Bioavailability of CCM added to phytase-treated, hydrothermally cooked soymilk						
CCM nontreated						
0.31	0.33 ± 0.01	0.50 ± 0.06	0.22	0.28	0.66	1.17
0.46	0.51 ± 0.05	0.48 ± 0.04	0.22 ²	0.26	1.06	1.96
0.61	0.67 ± 0.03	0.48 ± 0.06	0.22 ²	0.26	1.39	2.58
treated						
0.31	0.32 ± 0.03	0.48 ± 0.04	0.11	0.37	0.67	0.86
0.46	0.52 ± 0.05	0.53 ± 0.07	0.11 ²	0.42	0.98	1.24
0.61	0.69 ± 0.02	0.47 ± 0.03	0.11 ²	0.36	1.46	1.92
CaCO₃ nontreated						
0.76	0.99 ± 0.02	0.49 ± 0.04	0.22	0.27		
treated						
0.76	0.96 ± 0.02	0.54 ± 0.02	0.13	0.41		

²Phytate phosphorus values from the basal diet.

Table 3. Effects of supplemental microbial phytase (0.12 g/kg diet) on dry weight and mineral content of tibia (average of left and right) and performance (days 1 to 17) of broilers on corn/soybean meal diets¹

Formulated Ca content	Analyzed dietary content			Performance		Tibia mineral content		
	Ca (%)	nP ² (%)	Zn ppm	Weight gain (g)	Feed intake (g)	Tibia/ body wt. (%)	Ash (%)	Ash Ca (%)
CaCO ₃ ³ nontreated								
1.00	1.05	0.43		434 ± 24 ^x	619 ± 46 ^x	0.52 ± 0.03 ^x	46.3 ± 1.3 ^x	32.8 ± 2.1 ^x
treated								
1.00	0.99	0.43		483 ± 59 ^x	693 ± 79 ^x	0.52 ± 0.05 ^x	46.6 ± 0.7 ^x	35.4 ± 4.6 ^x
CCM nontreated								
0.45	0.48	0.29	33.3	439	645	0.43	39.1	34.4
0.55	0.59	0.24		419	631	0.48	39.5	36.4
0.65	0.69	0.21		440	646	0.48	42.4	38.1
0.75	0.81	0.26	33.5	438	646	0.54	38.5	34.6
treated								
0.45	0.49	0.24	33.2	449	658	0.45	39.5	35.6
0.55	0.57	0.22		430	633	0.49	39.8	33.7
0.65	0.68	0.24		452	647	0.48	42.7	34.6
0.75	0.82	0.22	32.1	457	657	0.49	41.4	36.6
Pooled SEM				9.23	2.08	0.011	0.93	1.49
Source of variation				Probabilities ⁴				
Phytase				0.18	0.51	0.38	0.30	0.66
Calcium				0.28	0.50	0.0009	0.08	0.89
Phytase × calcium				0.98	0.97	0.094	0.69	0.44

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¹Four pens of 4 birds each per treatment mean.

²Nonphytate phosphorus.

³Compares phytase treated vs. phytase nontreated CaCO₃ subgroups at 1% formulated Ca; means not sharing a common superscript are significantly different (P < 0.05).

⁴Compares phytase treated vs. nontreated CCM subgroups, probability at 0.05%.

Table 4. Effect of phytase-treated, hydrothermally cooked soymilk on dry weight, mineral content of tibias, and mineral content of liver, and performance (days 1 to 17) of broilers ¹

Analyzed dietary content				Weight Feed		Tibia/ body wt. (%)	Mineral content				Tibia		liver	
Ca ² (%)	Ca (%)	nP ³ (%)	Zn ppm	gain (g)	intake (g)		Ash (%)	Ash (%)	Ca ppm	Ash ppm	Zn ppm	Zn ppm	Fe ppm	
CaCO ₃ ⁴ nontreated														
0.76	0.99	0.27	35	293 ± 41 ^x	511 ± 32 ^x	0.29 ± 0.01 ^x	35.4 ± 0.9 ^x	36.3 ± 4.6 ^x	365 ± 23 ^y		21.6 ± 1.4 ^x	174 ± 56 ^x		
treated														
0.76	0.96	0.41	34	404 ± 35 ^y	614 ± 52 ^y	0.32 ± 0.01 ^y	40.7 ± 2.3 ^y	36.2 ± 9.6 ^x	306 ± 26 ^x		22.9 ± 0.6 ^x	177 ± 72 ^x		
CCM nontreated														
0.31	0.33	0.28	37	333	534	0.27	32.4	34.4	466		26.3	182		
0.46	0.51	-----	35	398	580	0.30	34.9	35.0	445		23.3	179		
0.61	0.67	-----	36	450	653	0.30	37.3	36.2	362		24.4	269		
treated														
0.31	0.32	0.37	35	292	504	0.27	31.9	34.2	489		23.7	272		
0.46	0.52	-----	39	446	638	0.29	35.4	35.4	415		22.5	228		
0.61	0.69	-----	35	471	673	0.31	39.2	36.4	358		23.9	163		
Pooled SEM				16.72	20.66	0.007	0.50	0.37	14.25		1.13	34.50		
Source of variation						Probabilities ⁵								
Phytase						0.54	0.39	0.97	0.17	0.73	0.76	0.22	0.72	
Ca						0.0001	0.0001	0.0007	0.0001	0.0001	0.0002	0.24	0.82	
Phytase × Ca						0.0651	0.17	0.32	0.10	0.71	0.24	0.68	0.038	

