

Studies on the Improvement of Mineral Availability
of Soybean Meal by the Fermentation with
Aspergillus Usamii in Animals

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Chapter 1

Introduction

Soybean products are important traditional sources of protein for many Asian populations. In recent years the consumption of soybean products has been increasing in Western countries (Torun et al., 1981). The increasing rate of consumption of soybean products in human diets has caused much concern about unfavorable effects on mineral availability. Many reports have shown the decrease of mineral absorption in diets containing soybeans was due to the large amount of phytic acid in soybean products (Cook et al., 1981; Forbes et al., 1979; Hurrell et al., 1992; Prattley et al., 1982a,b; Thompson and Erdman, 1984). Phytic acid is known to inhibit availabilities of minerals thorough forming insoluble complexes with minerals in the digestive tract (Cheryan, 1980). There have been trials to reduce phytic acid in feeds and foods using several methods. Phytic acid in wheat bran was degraded by extrusion cooking (Sandberg et al., 1987), by soaking to activate endogenous phytase (Morris and Ellis, 1980), by fermentation using yeast (Widdowson, 1941), or by adding phytase to feed (Lei et al., 1993; Mroz et al., 1994) or food (Sandberg and Anderson, 1988). Phytic acid in soy protein was removed by alkaline solution (Hartman, 1979) or by ion exchange resin (Niiyama et al., 1992). Phytic acid in soy plants was reduced by the sand culture technique (Zhou et al., 1992). Mineral availability was improved in some of these experiments. Fermentation is traditional method for food process in the Orient. Ilyas et al. (1995) reported that fermentation of soybean meal with *Aspergillus usarii* almost completely degraded phytic acid into inorganic phosphorus and inositol. Firstly, this study investigated the effects of

fermented soybean meal on mineral availability in rats.

Zhu et al. (1990) indicated that incubation of soybean meal with wheat bran partly degraded phytate in soybean meal, which was due to phytase activity (EC 3.1.3.8) from bran. Morris and Ellis (1980) observed that soaking of wheat bran increased zinc concentration in the femur of rats through degrading phytate by activating endogenous phytase in bran. Although non-fermented soybean products showed little or no phytase activity (Mollgard et al., 1946), phytase activity was found in soybean meal fermented by *Aspergillus usamii* (Ilyas et al., 1995). Secondly, this study examined the efficacy of phytase activity from the fermented soybean meal on mineral availability in diets containing phytate.

More than half of phosphorus in soybean products exists as phytate-phosphorus (Earle and Milner, 1938). The digestibility of phytate-phosphorus is low for monogastric animals and therefore the supplementation with inorganic phosphorus is needed to the soybean meal-based diets. However, the supplementation with inorganic phosphorus induces a large amount of phosphorus in animal waste, which causes an environmental pollutant. Furthermore, the supplementation with inorganic phosphorus increases animal feed costs. Thirdly, this study investigated the effects of soybean meal on phosphorus availability in chicks.

Chapter 2

Literature review**I. Properties of Protein in Soybean**

Soybean protein is good protein sources because it abounds in essential amino acids, especially glutamic acid, aspartic acid and lysine. Meat proteins contain a large amount of cholesterol and saturated fatty acids. On the other hand, soybean protein contains unsaturated, essential fatty acids such as linoleic acid and linolenic acid.

Main compositions of protein in soybeans are glycinin (11S globulin) and conglycinin (7S globulin), which account for 70 % of protein in soybeans and their molecular weights are about 350 kDa and 180 kDa, respectively, and molecular weights of subunits consisting of glycinin and conglycinin are more than 50 kDa.

II. Unfavorable Materials of Soybean for Foods

Soybeans contain unfavorable materials for foods and feeds, which reduce nutritional quality of the protein. Protease inhibitors induce the hypertrophy and the hyperplasia of the pancreas, which inhibit the growth by reducing available amino acids (Lyman and Lepkovsky, 1957; Booth et al., 1960). Protease inhibitors are inactivated by heat treatment (Rackis and Gumbmann, 1982). However, only partial inactivation by heat can be induced in some cases and it has not been unclear what residual level of protease inhibitor activity affect the human health. Lectins also inhibit the growth by lowering absorption of nutrients through binding glycoprotein receptors on the epithelial cells lining the intestinal mucosa (Ishiguro et al., 1992; Jindai, et al., 1984; Pusztai et al., 1990).

The soybean lectin is inactivated by moist heat treatment (Liener and Hill, 1953) but it is quite resistant to inactivation by dry heat treatment (de Muelenaere, 1964). Unheated soybeans were reported to cause an enlargement of the thyroid gland in rats (McCarrison, 1933; Sharpless et al., 1939) and chicks (Patton et al., 1939; Wilgus et al., 1941), which could be prevented by iodine supplementation (Block et al., 1961; Halverson et al., 1949) or partially eliminated by feeding heated soybeans as substitute for unheated soybeans (Wilgus et al., 1941). These substances, which exist in soybeans, showing goitrogenic property have not been clear. Raw soybeans also contain lipoxigenase, which is the cause for grassy odor in soybean products. Lipoxigenase oxidates and destroys carotene (Sumner, 1939). Furthermore, unheated soybeans contain heat-labile substances which increase the requirements for cyanocobalamin (Edelstein and Guggenheim 1970; Frohlich, 1954) and α -tocopherol (Fisher et al., 1969).

Tannins, glycoside, phytate and flatulence-producing factors are heat-stable components in soybeans. However, these components except for phytate do not severely affect nutritional quality of soybean proteins and physiological condition of animals. Tannins depress the growth by decreasing absorption of protein and carbohydrate (Deshpande and Salunkhe, 1982; Griffiths, 1986) but the contents are much less in soybeans compared with most other legumes (Liener, 1994). Glycoside gives obnoxious odor and taste to soybean products. Saponins are one of glycoside but have effects of anti-lipidemia, anti-oxidation and anti-cholesterol (Birk and Peri, 1980; Cheeke, 1976; Oakenfull, 1981; Oakenfull and Sidhu, 1989; Price et al., 1987). Isoflavones, which are the other

kind of glycoside in soybeans have anticarcinogenic properties, antioxidative activities and weak estrogenic properties (Carter et al., 1953; Drane et al., 1980; Naim et al., 1974; Walter, 1941).

III. Phytic Acid in Soybean

Phytic acid is normally found in the form of complexes with essential minerals and/or protein. In vitro studies have shown that phytic acid-protein complexes are formed by electrostatic interactions involving the terminal α -amino groups, the ϵ -amino group of lysine, the imidazole group of histidine, and the guanidyl groups of arginine (Cheryan, 1980). Many of these complexes are insoluble and are not biologically available for humans under normal physiological conditions (Cheryan, 1980; deRham and Jost, 1979; Hartman, 1979). In addition, these proteins are less subject to attack by proteolytic enzymes than free proteins (Champagne and Phyllippy, 1989; Frolich, 1984, 1985; Rodriguez et al., 1985).

Soybean seeds contain phytic acid of 0.72 to 1.8 % by weight of defatted meal and of 1 to 1.47 % by dry weight of soybean seeds (Lolas and Markakis, 1975). Phytic acid and its salts are considered to be one of the major forms of phosphorus in many plant seeds. Typically approximately 60 to 90 % of phosphorus in these seeds exist as phytate-phosphorus (Webster, 1928). In soybean seeds, 75 % of phosphorus exist as the form of phytic acid, 12 % as phosphatides, 4.5 % as inorganic phosphorus, and the rest is unknown composition, possibly including nucleic acid.

In contrast to many other oilseeds and cereals, there appears to be no specific location for phytic acid in soybean seeds. O'Dell et al. (1972) found that

90 % of phytic acid in corn concentrated in the germ. Most phytic acid in wheat and rice was reported to exist in the outer layers, the pericarp and aleurone. In other oilseeds such as peanut (Dieckert et al., 1962), cottonseed (Lui and Altschul, 1967) and sunflower (Saio et al., 1977), phytic acid was found to be concentrated within crystalloid-type substructures of globoids, which might serve as storage sites. Since protein bodies of the soybeans did not possess globoids, phytic acid was suggested to exist throughout the kernel of soybeans (Tomb, 1967). Thus it seems to be difficult to remove phytic acid from soybeans. On the other hand, phytic acid can be reduced in many plant seeds except for soybeans because phytic acid appears to be associated with specific component within plant seeds.

IV. Removal of Phytic Acid in Soybean

A large number of oilseeds and cereals except for soybean seeds can separate selectively phytic acid by mechanical processes such as milling and grinding operations. Since over 90 % of phytic acid in corn is in germ and most of phytic acid in wheat and rice are in the outer layers (O'Dell et al., 1972), milling and degerming processes were reported to reduce phytic acid in these seeds effectively (Donnelly and Tabekhia, 1977). Polishing was found to remove phytic acid in rice (Sathe and Krishnamurthy, 1953; Tabekhia and Luh, 1979).

IV-1. Water extraction and differential solubility Since phytic acid is present in native soybeans as an almost totally water-soluble form and the solubility profiles of protein and phytic acid do not exactly coincident (deRham and Jost, 1979; Fontaine et al., 1946), one of the methods to remove phytic acid is to carefully adjust pH of a water extract of soybeans to a point

where one component is far less soluble than the others and then to remove insoluble component by filtration or centrifugation. Solubility of phytic acid was reported to decrease between at pH 4.5 and 8, increase between at 8 and 10 and be minimum at pH above 11 (Gillberg and Tornell, 1976). Goodnight et al. (1978) reduced phytic acid in soy protein compounds by basification of aqueous extracts of defatted soy flakes to pH 11 to 12 and then by centrifugation to separate the insoluble phytic acid from the soluble compounds of the extract. However, careful control of temperature and centrifugal force are required to use this method successfully since phytate salts can exist in very fine suspension. At moderate pH between 6 to 10, phytic acid exists as ternary protein-cation-phytic acid complexes. Successful removal of phytic acid from the system at this pH range depends on the ability to dissociate the complexes. One method is the addition of competitive chelators such as EDTA to the system prior to processing. Since some cations bind EDTA preferentially rather than phytic acid, soluble cation-EDTA complexes are formed which prevented the formation of protein-cation-phytic acid complexes, and then phytic acid can be removed from the system by ultrafiltration (Okubo et al., 1975). However, methods based on molecular size differences such as dialysis or ultrafiltration are needed to separate phytic acid from protein in solution. DeRham and Jost (1979) separated protein from suspension containing phytic acid at pH 5.5 because protein was insoluble and phytic acid was soluble at this pH. However, separation was incomplete by usual centrifugal forces and residence times in commercial disc-type deluging centrifuges. As a result, methods based on differential solubility are on laboratory scale and not suitable for practical use.

DeRham and Jost (1979) found phytic acid precipitated by adding more than 8.5% of NaCl at pH 7.5 without forming complex with protein and thus protein existed in soluble fraction. However, a salt level in the final protein products is excessively high and the salt must be removed from the products to be useful. Hill and Tyler (1954) observed that the addition of extra calcium prevented phytic acid making complex with protein at pH 3 to 4 in soybean-water extracts. On the other hand, the addition of calcium ion to soybean-water extracts under low pH resulted in bitter off-flavors and darker colored products.

IV-2. Enzyme treatment Phytic acid is hydrolyzed by the enzyme, phytase, and the main three sources of this enzyme are plant phytase, microbial phytase and intestinal phytase. Wheat and rye contain a high activity of phytase, barley contains considerably less active phytase, and oats and maize possess negligible the enzyme (McCance and Widdowson, 1944). All soy preparations themselves show little or no phytase activity (Mollgard et al., 1946). The optimum pH for plant phytase is reported to be approximately 4.0 to 6.0 and the activity probably be depressed in the acid condition of the stomach (Irwing, 1980). On the other hand, microbial phytase is suggested to be active over a wide pH range than plant phytase (Ullah and Cummins, 1988). Phytase produced by *Aspergillus nigar*, which is frequently used for phytate hydrolysis in foods or feeds, has two pH optima; one at 2.5 to 3.0 and the other at 5.0 (Simell et al., 1991). The addition of *Aspergillus nigar* phytase to feeds has reported to increase absorption of phosphorus (Lei et al., 1993a, b; Nasi, 1990; Nelson et al., 1971; Pallauf et al., 1994; Simons et al., 1990), zinc (Lei et al., 1993c; Pallauf et al., 1994; Rimbach and Pallauf, 1992, 1993), calcium (Pallauf et al., 1994),

magnesium (Pallauf et al., 1994; Rimbach et al., 1995) and iron (Sandberg et al., 1996). Lei et al. (1993b) reported that 1200 U/kg of phytase activity was needed to maximally degrade phytate-phosphorus in pigs fed maize-soybean meal-based diet. Kornegay and Qian (1996) also reported that 1050 U/kg of phytase activity maximally degraded phytate in maize-soybean meal-diets containing 0.7 g/kg of available phosphorus and 700 U/kg of the enzyme activity in diets containing 1.6 g/kg of available phosphorus.

Phytic acid, i.e., inositol hexaphosphate (IP_6), in wheat bran was reported to be hydrolyzed to lesser phosphorylated derivatives of inositol such as inositol tri- (IP_3), tetra- (IP_4), and penta- (IP_5) phosphates during the digestion in the gut (Sandberg and Ahderinne, 1986; Sandberg et al., 1987; Sandberg and Andersson, 1988). However, phytic acid in extrusion cooked-bran was not hydrolyzed and IP_3 , IP_4 and IP_5 did not increase in the digestive tract, which was suggested to be due to degraded endogenous phytase activity by extrusion cooking (Sandberg et al., 1987; Sandberg and Andersson, 1988). Lesser phosphorylated derivatives of inositol were suggested to form more soluble complexes with minerals compared with phytic acid (Lonnerdal et al., 1989; Sandstrom and Sandberg, 1992; Simpson and Wise, 1990). Furthermore, Han et al. (1994) showed that solubilized IP_3 , IP_4 , IP_5 and IP_6 inhibited transports of zinc and iron but the inhibitory effects of IP_3 and IP_4 were weaker than those of IP_5 and IP_6 in human intestinal cell line.

Intestinal phytase and alkaline phosphatase present in the brush-border of the mucosal cells were not suggested to play an important role on phytate hydrolysis in rats (Miyazawa et al., 1996; Shinoda and Yoshida, 1989) and in

man (Iqbal et al., 1994; Sandberg and Andersson, 1988).

IV-3. Other treatment Autoclaving of soy isolates at 115°C for 4 hours reported to destroy phytic acid (O'Dell, 1969) but this excessive heat treatment was suggested to destroy essential amino acids and depress nutritive value of soybeans (Rackis, 1974). Zhou et al. (1992) reduced phytic acid in soy plants by the sand culture technique.

V. Phytic Acid and Mineral

Phytic acid has six strongly dissociated protons (pKs 1.1 to 2.1) and six weakly dissociated protons (pKs 4.6 to 10.0). Since these ionizable protons dissociate, phytate can make stable complexes with metallic cations which show at least five kinds of structures (Nolan et al., 1987). Phytic acid possibly makes complex with cation within a single phosphate group or between two phosphate groups on either the same or different molecules (Erdman, 1979; Fox and Tao 1989).

V-1. Calcium and phytic acid Phytic acid has been reported to make complex with calcium and reduce availability of calcium in vitro (Champagne and Phillippy, 1989; Nolan et al., 1987; Platt and Clydesdale, 1987). However, phytic acid was suggested to inhibit calcium availability when dietary phytic acid to calcium molar ratio was more than 0.2 in man (Morris and Ellis, 1985) and in chicks (Nelson and Kirby, 1987). Helander et al. (1996) reported the addition of microbial phytase improved calcium absorption in pigs fed a diet containing insufficient amount of available phosphorus but did not affect calcium balance in animals fed a diet containing a sufficient amount of available phosphorus.

On the other hand, it has been suggested that calcium has synergistic

effect of phytic acid on mineral availability. Calcium was reported to exhibit synergistic action on the precipitation of phytate-complex with zinc (Oberlease et al., 1966) and with iron (Platt and Clydesdale, 1987; Rao and Rao, 1983). Zinc absorption was observed to be greatly reduced by adding calcium to diets containing phytic acid (Forbes, 1964; Likuski and Forbes, 1964; Oberleas et al., 1966). Furthermore, Lantzsche et al. (1995) reported that increasing dietary calcium level progressively inhibited the rise of apparent phosphorus absorption by adding phytase. Soybean concentrates contain more calcium compared with other oilseeds such as groundnut, rapeseed meal, sunflower meal and cottonseed meal (Bamgbose, 1995).

V-2. Magnesium and phytic acid Roberts and Yudkin (1960)

found that rats fed diets containing sodium phytate developed magnesium deficiency which resulted in severe illnesses. Phytic acid was observed to decrease soluble magnesium in vitro (Champagne, 1988; Cheryan et al., 1983) and in the digestive tract (Shinoda and Yoshida, 1989). However, magnesium bioavailability was reported to be high in soybean as same as in skim milk (Guenter and Sell, 1974), casein and beef (Lo et al., 1980). Brink and Beynen (1992) suggested that the fall of apparent magnesium absorption caused by phytic acid may be compensated by increasing magnesium intake and reducing urinary magnesium excretion in rats fed practical diets containing a sufficient amount of magnesium. On the other hand, Miyazawa et al. (1996) reported that dietary phytate decreased apparent magnesium absorption in rats fed diets containing an enough amount of magnesium.

V-3. Zinc and phytic acid Zinc was reported to be essential

mineral more adversely affected by phytic acid (Fox and Tao, 1989; Erdman, 1979; Maga, 1982; Sandstrom et al., 1989). Numerous in vivo and in vitro studies have demonstrated an inverse relationship between phytic acid content and zinc availability. Nosworthy and Caldwell (1988), and Champagne and Phyllippy (1989) reported that phytate made complexes with zinc and/or glycinin in soybean products. Phytate-zinc-glycinin complexes in soybeans were suggested to decrease not only zinc availability but also to retard proteolysis and decrease net protein availability.

Lo et al. (1981) indicated that the dietary molar ratio of phytate to zinc became an indicator of zinc bioavailability. Fordyce et al. (1987) proposed that the dietary molar ratio of phytic acid and calcium to zinc was a better predictor of zinc bioavailability. However, static measurements such as molar ratio suggested that it is impossible to predict in vivo zinc availability since the zinc availability depended on a dietary amount and the source of zinc, and the interactions with other components in diets (Erdman et al., 1983; Fordyce et al., 1987; Hunt et al., 1989).

V-4. Iron and phytic acid The absorption of iron from diets takes place from two independent iron pools in the gastrointestinal tract. One is a heme iron pool formed by all heme iron compounds in a meal such as myoglobin and hemoglobin in meat and blood products. The other is a nonheme iron pool formed by various nonheme iron compounds present in cereals, pulses, fruits and vegetables. The nonheme iron usually constitutes more than 90 % of dietary iron intake and is the main source of absorbed iron, however absorbability of nonheme iron is much lower than that of heme iron. The iron absorption from

nonheme iron pool is increased by several factors in diets such as ascorbic acid (Hallberg, 1981; Hazell and Johnson, 1987), citric acid (Christopher et al., 1974; Gillooly et al., 1983; Hazell and Johnson, 1987) and meat/fish (Layrisse and Martinez-Torres, 1971). Hazell and Johnson (1987) showed that ascorbate and citrate prevented phytate from forming insoluble complex with iron and maintained iron in the ferrous form. Reddy et al. (1996) suggested that meat formed soluble complex with iron and facilitated iron absorption by preventing the precipitation of iron. On the other hand, dietary factors such as phytic acid (Apte and Venkatachalam, 1962; Sharpe et al., 1950), fiber (Fernandez and Phyllips, 1982; Kim and Attallah, 1993; Reinhold et al., 1975) and tannins (Disler et al., 1975) inhibit iron absorption from nonheme iron pool by forming insoluble complex with iron.

Hurrell et al. (1992) reported that a major inhibitory factor of iron absorption in soybean was phytate. Furthermore, Lynch et al. (1994) indicated that conglycinin fraction; 7S fraction of soybean protein, inhibited iron absorption in the absence of phytate.

V-5. Copper and phytic acid Phytic acid was reported to decrease copper availability in chicks fed soybean protein-based diets (Davies et al., 1962) and in rats fed soybean meal-based diets (Swick et al., 1982, 1984). Gahlawat and Sehgal (1993) indicated that roasting and malting of cereals and pulses improved copper bioavailability through reducing phytic acid content in diets. On the other hand, Lee et al. (1988) suggested that phytate improved copper absorption by binding zinc which had competitive antagonistic effect on copper absorption.

Chapter 3

Fermentation of Soybean Meal with *Aspergillus Usamii* Improves Phosphorus Absorption in Rats

Phytate-phosphorus is the predominant form of phosphorus in plant seeds (Lolas et al., 1976) which interferes with bioavailability of calcium (Nehapetian and Young, 1980) and trace elements (Erdman, 1979). Additionally, availability of phosphorus covalently bound to inositol is poor for monogastric animals (Reddy et al., 1982; Taylor, 1980). Soybean products are good protein sources for foods and feeds but more than half of total phosphorus in soybean meal exists as phytate-phosphorus. Many trials have been done to degrade phytate-phosphorus into inorganic phosphorus by germination of soybean (Bau et al., 1997) or by the autoclaving treatment (O'Dell, 1969). Phytate-phosphorus has been reported to separate from a water extract of soybean by the ion exchange (Brooks and Morr, 1982), by the careful pH adjustment (DeRham and Jost, 1979; Gillberg and Tornell, 1976; Goodnight et al., 1978), by the addition of competitive chelators such as EDTA (Okubo et al., 1975), the addition of more than 8.5% of NaCl at pH 7.5 (DeRham and Jost, 1979) or the addition of extra calcium at pH 3 to 4 (Hill and Tyler, 1954) and the addition of microbial phytase to feeds (Lei et al., 1993a,b; Nasi, 1990; Nelson et al., 1971; Pallauf et al., 1994; Simons et al., 1990). Some of them improved phosphorus absorption.

Fermentation is a traditional method for food process in the East Asian countries. In the previous study the fermentation of soybean meal with *Aspergillus usamii*

almost completely degraded phytate-phosphorus into inorganic phosphorus (Ilyas et al., 1995). The present study examined the effects of fermented soybean meal on phosphorus absorption in rats.