¹Formulated calcium content.

²Five pens of 4 birds each per treatment mean.

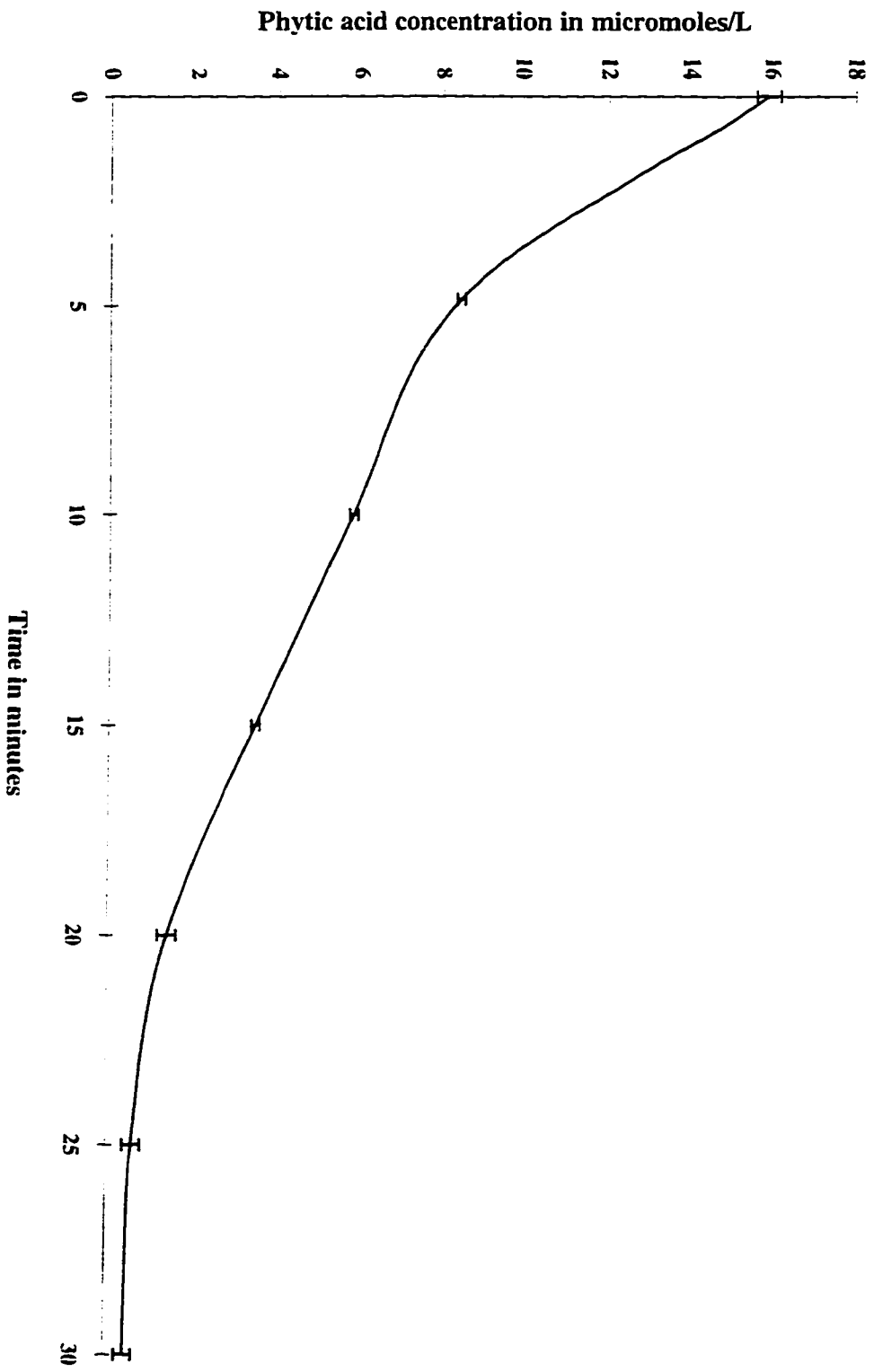
³Nonphytate phosphorus.