Although soybean products themselves showed little or no phytase activity (Mollgard et al., 1946). However, phytase activity was found in fermented soybean meal (Ilyas et al., 1995). Thus the efficacy of phytase in fermented soybean meal was also investigated on phosphorus absorption.

MATERIALS AND METHODS

Diet Preparation. Fermented soybean meal (FS) was prepared by the method described in the previous report (Ilyas et al., 1995). Briefly, commercial defatted soybean meal was steamed, and approximately 8×10^7 spores of *Aspergillus usamii* were added to 100 g of steamed soybean meal. Then the soybean meal was fermented for 48 h. Following the first fermentation, the water was added to the product until moisture became 50 % and fermented again for 12 h. After the second fermentation, FS was dried at 45 °C. Three diets were prepared; regular soybean meal (RS) -based diet, FS-based diet, and FS-based diet supplemented with sodium phytate to adjust the phytate level as in the RS diet (FS+PA diet) (Table 3-1).

Feeding Study. Eighteen male Wistar rats aged 6 weeks and weighing approximately 100 g were purchased from Japan SLC Inc.(Shizuoka, Japan) and were cared for according to the Guide for the Care and Use of Laboratory Animals (Kyoto University Animal Care Committee). Rats were individually housed in stainless steel metabolism cages for collection of feces, and in a room with controlled temperature (22 °C), relative humidity (60 %), and

lighting (12-h light). The rats were given distilled water and experimental diets ad libitum. All rats were fed the RS diet for a 7-day preliminary period. Then the rats were randomly allotted to three dietary groups of six animals each and were fed one of three experimental diets. Feed intake was recorded and feces were collected in the last 5 days of the feeding trial. The rats were exsanguinated under pentobarbital anesthesia and the blood was collected with heparinized tubes from the aorta abdominalis at the end of the trial. The small intestine was removed and divided into two segments of equal length. Digesta in the upper and the lower segments of small intestine were collected by flushing with 10 ml of ice cold saline and were diluted to 20 ml. The right femur was also collected.

Analyses. Dietary phytic acid content was measured by the AOAC procedure (1990). Phytase activity in RS and FS was determined by the following method. RS and FS were homogenized at 1000 rpm for 1 min in 0.1mol/l sodium acetate buffer (pH 5.0). The homogenates were centrifuged for 15 min at 12000 X g. The solution used in the assay was 0.1 mol/l sodium acetate buffer (pH 5) with 2.1 mmol/l sodium phytate (Han et al., 1987). After a 15 minute incubation, true inorganic phosphorus concentration in the solution was measured by the method of Takahashi (1955). One unit of enzyme activity was defined as nmol phosphorus production in 1 min.

The diluted digesta was homogenized at 1000 rpm for 1 min and 10 ml of the homogenate were centrifuged at 10000 x g for 30 min to collect soluble fraction. Femora were cleaned of adhering tissues. All samples were digested by nitric acid and perchloric acid for mineral analysis. Phosphorus contents were measured by Gomori's method (Gomori, 1942). Calcium contents were

measured using an atomic absorption spectrophotometry (AA-782, Nippon Jarrel Ash, Kyoto, Japan). Solubilities of phosphorus and calcium were determined according to the ratio of minerals in the soluble fraction to minerals in the whole digesta.

Statistical Analysis. The data from digesta were tested by three-way ANOVA which was employed to establish significant parameters among the diets (RS, FS, and FS+PA), the segments of small intestine (the upper small intestine and the lower small intestine), the animals nested in the diet's effect, and their interactions. The other data were tested by one-way ANOVA. All data were analyzed using GLM procedure of SAS (1985) at the probability level of $P < 0.05$. Significant differences among the means of each group were determined using Duncan's new multiple range test (Duncan, 1955) where the dietary effects were significant.

RESULTS

The amount of excreted phosphorus in feces was much more in the RS group than in the FS and the FS+PA groups and did not differ between the FS group and the FS+PA group (Table 3-2). Apparent phosphorus absorption was higher in the FS and the FS+PA groups than in the RS group. Excreted calcium in feces did not differ among the dietary groups (Table 3-3). Apparent calcium absorption were not different between the RS group and the FS group. Apparent calcium absorption in the FS+PA group was higher than the other two groups

Contents of dry matter, phosphorus and calcium in the femur did not differ among the dietary groups (Table 3-4). Concentrations of phosphorus and calcium in the plasma were similar among the dietary groups (Table 3-5).

Solubility of phosphorus in the upper segment of small intestine was not different among the dietary groups (Table 3-6). Phosphorus solubility in the lower small intestine was higher in the FS and the FS+PA groups than in the RS group and was not different between the FS group and the FS+PA group. Solubility of calcium in each segment of the small intestine was similar among the dietary groups.

DISCUSSION

Phytate-phosphorus is a major form of phosphorus in soybean meal and other plant seeds, which is poorly available in the digestive tract of monogastric animals (Reddy et al., 1982; Taylor, 1980). The amount of excreted phosphorus in feces was less in the FS group than in the RS group and thus apparent phosphorus absorption was higher in the FS group than in the RS group. The RS diet contained approximately 1.6 g/kg diet of phytate-phosphorus because regular soybean meal used in this study contained 3.9 g/kg of phytic acid, *myo*-inositolhexaphosphate. On the other hand, phytate-phosphorus was not detected in FS since fermentation degraded phytate-phosphorus in soybean meal into inorganic phosphorus. The large amount of excreted phosphorus in the RS group was due to the existence of phytate-phosphorus in RS and the degradation of phytate-phosphorus by the fermentation reduced fecal phosphorus excretion in the FS group.

Solubility of phosphorus in the upper small intestine did not differ among the dietary groups. Phytate-phosphorus was reported to make insoluble complexes with zinc at pH above 6.3, with iron above 7.33, with calcium above 10.4 (Jackman and Black, 1951) and with magnesium above 7.2 (Champagne et

al., 1985). The value of pH in the upper small intestine of rats under similar condition was 6.8. Thus most of phytic acid-mineral complexes was considered to be soluble in the upper small intestine. On the other hand, phosphorus solubility was depressed in the lower small intestine of the RS group compared with the FS group, which may be result from increasing insoluble phytic acid-mineral complexes in the lower small intestine. Thus, higher phosphorus solubility in the lower small intestine may result in the increase of phosphorus absorption in the FS group compared with the RS group.

Contents of dry matter and phosphorus in the femur, and phosphorus concentration in the plasma did not differ between the RS group and the FS group. Since inorganic phosphorus content in the RS diet was 0.59 % which was enough to meet requirement of phosphorus for rats (NRC, 1995), the RS rats were not deficient in phosphorus. As a result, phosphorus availability in the RS diet may not be depressed.

Apparent calcium absorption, calcium content in the femur and calcium concentration in the plasma were not affected by dietary treatment. Calcium absorption was reported to be inhibited by phytic acid when the molar ratio of phytic acid to calcium was more than 0.2 in human (Morris and Ellis, 1985) and in chicks (Nelson and Kirby, 1987). Because the molar ratio of phytic acid to calcium was approximately 0.025 in the RS diet, phytic acid might not inhibit absorption and availability of calcium in the RS diet.

The addition of phytate as much as which contained in the RS diet did not affect solubility and absorption of phosphorus in the FS+PA group. Phytase activity was found in FS though the activity could not be detected in RS (Ilyas et

al., 1995). Phytase in FS may degrade added phytate in FS+PA diet which resulted in improving phosphorus solubility and apparent phosphorus absorption in the FS+PA group. However, phytase in the FS+PA diet was only 67.1 U/g, which might be low to degrade phytate completely because dietary phytase effectively improved phosphorus availability when the enzyme was added more than 250 U/g diet (Cromwell et al., 1993; Rimbach and Pallauf, 1993). Nevertheless, this effective level of phytase activity appeared to be that of the degradation of intrinsic phytate not to be that of the degradation of extrinsically added phytate. Phytase might degrade extrinsically added phytate more easily than intrinsic phytate because intrinsic phytate was reported to exist as stable complexes with protein (Rackis et al., 1975; Rackis and Anderson, 1977). Thus, in the next experiment effects of phytase in FS were investigated on mineral availability in diets containing intrinsic phytate.

The FS+PA group ingested more calcium than did the FS group and tended to excrete less calcium ($P < 0.10$) than did the RS group. As a result, the FS+PA group showed higher calcium absorption than did the other two groups. However the cause of the result was not clear.

In conclusion, fermentation of soybean meal reduces phosphorus excretion and improves phosphorus absorption through the increase of phosphorus solubility in the lower small intestine by the degradation of phytic acid.

Table 3-1. Composition of the experimental diets

	RS ¹	FS ²	FS+PA ³
Ingredient, g/kg			
Soybean meal	400	0	0
Fermented soybean meal	0	400	400
Sucrose	440.4	440.4	440.7
Corn oil	50	50	50
Cellulose	5	5	5
Vitamin mixture ⁴	20	20	20
Mineral mixture ⁵	45	45	45
CaCO ₃	14.5	14.5	14.5
NaH ₂ PO ₄ ·2H ₂ O	21.1	21.1	15.3
Sodium phytate	0	0	5.5
Methionine	3	3	3
Choline chloride	1	1	1
Chemical analysis ⁶			
Calcium, g/kg	6.9	6.9	6.9
Phosphorus, g/kg	7.1	6.7	6.5
Magnesium, g/kg	1.5	1.6	1.7
Zinc, mg/kg	58	62	62
Phytic acid, g/kg	4.0	Not detected	3.9
Phytase activity, phytase unit/kg	Not detected	67.1	67.1

¹ Diet containing regular soybean meal. ² Diet containing fermented soybean meal. ³ Diet containing fermented soybean meal with sodium phytate. ⁴ AIN-76 (AIN 1977). ⁵ Calcium carbonate, 177 g/kg; Sodium phosphate, monobasic, dihydrate, 188 g/kg; Sodium chloride, 30 g/kg; Potassium citrate, monohydrate, 91 g/kg; Potassium sulfate, 22 g/kg; Magnesium oxide, 9.9 g/kg; Manganese carbonate (43-48% Mn), 1.5 g/kg; Ferric citrate (16-17% Fe), 2.5 g/kg; Zinc carbonate, 660 mg/kg; Cupric carbonate, 124 mg/kg; Potassium iodate, 4.1 mg/kg; Sodium selenite 5-hydrate, 4.1 mg/kg; Chromium potassium sulfate, 12-hydrate, 226 mg/kg; Polyethylene glycol 4000, 222 g/kg. ⁶ Analytical values.

Table 3-2. Apparent phosphorus absorption in rats fed diets containing regular or fermented soybean meal ¹

	Dietary treatment			
	RS ²	FS ³	FS+PA ⁴	Effect ⁵
Phosphorus intake, mg/day	163±5 ^a	148±3 ^b	157±2 ^{ab}	*
Fecal phosphorus excretion, mg/day	42.7±1.0 ^a	17.0±1.4 ^b	15.2±0.8 ^b	**
Apparent phosphorus absorption, % of intake	73.7±0.7 ^b	88.6±0.8 ^a	89.7±0.3 ^a	**

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, P<0.05). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, *, P<0.05, **, P<0.01.

Table 3-3. Apparent calcium absorption in rats fed diets containing regular or fermented soybean meal ¹

	Dietary treatment			
	RS ²	FS ³	FS+PA ⁴	Effect ⁵
Calcium intake, mg/day	158±5 ^{ab}	152±3 ^b	166±3 ^a	*
Fecal calcium excretion, mg/day	85.5±3.7	81.9±3.0	75.9±4.7	NS
Apparent calcium absorption, % of intake	45.8±1.0 ^b	46.2±1.9 ^b	54.0±3.2 ^a	**

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, P<0.05). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, *, P<0.05, **, P<0.01, NS; not significant (P>0.05).

Table 3-4. Femoral dry weight, contents of phosphorus and calcium in rats fed diets containing regular or fermented soybean meal¹

	Dietary treatment			
	RS ²	FS ³	FS+PA ⁴	Effect ⁵
Dry weight, mg	386±9	389±5	371±13	NS
Phosphorus, mg	42.3±1.0	42.5±0.3	40.8±1.3	NS
Calcium, mg	81.2±1.1	77.1±1.4	76.6±2.3	NS

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, P<0.05). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, NS; not significant (p>0.05).

Table 3-5. Plasma concentrations of phosphorus and calcium in rats fed diets containing regular or fermented soybean meal¹

	Dietary treatment			
	RS ²	FS ³	FS+PA ⁴	Effect ⁵
Phosphorus, mg/l	85.2±5.9	83.6±2.3	89.4±5.0	NS
Calcium, mg/l	96.8±7.6	109.4±19.3	97.5±9.7	NS

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, P<0.05). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, NS; not significant (p>0.05).

Table 3-6. Solubilities of phosphorus and calcium in the upper and lower small intestine of rats fed diets containing regular or fermented soybean meal¹

	Dietary treatment			Effect ⁵		
	RS ²	FS ³	FS+PA ⁴	D	S	DxS
Soluble phosphorus, %						
The upper segment	66.0±4.1	63.7±4.9	63.6±2.5	**	NS	NS
The lower segment	9.8±1.1 ^b	44.4±4.6 ^a	45.8±4.9 ^a			
Soluble calcium, %						
The upper segment	49.4±2.2	26.9±6.6	32.9±11.5	0.12	*	0.06
The lower segment	14.2±1.5	15.2±2.2	18.1±3.5			

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, $P < 0.05$). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, *, $P < 0.05$, **, $P < 0.01$, NS; not significant ($P > 0.05$). D, diet; S, segment of the small intestine; DxS, interaction of diet and segment.

Chapter 4

Fermentation of Soybean Meal with *Aspergillus Usamii* Improves Magnesium Availability in Rats

Soybean products are good protein sources for food and feed but contain a large amount of phytic acid, which reduces mineral absorption through forming insoluble complexes with minerals in the digestive tract (Cheryan, 1980). Roberts and Yudkin (1960) found that rats fed diets containing sodium phytate developed magnesium deficiency which resulted in severe illnesses. Phytic acid was observed to form insoluble complexes with magnesium in vitro (Champagne, 1988; Cheryan et al., 1983) and in vivo (Shinoda and Yoshida, 1989). Fermentation of soybean meal with *Aspergillus usamii* almost completely degraded phytic acid into inorganic phosphorus and inositol (Ilyas et al., 1995). The present study examined the effects of fermented soybean meal on magnesium availability in rats.

MATERIALS AND METHODS

Diet Preparation. Fermented soybean meal (FS) was prepared by the method described in the previous report (Ilyas et al., 1995). Three diets were prepared; regular soybean meal (RS) -based diet, FS-based diet and FS-based diet supplemented with sodium phytate to adjust the phytate level as in the RS diet (FS+PA diet) (Table 3-1).

Feeding Study. The protocol of feeding study and a manner for the use of animals were the same as those described previously (Chapter 3) .

Analyses. Dietary phytic acid content and phytase activity were measured by the same method described previously (Chapter 3). One unit of phytase activity was defined as nmol phosphorus production by 1 min.

Pre-treatment of samples for analysis was also the same as that described previously (Chapter 3). Magnesium contents were measured with an atomic absorption spectrophotometry (AA-782, Nippon Jarrel Ash, Kyoto, Japan). Solubility of magnesium was determined according to the ratio of minerals in the soluble fraction to minerals in the whole digesta.

Statistical Analysis. Statistical analysis were the same as those described previously (Chapter 3), i.e., the data from digesta were tested by three-way ANOVA and the other data were tested by one-way ANOVA using GLM procedure of SAS (1985) at the probability level of $P < 0.05$.

RESULTS

The amount of excreted magnesium in feces was much more in the RS group than in the FS and the FS+PA groups and did not differ between the FS group and the FS+PA group (Table 4-1). Thus, apparent magnesium absorption was higher in the FS and the FS+PA groups than in the RS group and was not different between the FS group and the FS+PA group.

Magnesium concentration in the femur was higher in the FS and the RF groups than in the RS group and did not differ between the FS group and the RF group (Table 4-2). Magnesium concentration in the plasma was similar among the dietary groups.

Solubility of magnesium in the upper segment of small intestine was similar among the dietary groups (Table 4-3). Magnesium solubility in the lower

small intestine was higher in the FS and the FS+PA groups than in the RS group and did not differ between the FS group and the FS+PA group.

DISCUSSION

Phytic acid was reported to form insoluble complexes with magnesium in vitro (Champagne, 1988; Cheryan et al., 1983) and in vivo (Shinoda and Yoshida, 1989). Magnesium solubility in the upper small intestine did not differ among the dietary groups. Champagne et al. (1985) observed that phytic acid complex with magnesium was increased to precipitate at pH above 7.2. Since the value of pH was 6.8 in the upper small intestine of rats fed the RS diet under similar condition of this study, most of phytic acid-magnesium complex may be soluble in the upper small intestine of the RS group. On the other hand, magnesium solubility was lowered in the lower small intestine of the RS group. The results might be induced from the precipitation of phytic acid-magnesium complex because the value of pH in the lower small intestine of rats fed the RS diet was 7.69. Magnesium absorption depends on the concentration of soluble magnesium in the intestine (Care and Van't Klooster, 1965; Ross, 1962). Furthermore, the ileum is predominant site of magnesium absorption (Hardwick et al., 1991). Apparent magnesium absorption was higher in the FS group than in the RS group, which was probably due to the rise of magnesium solubility in the lower small intestine of the FS group. Furthermore, magnesium concentration in the femur was higher in the FS group than in the RS group. These results suggested that the dephytinization of soybean meal by the fermentation improved magnesium availability through increasing magnesium solubility in the lower small intestine.

The addition of phytate as much as which contained in the RS diet did not affect solubility, absorption and availability of magnesium in the FS+PA group. Since phytase activity was found in FS though the activity could not be detected in RS (Ilyas et al. 1995), the phytase derived from FS may degrade added phytate in FS+PA diet. Furthermore, phosphorus solubility in the lower small intestine was higher in the FS+PA group than in the RS group and did not differ between the FS+PA group and the FS group (Chapter 3). These results suggested that added phytate may be degraded by the phytase derived from FS which induced the rise of solubility of phosphorus and magnesium in the lower small intestine.

In conclusion, fermentation of soybean meal improves magnesium availability through increasing magnesium solubility in the lower small intestine by the degradation of phytic acid. Fermented soybean meal improves magnesium availability even if phytate is added which probably results from the phytase activity in fermented soybean meal degrades added phytate.

Table 4-1. Apparent magnesium absorption in rats fed diets containing regular or fermented soybean meal ¹

	Dietary treatment			
	RS ²	FS ³	FS+PA ⁴	Effect ⁵
Magnesium intake, mg/day	35.3±1.0 ^b	35.8±0.7 ^b	42.0±0.6 ^a	**
Fecal magnesium excretion, mg/day	14.1±0.5 ^a	10.5±1.5 ^b	10.1±0.4 ^b	*
Apparent magnesium absorption, % of intake	60.1±0.6 ^b	71.0±3.8 ^a	75.9±1.0 ^a	**

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, P<0.05). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, *, P<0.05, **, P<0.01, NS; not significant (P>0.05).

Table 4-2. Magnesium concentrations in the femur and plasma in rats fed diets containing regular or fermented soybean meal ¹

	Dietary treatment			
	RS ²	FS ³	FS+PA ⁴	Effect ⁵
Femoral magnesium, mg/g dry matter	4.49±0.08 ^b	5.11±0.09 ^a	5.10±0.10 ^a	*
Plasma magnesium, mg/l	17.6±1.1	19.8±0.9	18.3±1.5	NS

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, P<0.05). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, *, P<0.05, NS; not significant (p>0.05).

Table 4-3. Magnesium solubilities in the upper and lower small intestine of rats fed diets containing regular or fermented soybean meal¹

	Dietary treatment					
	RS ²	FS ³	FS+PA ⁴	D	Effect ⁵ S	DxS
Soluble magnesium, %						
The upper segment	84.6±2.2	78.0±8.1	90.7±2.5	**	**	**
The lower segment	62.9±3.1 ^b	79.1±4.6 ^a	75.7±4.6 ^a			

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, P<0.05). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, **, P<0.01. D, diet; S, segment of the small intestine; DxS, interaction of diet and segment.

Chapter 5

Fermentation of Soybean Meal with *Aspergillus Usamii* Improves Zinc Availability in Rats

Although soybean products are good protein sources as food and feed, soybean products inhibit zinc availability owing to a large amount of phytic acid (Forbes et al., 1979; Lei et al., 1993; Lo et al., 1981). There have been trials to reduce phytic acid in feed and food using several methods. Phytic acid in wheat bran was degraded by extrusion cooking (Sandberg et al., 1987), by soaking to activate endogenous phytase (Morris and Ellis, 1980) or by fermentation using yeast (Widdowson, 1941), by adding phytase to feed (Lei et al., 1993; Mroz et al., 1994) or food (Sandberg and Andersson, 1988). Phytic acid in soy protein was removed by alkaline solution (Hartman, 1979) or by ion exchange resin (Niiyama et al., 1992). Phytic acid in soy plants was reduced by the sand culture technique (Zhou et al., 1992). Zinc availability was improved in some of these experiments.

The previous study showed that fermentation of soybean meal with *Aspergillus usamii* almost completely degraded phytic acid into inorganic phosphorus and inositol (Ilyas et al., 1995). The present experiment indicated that fermentation of soybean meal improved zinc availability in rats.

MATERIALS AND METHODS

Diet Preparation. Fermented soybean meal (FS) was prepared by the method described in the previous report (Ilyas et al., 1995). Three diets were

prepared; regular soybean meal (RS) -based diet, FS-based diet and FS-based diet supplemented with sodium phytate to adjust the phytate level as in the RS diet (FS+PA diet) (Table 3-1).

Feeding Study. The protocol of feeding study and a manner for the use of animals were the same as those described previously (Chapter 3) .

Analyses. Dietary phytic acid content and phytase activity were measured by the same methods as described previously (Chapter 3). One unit of phytase activity was defined as nmol phosphorus production by 1 min.

Pre-treatment of samples for analysis was also the same that described previously (Chapter 3). Zinc contents were measured with an atomic absorption spectrophotometry (AA-782, Nippon Jarrel Ash, Kyoto, Japan). Solubility of zinc was determined according to the ratio of minerals in the soluble fraction to minerals in the whole digesta.