⁴Compares phytase treated vs. nontreated CaCO₃ subgroups at 0.76% formulated Ca; means not sharing a common superscript are significantly different (P < 0.05).

⁵Compares phytase treated vs. nontreated CCM subgroups, probability at 0.05%.

Fig. 1. Residual phytic acid from incubated soyflakes

Dehulled soyflakes (Nichii Company, Jefferson, IA) were suspended at 10% by weight in 1 liter of water and incubated with 0.1 g (\approx 500 units) of microbial phytase (Natuphos, BASF Corp., Parsippany, NJ) at pH 5.6 and 37°C. Each point represents the mean \pm SD of 2 samples analyzed for phytic acid after the incubation reaction was terminated.



GENERAL CONCLUSIONS

In the first paper, protein utilization and zinc bioavailability from HTC soymilk as compared with a conventionally processed soymilk were assessed when fed to rats. HTC processed for 20 sec had a PER at least equal to that of casein and higher than that of a conventional soymilk when dietary zinc was at the recommended value of 50 mg/kg. When dietary zinc was 20 mg/kg, PER was less for HTC soymilk processed for 40 sec than for conventional soymilk. Zinc bioavailability of both types of soymilk was not different by the slope ratio bioassay procedure. Feeding of spray-dried HTC processed soymilk did not result in pancreatic hypertrophy in rats.

In the second paper, bioavailability of calcium from CCM added to microbial phytase-treated, hydrothermally cooked soymilk or corn-soybean meal diets was determined by a bioassay procedure in broiler chickens. No effect of the microbial phytase was found on the bioavailability of CCM added to corn-soybean or HTC soymilk diets. Chicks fed with calcium at 0.61% provided as CCM and with or without phytase treatment had weight gain, tibia ash, and mineral content similar to those of chicks fed phytase-treated diets with calcium at 0.76% as the carbonate. It seems that CCM has eliminated the effect of phytase on calcium bioavailability,

although that effect can still be observed when calcium is provided as CaCO_3 . More experiments will be needed to assess the bioavailability of calcium from CCM as compared to that from CaCO_3 in broilers.

These are the first *in vivo* experiments to investigate the nutritional characteristics of HTC soymilk. Results from the PER studies suggest that the protein quality for rats of the HTC soymilk diet is more related to its zinc content than is that of the conventional soymilk diet. Zinc bioavailability results confirm that fortification with inorganic zinc of soy protein foods improves the zinc bioavailability from the soy products to rats. Fortification of soymilk with calcium and zinc is desirable. CaCM presents solubility characteristics in water, absorption properties in humans, and, if confirmed, bioavailability characteristics in broilers that are strongly supportive of its use in the formulation of acceptable, stable, and nutritious fluid products from HTC soymilk.

APPENDIX

Effects of supplemental microbial phytase at 600 units/kg on dry weight and mineral content of tibia (average of left and right) and body weight (days 1 to 14) of broilers, preliminary trial bioavailability of calcium from CaCM added to corn/soybean meal diets¹

Formulated Ca content	Analyzed dietary content			Tibia mineral content				
	Ca (%)	nP ² (%)	Zn (ppm)	Final body weight (g)	Tibia/ body wt. (%)	Ash (%)	Ca (%)	P (%)
CaCO ₃ non-treated 1.00	1.05	0.43		416 ± 26	0.50 ± 0.04	48.0 ± 0.7	35.3 ± 0.3	4.2 ± 0.5
CCM non-treated 1.00	0.95			398 ± 24	0.51 ± 0.06	47.3 ± 1.3	35.3 ± 0.6	4.2 ± 0.2
CCM non-treated 0.45	0.48	0.29	33.3	406	0.40	40.0	34.5	3.6
0.55	0.59	0.24	----	415	0.43	40.9	34.7	3.6
0.65	0.69	0.21	----	403	0.44	46.0	36.0	4.2
0.75	0.81	0.26	33.5	400	0.42	41.8	35.7	3.7
treated 0.45	0.49	0.24	33.2	447	0.43	40.9	34.2	3.8
0.55	0.57	0.22	----	441	0.43	42.3	34.8	3.8
0.65	0.68	0.24	----	453	0.44	45.3	35.8	4.1
0.75	0.82	0.22	32.1	397	0.44	42.8	35.4	3.9
Pooled SEM				1.03	0.002	0.50	0.51	0.06
Source of variation	Probabilities ³							
Phytase				0.0001	0.17	0.21	0.74	0.23
Calcium				0.0007	0.32	0.0001	0.16	0.008
Phytase × calcium				0.0063	0.54	0.47	0.99	0.86

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¹Four pens of 4 birds each per treatment mean.

²Non phytate phosphorus.

³Compares phytase treated vs. non-treated of CCM subgroups, probability at 0.05%

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