Statistical Analysis. Statistical analysis were the same those described previously (Chapter 3), i.e., the data from digesta were tested by three-way ANOVA and the other data were tested by one-way ANOVA using GLM procedure of SAS (1985) at the probability level of $P < 0.05$.

RESULTS AND DISCUSSION

Dietary zinc requirement was reported to be 12 mg/kg diet in rats fed egg white- or casein-based diets (NRC, 1995). Dietary zinc requirement was increased to 18 mg/kg diet when rats were fed a soybean protein-based diets because soybean protein contained phytic acid, which suppressed zinc absorption (Forbes et al., 1979; Lei et al., 1993c; Lo et al., 1981). The dietary requirement of zinc has not been reported in rats fed soybean meal-based diets.

Shanklin et al. (1968) indicated that the zinc requirement was 14 mg/kg diet in piglets weighting between 2.8 and 9.6 kg when piglets were fed casein-based diets. However, the zinc requirement recommended by the NRC (1988) was 100 mg/kg diet in pigs weighing 1 to 10 kg when pigs were fed soybean meal-based diets. The large difference in zinc requirement between piglets fed a soybean meal-based diet and rats fed a soy protein based-diet may result from the difference in the dietary phytate content. Since soybean meal contained approximately 48 % crude protein, diets must contain twice as much soybean meal than soy protein when these soy products were used as the sole protein source. DeBoland et al. (1975) showed that phytic acid concentration did not largely differ between soybean meal and soy protein. The zinc requirement therefore must be higher in soybean meal-based diets than soy protein-based diets. Analogous to the research on piglets, the dietary zinc level in the present experiment of approximately 60 mg/kg diet may not be sufficient for rats fed a soybean meal-based diet.

Feed intake was not different between the RS group and the FS group (Table 5-1). Additionally, the dietary treatments did not affect body weight gain. The SBM group thereby did not show the signs of zinc deficiency. However, the RS group showed lower zinc content in the femur than did the FS group. Bone zinc content was suggested to be a more sensitive indicator for nutritional status of zinc than body weight gain (Forbes et al., 1984; Morris and Ellis, 1980; Yoshida et al., 1985). The present research clearly indicated that fermentation of soybean meal improved zinc availability. Ilyas et al. (1995) demonstrated that the fermentation with *Aspergillus usarii* almost completely degraded phytic acid

in soybean meal. These results suggested that zinc availability was suppressed by phytic acid in the RS group and was improved by the degradation of phytic acid in the FS group.

The FS group showed higher solubility of zinc in each segment of the small intestine than did the RS group (Table 5-2), which suggested that phytic acid decreased zinc solubility both in the upper and lower segments of small intestine. Champagne and Phyllippy (1989) observed that insoluble zinc complexes were formed under intraluminal gastric pH values of 5.0 or higher after pepsin-pancreatin digestion of soy protein isolate. Furthermore, Shinoda and Yoshida (1989) reported that dietary phytate decreased zinc solubility in the digesta of the lower small intestine in rats, and they suggested that phytic acid decreased bone zinc deposition through lowering zinc solubility. The results of the present study showed that the degradation of phytic acid by the fermentation increased zinc solubility, resulting in the improvement of zinc availability.

Adding the same amount of phytate that was contained in the RS diet did not affect availability of zinc in the FS+PA group (Table 5-1). Phytase activity was found in FS though the activity could not be detected in RS (Ilyas et al. 1995) and this activity may degrade added phytate in the FS+PA diet. Zinc solubility in the upper small intestine was not higher in the FS+PA group than in the RS group (Table 5-2). However, zinc solubility in the lower small intestine of the FS+PA group was significantly ($P<0.01$) higher than of the RS group and was not different from that of the FS group. These results suggested that added phytate depressed solubility of zinc in the upper small intestine, and phytate may be degraded by phytase in the FS+PA diet during the passage of the small

intestine. The ileum was shown to be the major site of zinc absorption (Antonson et al., 1979), which supported the positive relationship between zinc solubility in the lower small intestine and zinc availability. However, phytase in the FS diet was only 67.1 U/g, which might be too low to degrade phytate completely, because dietary phytase effectively improved phosphorus availability when the enzyme was added at more than 250 U/g diet (Cromwell et al. 1993; Rimbach and Pallauf 1993). Nevertheless, this effective level of phytase activity was for the degradation of intrinsic phytate and not for that of extrinsic phytate. Phytase might degrade extrinsically added phytate more easily than intrinsic phytate, because intrinsic phytate was reported to exist as stable complexes with protein (Rackis et al., 1975; Rackis and Anderson, 1977).

On the other hand, O'Dell and Savage (1960) observed that casein-phytate complex inhibited zinc availability, whereas the calcium-phytate complex did not in chick, and thus they suggested that phytic acid must be in combination with protein to make zinc unavailable. Because fermentation partly degraded protein in RS to less than 50000 molecular weight (Ilyas et al., 1995), the digestibility of protein may be higher in FS than in RS. Additionally, the degradation of phytic acid is considered to stimulate further protein digestion in FS, because phytic acid was reported to inhibit protein digestibility (Carnovale et al., 1990; Nyman et al., 1989). Thus, the fermentation may stimulate protein digestion in the gut by the reduction of phytic acid and the partial degradation of protein before ingestion. Added phytate therefore could not inhibit zinc availability in the FS+PA group, because added phytate may not make complexes with protein and zinc in the lower small intestine.

In conclusion, fermentation of soybean meal improved zinc availability through the increase of zinc solubility in the small intestine, which resulted from the degradation of phytic acid in soybean meal. Furthermore, phytase and/or partially degraded protein in the fermented soybean meal also improved zinc availability. Zinc supplement can be reduced when fermented soybean meal is fed as a substitute for regular soybean meal.

Table 5-1. Daily intake, body weight gain and femoral zinc content in rats fed diets containing regular or fermented soybean meal¹

	Dietary treatment			
	RS ²	FS ³	FS+PA ⁴	Effect ⁵
Daily intake, g/day	22.9±0.7	22.1±0.5	24.1±0.4	NS
Body weight, g				
Initial	108±2	108±2	105±3	NS
Final	293±7	281±5	289±8	NS
Gain ⁶	186±6	173±6	184±9	NS
Femoral zinc, µg	91±4 ^b	107±3 ^a	117±3 ^a	**

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, P<0.05). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, NS; not significant (p>0.05). ⁶ Gain was calculated from the difference between initial and final values.

Table 5-2. Zinc solubilities in the upper and lower small intestine of rats fed diets containing regular or fermented soybean meal¹

	Dietary treatment					
	RS ²	FS ³	FS+PA ⁴	D	Effect ⁵ S	DxS
Soluble zinc, %						
The upper segment	38.9±4.1 ^b	67.7±6.9 ^a	56.8±10.4 ^{ab}	**	**	NS
The lower segment	20.9±3.5 ^b	50.4±4.0 ^a	48.3±4.1 ^a			

¹ Values are means ± SEM. Means within a row not sharing a common letter in their superscripts are significantly different (Duncan's multiple range test, P<0.05). ² Diet containing regular soybean meal. ³ Diet containing fermented soybean meal. ⁴ Diet containing fermented soybean meal with sodium phytate. ⁵ Statistical effect, *, P<0.05, **, P<0.01, NS; not significant (P>0.05). D, diet; S, segment of the small intestine; DxS, interaction of diet and segment.

Chapter 6

Degradation of Intrinsic Phytate Using Fermented Soybean Meal Improves Magnesium Availability in Rats

Although soybean products are good protein sources as food and feed, more than half of total phosphorus in soybean meal exists as phytate-phosphorus. The digestibility of phytate-phosphorus is poor for monogastric animals. Furthermore, Roberts and Yudkin (1960) found that rats fed diets containing phytate developed magnesium deficiency which resulted in severe illnesses. The addition of microbial phytase to diets containing phytate was reported to improve digestibility of phytate-phosphorus (Lei et al., 1993a, b; Nasi, 1990; Nelson et al., 1971; Pallauf et al., 1994; Simons et al., 1990). Furthermore, Morris and Ellis (1980) observed that the degradation of phytic acid in wheat bran improved zinc availability by soaking to activate endogenous phytase. Although soybean products themselves showed little or no phytase activity (Mollgard et al., 1946), phytase activity was found in the soybean meal fermented with *Aspergillus usamii* (Ilyas et al., 1995). Zhu et al. (1990) indicated that incubation of soybean meal with wheat bran partly degraded phytate in soybean meal, which was due to the phytase activity from the bran. In the previous study the addition of sodium phytate to the fermented soybean meal did not affect magnesium availability, which may result from phytase activity originated from the fermented soybean meal degrading added phytate. However, phytase might degrade extrinsically added phytate more easily than intrinsic

phytate because intrinsic phytate was reported to exist as stable complexes with protein (Rackis et al., 1975; Rackis and Anderson, 1977). This study examined the efficacy of phytase activity from fermented soybean meal on magnesium availability in a diet consisting of both regular and fermented soybean meals.

MATERIALS AND METHODS

Diet Preparation. Deffated soybean meal was fermented by the method described in the previous report (Ilyas et al., 1995). Briefly, commercial deffated soybean meal was steamed, and approximately 8×10^7 spores of *Aspergillus usamii* were added to 100 g of steamed soybean meal. Then the soybean meal was fermented for 48 h. Following the first fermentation, the water was added to the product until moisture became 50 % and fermented again for 12 h. After the second fermentation, fermented soybean meal was dried at 45 °C. Three diets were prepared, a diet consisting of 40 % regular soybean meal (RS diet), a diet consisting of 40 % fermented soybean meal (FS diet), and a diet consisting of 20 % RS and 20 % FS (RF diet) (Table 6-1).

Feeding Study. Eighteen male Wistar rats aged 6 weeks and weighing approximately 100 g were purchased from Japan SLC Inc. (Shizuoka, Japan) and were cared for according to the Guide for the Care and Use of Laboratory Animals (Kyoto University Animal Care Committee). Rats were individually housed in stainless steel cages, and in a room with controlled temperature (22 °C), relative humidity (60 %), and lighting (12-h light). The rats were given distilled water and experimental diets ad libitum. All rats were fed the RS diet for a 7-day of preliminary period. Then the rats were randomly allotted to three dietary groups of six animals each and fed one of three experimental diets

for 4 weeks. The rats were exsanguinated under pentobarbital anesthesia and the blood was collected with heparinized tubes from the aorta abdominalis at the end of the trial. The small intestine was removed and divided into two segments of equal length. Digesta in the upper and the lower segments of small intestine were collected by flushing with 10 ml of ice cold saline and were diluted to 20 ml. The right femur was also collected.

Analyses. Phytic acid contents in diets were measured by the AOAC (1990) procedure. Phytase activities in RS and FS were determined by the method of Han et al. (1987) with minimal modification, i.e., true inorganic phosphorus concentration was measured by the method of Takahashi (1955). One unit of enzyme activity was expressed as nmol phosphorus production in 1 min.

After the value of pH in diluted digesta was measured, the digesta was homogenized at 1000 rpm for 1 min and 10 ml of the homogenate were centrifuged at 10000 x g for 30 min to collect soluble fraction. Femora were cleaned of adhering tissues. All samples were digested by nitric acid and perchloric acid for mineral analysis. Phosphorus content was measured by Gomori's method (Gomori, 1942). Magnesium content was measured using an atomic absorption spectrophotometry (AA-782, Nippon Jarrel Ash, Kyoto, Japan). Magnesium solubility was determined as the ratio of mineral concentration in the soluble fraction to that in the whole digesta.

Statistical Analysis. The data from digesta were tested by three-way ANOVA, which was employed to establish significant parameters among the diets (RS, RF, and FS), the segments of small intestine (the upper

small intestine and the lower small intestine), the animals nested in the diet's effect, and their interactions. The other data were tested by one-way ANOVA. All data were analyzed using GLM procedure of SAS (1985) at the probability level of $P < 0.05$. Significant differences among the means of each group were determined using Duncan's new multiple range test (Duncan, 1955) where the dietary effects were significant.

RESULTS

Daily intake was not different among the dietary groups (Table 6-2). The FS group excreted more feces than did the other two groups. Body weight gain was similar among the dietary groups. The amount of excreted phosphorus in feces (mg/day) was more in the RS group than in the RF and the FS groups and did not differ between the RF group and the FS group (Table 6-3). However, fecal phosphorus excretion (% of intake) tended to be higher ($P = 0.07$) in the RF group than in the FS group. As a result, apparent phosphorus absorption (% of intake) in the RF group was higher than in the RS group but lower than in the FS group.

Fecal magnesium excretion (% of intake) was higher in the RS group than in the RF and the FS groups and was not different between the RF group and the FS group (Table 6-4). Apparent magnesium absorption (% of intake) was higher in the RF and the FS groups than in the RS group and did not differ between the RF group and the FS group.

Phosphorus concentrations in the femur and plasma were not different among the dietary groups (Table 6-5). Femoral and plasma magnesium concentrations were higher in the RF and the FS groups than in the RS group

and did not differ between the RF group and the FS group.

The value of pH in each segment of the small intestine was similar among the dietary groups (Table 6-6). Magnesium solubility in the upper small intestine was not different among the dietary groups. On the other hand, magnesium solubility in the lower small intestine was higher in the RF and the FS groups than in the RS group and did not differ between the RF group and the FS group.

DISCUSSION

The FS group showed higher magnesium solubility in the lower small intestine and apparent magnesium absorption than did the RS group. Phytic acid was reported to make insoluble complex with magnesium in vitro (Champagne, 1988; Cheryan et al., 1983) and in vivo (Shinoda and Yoshida, 1989). Magnesium absorption was suggested to depend on the concentration of soluble magnesium in the intestine (Care and Van't Klooster, 1965; Ross, 1962). Thus phytic acid in the RS diet probably made insoluble complex with magnesium in the lower small intestine which resulted in decreasing of magnesium absorption in the RS group because lower segment of the small intestine was the main absorption site of magnesium (Hardwick et al., 1991). On the other hand, magnesium did not form insoluble complex with phytic acid in the digestive tract of the FS group, which was due to the dephytinization of soybean meal by the fermentation. Magnesium concentrations in the femur and plasma were higher in the FS group than in the RS group. As a result, fermentation of soybean meal improved magnesium availability through raising magnesium solubility in the lower small intestine. These results were in agreement with the

results described previously (Chapter 4).

In the previous experiment, the addition of sodium phytate to the FS diet did not inhibit magnesium availability (Chapter 4). Phytase activity was detected in FS though the activity was not detected in RS (Ilyas et al., 1995). Thus, phytase originated from FS may degrade added phytate (Chapter 4). However, phytase was considered to degrade extrinsically added phytate more easily than intrinsic phytate because intrinsic phytate in soybean meal existed as stable complexes with protein (Rackis et al., 1975; Rackis and Anderson, 1977). To investigate the possibility of phytase originated from FS to degrade intrinsic phytate, a diet consisting of both regular and fermented soybean meal was prepared in this study.

Magnesium solubility in the lower small intestine of the RF group was as high as the FS group. Furthermore, apparent magnesium absorption was not different between the RF group and the FS group. Magnesium concentrations in the femur and plasma also did not differ between the RF group and the FS group. These results suggested that phytase originated from FS might degrade intrinsic phytate from RS in the digestive tract, which resulted in improving magnesium availability through raising magnesium solubility in the lower small intestine.

On the other hand, fecal phosphorus excretion in the RF group was lower than in the RS group but tended to be higher ($P=0.07$) than in the FS group. As a result, apparent phosphorus absorption in the RF group was lower than in the FS group. Dietary phytase was reported to improve phosphorus availability effectively when the enzyme was added more than 250 U/g (Cromwell et al. 1993; Rimbach and Pallauf 1993). However, phytase activity of

126.1 U/g in the RF diet might not be high enough for degrading phytate completely because 1200 U/g phytase activity was reported to need to maximally degrade phytate in the digestive tract (Lei et al., 1993b; Rimbach and Pallauf, 1992). Thus lower absorption of phosphorus in the RF group compared with the FS group may be due to the incomplete degradation of phytate. Phytic acid in soybean products was shown to inhibit availabilities of cations, especially zinc compared with other minerals (Forbes and Parker, 1977; Forbes et al., 1979). The relative order of affinities of various metals to phytate were reported: $Zn > Mn > Fe > Ca > Mg$ (Maddaish et al., 1964; Rao and Rao, 1983; Vohra et al., 1965). Thus inhibitory effects of phytic acid might be weaker on magnesium availability than zinc availability. Furthermore, incomplete degradation of phytic acid was considered not to inhibit magnesium availability but inhibit other mineral availability in the RF diet because phosphorus absorption was lowered in the RF group.

The amount of excreted feces in the FS group was more than the other two groups, which probably resulted from the FS group showing mild diarrhea. The phytase activity was stronger in fermented soybean meal used in this experiment than in the previous study. Since fermented soybean meal with stronger phytase activity might have unfavorable effects on health for animals further investigation is needed for practical use of fermented soybean meal.

Table 6-1. Composition of the experimental diets

	RS ¹	RF ²	FS ³
Ingredient, g/kg			
Soybean meal	400	200	0
Fermented soybean meal	0	200	400
Sucrose	459	459.7	460.6
Corn oil	50	50	50
Vitamin mixture ⁴	10	10	10
Mineral mixture ⁵	45	45	45
CaCO ₃	14.2	14.1	13.9
NaH ₂ PO ₄ ·2H ₂ O	18.8	18.2	17.5
Methionine	3	3	3
Chemical analysis ⁶			
Calcium, g/kg	7.2	7.1	7.4
Phosphorus, g/kg	6.4	6.5	6.6
Magnesium, g/kg	1.6	1.6	1.7
Zinc, mg/kg	50	50	50
Iron, mg/kg	130	140	140
Copper, mg/kg	10	10	10
Phytic acid, g/kg	4.0	2.1	0.1
Phytase activity, phytase unit/kg	5	126.1	247.2

¹ Diet containing regular soybean meal. ² Diet containing regular and fermented soybean meal. ³ Diet containing fermented soybean meal. ⁴ AIN-76 (AIN 1977) ⁵ Calcium carbonate, 177 g/kg; Sodium phosphate, monobasic, dihydrate, 188 g/kg; Sodium chloride, 30 g/kg; Potassium citrate, monohydrate, 91 g/kg; Potassium sulfate, 22 g/kg; Magnesium oxide, 9.9 g/ kg; Manganese carbonate (43-48% Mn), 1.5 g/kg; Ferric citrate (16-17% Fe), 2.5 g/kg; Zinc carbonate, 660 mg/kg; Cupric carbonate, 124 mg/kg; Potassium iodate, 4.1 mg/kg; Sodium selenite 5-hydrate, 4.1 mg/kg; Chromium potassium sulfate, 12-hydrate, 226 mg/kg; Polyethylene glycol 4000, 222 g/kg. ⁶ Analytical values.

Table 6-2. Daily intake, the amount of fecal excretion and body weight gain in rats fed diets containing regular and/or fermented soybean meal¹

	Dietary treatment			
	RS ²	RF ³	FS ⁴	Effect ⁵
Daily intake, g/day	21.8±0.2	21.9±0.2	22.7±1.2	NS
Fecal excretion, g/day	2.86±0.02 ^b	3.07±0.03 ^b	3.24±0.18 ^a	**
Initial body weight, g	144.4±4.6	145.8±1.9	146.0±1.6	NS
Body weight gain, g	125.2±3.8	113.2±8.3	111.9±8.0	NS

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular and fermented soybean meal. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, **, P<0.01, NS; not significant (P>0.05).

Table 6-3. Fecal phosphorus excretion and apparent phosphorus absorption in rats fed diets containing regular and/or fermented soybean meal ¹

	Dietary treatment			
	RS ²	RF ³	FS ⁴	Effect ⁵
Phosphorus intake, mg/day	139±1 ^a	121±1 ^b	149±8 ^a	**
Fecal phosphorus excretion, mg/day	23.9±1.2 ^a	8.5±0.4 ^b	8.2±0.9 ^b	**
Fecal phosphorus excretion, %	17.1±0.8 ^a	7.20±0.32 ^b	5.43±0.38 ^b	**
Apparent phosphorus absorption, mg/day	116±1 ^b	108±3 ^b	141±7 ^a	**
Apparent phosphorus absorption, %	82.9±0.8 ^c	92.8±0.3 ^b	94.6±0.4 ^a	**

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular and fermented soybean meal. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, **, P<0.01.

Table 6-4. Fecal magnesium excretion and apparent magnesium absorption in rats fed diets containing regular and/or fermented soybean meal ¹

	Dietary treatment			Effect ⁵
	RS ²	RF ³	FS ⁴	
Magnesium intake, mg/day	34.3±0.3	35.0±0.3	38.8±2.1	NS
Fecal magnesium excretion, mg/day	9.24±0.59 ^a	4.89±0.78 ^b	7.97±1.24 ^a	*
Fecal magnesium excretion, %	27.0±1.6 ^a	14.7±2.7 ^b	20.0±2.4 ^b	**
Apparent magnesium absorption, mg/day	25.0±0.5 ^b	29.1±1.2 ^a	30.8±1.1 ^a	**
Apparent magnesium absorption, %	73.0±1.6 ^b	85.6±2.2 ^a	80.0±2.4 ^a	**

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular and fermented soybean meal. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, *, P<0.05, **, P<0.01, NS; not significant (P>0.05).

Table 6-5. Concentrations of phosphorus and magnesium in the femur and plasma of rats fed diets containing regular and /or fermented soybean meal ¹

	Dietary treatment			Effect ⁵
	RS ²	RF ³	FS ⁴	
Femur, mg/g				
Phosphorus	115±2	111±2	109±2	NS
Magnesium	5.01±0.11 ^b	5.53±0.10 ^a	5.49±0.15 ^a	*
Plasma, mg/l				
Phosphorus	68.1±3.2	70.1±2.4	62.3±2.7	NS
Magnesium	19.1±0.7 ^b	23.0±0.2 ^a	24.0±0.9 ^a	**

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular and fermented soybean meal. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, *, P<0.05, **, P<0.01, NS; not significant (P>0.05).

Table 6-6. The value of pH and magnesium solubility in the upper and lower small intestine in rats fed regular and/or fermented soybean meal ¹

	Dietary treatment					
	RS ²	RF ³	FS ⁴	Effect ⁵		
				D	S	DxS
pH						
The upper segment	6.84±0.11	6.91±0.04	6.82±0.12	NS	**	NS
The lower segment	7.69±0.10	7.24±0.40	7.09±0.07			
Soluble magnesium, %						
The upper segment	83.6±3.1	89.2±1.9	90.2±1.5	**	**	NS
The lower segment	61.3±2.5 ^b	80.7±5.0 ^a	77.2±3.8 ^a			(0.14)

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular and fermented soybean meal. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, **, P<0.01, NS; not significant (P>0.05). D, diet; S, segment of the small intestine; DxS, interaction of diet and segment.

Chapter 7

Fermentation of Soybean Meal with *Aspergillus Usamii* Improves Availabilities of Zinc and Iron in Rats

The increase in water and soil polluted by heavy metals such as zinc and iron has been discussing (Baars et al., 1988; Brennan, 1991; Hart et al., 1985; Picket et al., 1992; Sterrett et al., 1982, 1983). Heavy metals from domestic waste spread on the soil, accumulate or flow into the ground water and then into the ponds, streams, rivers, lakes and oceans. Diffused heavy metals in the environment contaminate water and crop from farms. Thus the necessity for decreasing excretion of heavy metals through improving the bioavailabilities has increasing.

Soybean products are frequency used for foods and feeds as protein sources but contain a large amount of phytate which suppresses availabilities of zinc (Champagne and Phyllippy, 1989; Shinoda and Yoshida, 1989; Torre et al., 1991) and iron (Svanberg et al., 1993; Wyatt and Triana, 1994) through making insoluble complexes with these elements in the digestive tract. In the previous report fermentation of soybean meal with *Aspergillus usamii* almost completely degraded phytate into inorganic phosphorus and inositol (Ilyas et al., 1995). Kasaoka et al. (1997) observed that tempeh, an Indonesian non-salted fermented soybean product, increased iron concentration in the liver of rats. Firstly, the present study examined the effects of fermented soybean meal with *Aspergillus usamii* on availabilities of dietary zinc and iron.

The addition of microbial phytase (EC 3.1.3.26) to diets containing phytate was reported to improve bioavailability of zinc (Lei et al., 1993; Pallauf et al., 1994; Rimbach and Pallauf, 1993) and iron (Sandberg et al., 1996). Zhu et al (1990) indicated that incubation of soybean meal with wheat bran partly degraded phytate in soybean meal, which was due to phytase activity (EC 3.1.3.8) from bran. Morris and Ellis (1980) observed soaking of wheat bran increased zinc concentration in the femur of rats through degrading phytate by activating endogenous phytase in bran. Although non-fermented soybean products showed little or no phytase activity (Mollgard et al., 1946), phytase activity was found in soybean meal fermented by *Aspergillus usarii* (Ilyas et al., 1995). Secondly, this study examined the efficacy of phytase activity from the fermented soybean meal on bioavailabilities of zinc and iron in a diet consisting of both regular and fermented soybean meal.

MATERIALS AND METHODS

Diet Preparation. Deffated soybean meal was fermented by the method described in the previous report (Ilyas et al., 1995). Three diets were prepared, a diet consisting of 40 % regular soybean meal (RS diet), a diet consisting of 40 % fermented soybean meal (FS diet), and a diet consisting of 20 % RS and 20 % FS (RF diet) (Table 6-1).

Feeding Study. The protocol of feeding study and a manner for the use of animals were the same as those described previously (Chapter 6). The rats were given distilled water and experimental diets ad libitum. All rats were fed the RS diet for a 7-day preliminary period. Then the rats were randomly allotted to three dietary groups of six animals each and fed one of three

experimental diets for 4 weeks. The rats were exsanguinated under pentobarbital anesthesia and the blood was collected with heparinized tubes from the aorta abdominalis at the end of the trial. The small intestine was removed and divided into two segments of equal length. Digesta in the upper and the lower segments of small intestine were collected by flushing with 10 ml of ice cold saline and were diluted to 20 ml. The right femur and the liver were also collected.

Analyses. Dietary phytic acid contents and phytase activities were measured by the same methods described previously (Chapter 6). One unit of the enzyme activity was expressed as nmol phosphorus production in 1 min.

Pre-treatment of samples for analysis was also the same that described previously (Chapter 6). Zinc and iron contents were measured with an atomic absorption spectrophotometry (AA-782, Nippon Jarrel Ash, Kyoto, Japan). Solubilities of zinc and iron were determined as the ratio of mineral concentration in the soluble fraction to that in the whole digesta.

Statistical Analysis. Statistical analysis were the same as those described previously (Chapter 6), i.e., the data from digesta were tested by three-way ANOVA, and the other data were tested by one-way ANOVA using the GLM procedure of SAS (1985) at the probability level of $P < 0.05$.

RESULTS

Zinc concentrations in the femur and plasma were higher in the FS and the RF groups than in the RS group (Table 7-1). Furthermore, the higher zinc concentrations were shown in the femur and plasma of the FS group compared with the RF group. Iron concentrations in the liver and plasma were higher in the

FS and the RF groups than in the RS group and did not differ between the FS group and the RF group (Table 7-2).

The value of pH in each segment of the small intestine did not differ among the dietary groups (Table 7-3). Zinc solubilities in the upper and the lower small intestine were higher in the FS and the RF groups than in the RS group and were not different between the FS group and the RF group. Iron solubility in the upper small intestine did not differ among the dietary groups. On the other hand, iron solubility in the lower small intestine was lower in the RS group than the other groups.

DISCUSSION

The RS diet contained 50 mg zinc /kg diet, which might be adequate because dietary zinc requirement was estimated to be 12 mg/kg diet in rats fed egg white- or casein-based diets (NRC, 1995). However, Sandstrom et al. (1980) suggested that daily dietary requirement of zinc depended not only on the physiological requirement of zinc but also on the composition of the meals. Furthermore, Mclaughlan (1977) showed that 50 mg/kg zinc was insufficient for normal growth of rats fed a diet consisting of 20% rapeseed protein concentrate containing approximately 1.46 g/kg phytic acid. It has been suggested that less than 0.9 mg/l of zinc concentration in plasma indicated zinc deficiency in rats (Walker and Kelleher, 1978). Since the plasma zinc concentration was 0.86 mg/l in the RS group, the dietary zinc level appeared to be insufficient for rats fed the RS diet containing 4 g/kg phytic acid.

Zinc solubilities were lower in the upper and lower small intestine of the RS group compared with the FS group. Phytate-zinc complex was reported to

precipitate at pH above 6.3 (Vohra et al., 1965). The value of pH was 6.84 in the upper small intestine of the RS group. Thus phytate in RS probably made insoluble complex with zinc and decreased zinc solubility in the upper small intestine of the RS group. Since the fermentation with *Aspergillus usarii* almost completely degraded phytate into inorganic phosphorus and inositol in soybean meal (Ilyas et al., 1995), zinc did not form insoluble complex with phytate in the small intestine of the FS group. Concentrations of zinc in the femur and plasma were higher in the FS group than in the RS group. Femoral and plasma zinc concentrations were suggested to be sensitive indicators for zinc bioavailability (Dreosti et al., 1986). Shinoda and Yoshida (1989) reported that dietary phytic acid decreased zinc solubility in the small intestine of rats, which resulted in decreasing bone zinc concentration. Fermentation of soybean meal improved zinc bioavailability, which probably resulted from dephytinization improving zinc solubility in the small intestine.

Dietary iron content in the present diets appeared to be adequate because dietary iron requirement was 35 mg/kg diet in rats fed egg white- or casein-based diets according to NRC (1995). However, the amount and type of protein were suggested to play an important role on iron absorption (Perez-Llamas et al., 1996; Snedeker and Greger, 1983) and iron requirement has not been well defined when protein sources differ. Iron absorption was reported to be lower in rats fed a soybean protein-based diet than in those fed a casein-based diet because soybean protein contained phytate (Hunter, 1981). Kasaoka et al. (1997) reported that iron concentration in the liver was 127 $\mu\text{g/g}$ in iron-deficient anemic rats. Since liver iron concentration was 79 $\mu\text{g/g}$ in the RS rats,

dietary iron level in the RS diet might be insufficient.

Concentrations of iron in the liver and plasma were higher in the FS group than in the RS group. Thus iron absorption was considered to be higher in the FS group compared with the RS group. Salz et al. (1993) reported that iron was absorbed in whole small intestine and main absorption site of iron was duodenum. Monson and Cook (1976) suggested that iron solubilization was a primary step before iron could be absorbed in the intestine. However, iron solubility in the upper small intestine did not differ between the RS group and the FS group. Vohra et al. (1965) reported that phytate-iron complex increased to precipitate at pH above 7.33. Because the value of pH in digesta of the upper small intestine was 6.84 in the RS group, phytate-iron complex was not considered to precipitate in the upper small intestine of the RS group. Rao and Rao (1983) reported that phytate formed soluble complexes with iron. Kim and Atallah (1993) indicated that solubility of iron did not completely reflect its absorbability and suggested that iron bioavailability depended on the solubility of iron complex and the binding characteristics of that complex. Phytate in the RS diet possibly decreased iron absorption through making soluble complex with iron in the upper small intestine of the RS group, which may lower iron concentrations in the liver and plasma. On the other hand, the fermentation of soybean meal improved iron bioavailability, which may be due to the increase in iron absorption by the degradation of phytate.

Iron solubility in the lower small intestine was higher in the FS group than in the RS group. The value of pH in the lower small intestine of the RS group was 7.69 and thus phytic acid-iron complex probably precipitated in the

lower small intestine of the RS group. Higher iron concentrations in the liver and plasma of the FS group as compared with the RS group may be resulted from the increase of iron absorption in the lower segment of the small intestine through the higher iron solubility in this segment of the FS group.

Solubility of zinc in each segment of the small intestine did not differ between the RF group and the FS group. These results suggested that phytase originated from the fermented soybean meal degraded phytate from the regular soybean meal in the small intestine of the RF group. However, femoral and plasma zinc concentrations were lower in the RF group than in the FS group. Phytase activity of 126.1 U/g in the RF diet might not be high enough for degrading phytate completely because 1200 U/g phytase activity was reported to need to maximally degrade phytate in the digestive tract (Lei et al., 1993b; Rimbach and Pallauf, 1992). Phytic acid, i.e., inositol hexaphosphate (IP_6), in wheat bran was reported to be hydrolyzed to lesser phosphorylated derivatives of inositol such as inositol tri- (IP_3), tetra- (IP_4), and penta- (IP_5) phosphates during the digestion in the gut (Sandberg and Ahderinne, 1986; Sandberg and Andersson, 1988; Sandberg et al., 1987). However, phytic acid in extrusion cooked-bran was not hydrolyzed and IP_3 , IP_4 and IP_5 did not increase in the digestive tract, which was suggested to be due to the degraded endogenous phytase activity by extrusion cooking (Sandberg and Andersson, 1988; Sandberg et al., 1987). The digesta of the RF group was considered to contain these lesser phosphorylated derivatives of inositol. Simpson and Wise (1990) indicated that zinc bound lesser phosphorylated derivatives of inositol became more soluble as the number of phosphate groups per molecule decreased. Han

et al. (1994) showed that solubilized IP_3 , IP_4 , IP_5 and IP_6 inhibited zinc transport but the inhibitory effects of IP_3 and IP_4 were weaker than those of IP_5 and IP_6 in human intestinal cell line. These results suggested that the lower bioavailability with high solubility of zinc in the RF group might be due to the production of lesser phosphorylated inositols resulted from partial degradation of phytate by phytase originated from FS in the RF diet.

Iron solubility in the lower small intestine, and concentrations of iron in the liver and plasma were higher in the RF group than in the RS group and did not differ between the RF group and the FS group. Iron bioavailability did not differ between the RF group and the FS group although zinc bioavailability was lower in the RF group than in the FS group. Phytic acid was shown to more strongly make complex with zinc than iron (Torre et al., 1991), and thus lesser phosphorylated inositols are considered to more easily form complex with zinc than iron. Han et al. (1994) observed that inhibitory effects of IP_3 and IP_4 were stronger on zinc transport than on iron transport across the model cells for human intestinal absorptive epithelium. These results suggested that the produced lesser phosphorylated inositols during digestion possibly decreased zinc bioavailability but not iron bioavailability in the RF group. Phytase originated from fermented soybean meal may degrade a measure of intrinsic phytate in regular soybean meal but the activity appeared to be insufficient for complete degradation.

The environmental pollution by heavy metals such as zinc and iron from animal waste have been increasing (Baars et al., 1988; Brennan, 1991; Hart et al., 1985; Pickett et al., 1992; Sterrett et al., 1982, 1983). Dietary zinc and iron

thus can be reduced by feeding fermented soybean meal as substitute for regular soybean meal through improving availabilities of zinc and iron, which may result in decreasing excreted heavy metals.

Table 7-1. Concentrations of zinc in the femur and plasma in rats fed diets containing regular and/or fermented soybean meal ¹

	Dietary treatment			
	RS ²	RF ³	FS ⁴	Effect ⁵
Femur, µg/g	218±7 ^c	251±6 ^b	287±6 ^a	**
Plasma, mg/l	0.86±0.01 ^c	1.49±0.05 ^b	1.76±0.08 ^a	**

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular and fermented soybean meal. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, **, P<0.01.

Table 7-2. Concentrations of iron in the liver and plasma in rats fed diets containing regular and /or fermented soybean meal ¹

	Dietary treatment			
	RS ²	RF ³	FS ⁴	Effect ⁵
Liver, µg/g	79±4 ^b	194±28 ^a	159±16 ^a	**
Plasma, mg/l	3.60±0.19 ^b	5.75±0.38 ^a	6.49±0.26 ^a	**

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular and fermented soybean meal. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, **, P<0.01.

Table 7-3. The value of pH and solubilities of zinc and iron in the upper and lower small intestine in rats fed regular and/or fermented soybean meal ¹

	Dietary treatment					
	RS ²	RF ³	FS ⁴	Effect ⁵		
				D	S	DxS
pH						
The upper segment	6.84±0.11	6.91±0.04	6.82±0.12	NS	**	NS
The lower segment	7.69±0.10	7.24±0.40	7.09±0.07			
Soluble zinc, %						
The upper segment	32.4±6.3 ^b	60.5±2.5 ^a	66.1±6.2 ^a	**	**	NS
The lower segment	23.4±1.0 ^b	46.5±3.3 ^a	44.3±2.5 ^a			
Soluble iron, %						
The upper segment	25.6±3.7	34.6±4.9	31.1±1.4	**	**	NS
The lower segment	10.5±0.7 ^b	23.3±2.3 ^a	24.7±3.1 ^a			

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular and fermented soybean meal. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, **, P<0.01, NS; not significant (P>0.05). D, diet; S, segment of the small intestine; DxS, interaction of diet and segment.

Chapter 8

Fermentation of Soybean Meal with *Aspergillus Usamii* Improves Phosphorus Availability in Chicks

Soybean meal is basic protein source in poultry feeds. However, more than half of total phosphorus in soybean meal exists as phytate phosphorus, the availability of which is very low (Nelson et al., 1971). Many researchers have improved availability of phytate phosphorus by using phytase in simple-stomached animals, including chicks (Nelson et al., 1971; Simsons et al., 1990). There are also reports that microbial phytase increased phosphorus availability in pigs fed maize-soybean meal based diets (Cromwell et al., 1993; Lei et al., 1993a; Simpsons et al., 1990). Zhu et al. (1990) indicated that incubation of soybean meal with bran partly degraded phytate in soybean meal, due to phytase activity from the bran, with corresponding increased phosphorus availability in chicks.

In a previous report, fermentation using *Aspergillus usamii* almost completely degraded phytate in soybean meal with a slight increase in crude protein and crude fiber contents, minimal change in amino acid composition and 7% of dry matter loss due to a reduction in nitrogen-free extract content (Ilyas et al., 19959. Fermentation increased phosphorus absorption of soybean meal in rats (Chapter 3 and 6). In the present study, effects of fermentation of soybean meal were investigated on phosphorus availability in chicks.

MATERIALS AND METHODS

Diet Preparation. Fermented soybean meal (FS) was prepared by the method described in the previous report (Ilyas et al., 1995). Briefly, commercial soybean meal was steamed and approximately 8×10^7 spores of *Aspergillus usamii* were added to 100g of steamed soybean meal. Then the soybean meal was fermented for 48 h. Following the first fermentation, the product was added water until moisture became 50 % and fermented again for 12 h. After the second fermentation, FS was dried at 45 °C. The following three diets were prepared; regular soybean meal (RS)-based diet, RS-based diet supplemented with inorganic phosphorus (RS+Pi) and FS-based diet (Table 8-1).

Feeding Study. Thirty 1-week-old male White Leghorn chicks with an average body weight of 64.1 g were used. Chicks were divided into three groups of ten chicks each and were fed one of three experimental diets for 4 weeks. The chicks were housed in electrically heated brooder battery cages for 2 weeks, and moved to unheated cages for the second 2-week period. The chicks were allowed free access to distilled water and experimental diets. Individual body weight and feed intake for each group were determined during the feeding trials, and feed/gain ratio calculated. The chicks were exsanguinated under diethyl ether anesthesia and the left femur was collected at the end of feeding trial.

Analyses. Femora were cleaned of adhering tissues, dried at 100°C for 24 h and weighted. The volume of the dried femur was measured using an Archimedean theorem (Whiting and Draper, 1981). Specific gravity of the femur was calculated from the dry weight and the volume. The bone was then ashed

at 600°C for 24h and ash content determined. Bone ash density was calculated from the ash content and the volume of bone. The ash was dissolved in 0.1M of HCl for calcium and phosphorus analysis. The diets were dried at 135°C for 2h and digested by concentrated nitric acid and 60% perchloric acid at approximately 600°C for calcium and total phosphorus analyses. Phosphorus contents in the femur and diets were measured by Gomori's method (Gomori, 1942). Calcium contents in the femur and diets were measured with an atomic absorption spectrophotometry (AA-782, Nippon Jarrel Ash, Kyoto, Japan). Dietary phytate phosphorus content was measured by AOAC procedure (1990). Nonphytate phosphorus content was calculated from the contents of total phosphorus and phytate phosphorus.

Statistical Analysis. All data except for feed intake and feed/gain ratio were tested by one-way ANOVA, which were analyzed using GLM procedure of SAS (1985) at the probability level of $P < 0.05$. Significant differences among the means of each group were determined using Duncan's new multiple range test (Duncan, 1955) where the dietary effects were significant.

RESULTS AND DISCUSSION

Most of the phosphorus in cereals and byproducts of seed origin exists as phytate phosphorus (Nelson et al., 1968). The RS used in the present experiment contained approximately 5.6 g/kg phytate phosphorus which account for 72% of total phosphorus in RS. Phytate phosphorus was not detected in the FS. A previous report similarly indicated that fermentation with *Aspergillus*

usamii almost completely degraded phytate in soybean meal (Ilyas et al., 1995).

The total phosphorus content was the same in the RS diet and the FS diet (Table 8-1). Because of the high phytate content in soybean meal, the nonphytate phosphorus content in the RS diet was 60% of that in the FS diet. The estimated requirements for nonphytate phosphorus are 4 g/kg in Leghorn-type chicks of 0-6 weeks of age (NRC, 1994). In the present experiment, dietary nonphytate phosphorus content was 3.6 g/kg in the FS and 4.2 g/kg in the RS+Pi diets. The diets of FS and RS+Pi contained almost a sufficient amount of nonphytate phosphorus for these chicks. The chicks fed the RS diet were phosphorus deficient, with a nonphytate phosphorus content of only 2.0 g/kg.

Femoral dry weight, ash, calcium and phosphorus contents, and bone ash density were significantly lower ($P<0.01$) in the RS group than in the FS and the RS+Pi groups (Table 8-3) and did not differ between the FS group and the RS+Pi group. Degradation of phytate in soybean meal apparently improved phosphorus availability. A second possibility was that the improvement was not due to the increase in phosphorus availability but rather to the increased calcium availability. Calcium absorption might be suppressed by phytic acid, which induced a slight deficiency of calcium with an accompanying decrease in mineral deposition including phosphorus deposition. Degradation of phytate might prevent the calcium deficiency. However, phytic acid decreases calcium absorption when the molar ratio between phytic acid and calcium is greater than 1.0 (Lonnerdal et al., 1989). Because the molar ratio was only 0.06 in the RS diet, calcium absorption should not have been suppressed in chicks fed the RS diet. The femoral parameters were improved when inorganic phosphorus was

added to the RS-based diet. The degradation of phytate directly increased phosphorus availability and the improvement in phosphorus availability increased the bone ash, phosphorus and calcium contents.

Body weight gain was significantly ($P<0.01$) higher in the FS group than in the RS group and similar to that in the RS+Pi group (Table 8-2). The chicks fed the RS diet suffered from phosphorus deficiency, which was the major reason for the suppression of body weight gain in the chicks fed the RS diet, and the feeding of FS or the supplementation of inorganic phosphorus to the RS-based diet prevented phosphorus deficiency. The feed/gain ratio tended to be higher in the RS group than in the FS group. There was a tendency for the feed/gain ratio to be lower in the FS group than in the RS+Pi group although there was not a large difference in the intake of nonphytate phosphorus between these groups. Phytic acid has been reported to decrease apparent digestibility of organic matter and protein (Mroz et al., 1994). The adverse actions of phytate on some nutrient digestion might partly explain the higher feed/gain ratio in chicks fed the diets containing RS than in those fed the FS diet. A further investigation is required to clarify the reduction of dietary phytate content increasing such nutrient digestion in chicks.

The present study has shown that the fermentation improves phosphorus availability of soybean meal in chicks. It is concluded that dietary supplementation of inorganic phosphorus is not necessary for chicks fed FS.

Table 8-1. Composition of the experimental diets

	RS ¹	RS+Pi ²	FS ³
Ingredient, g/kg			
Yellow maize	644	639	644
Soybean meal	260	260	0
Fermented soybean meal	0	0	260
Rice bran	50	50	50
Soybean oil	10	10	10
Calcium carbonate	18	11	18
Dicalcium phosphate	0	12	0
Sodium chloride	3	3	3
Vitamin-trace mineral pre-mix ⁴	14	14	14
DL-methionine	1	1	1
Chemical analysis ⁵			
Calcium, g/kg	9.1	9.3	9.4
Phosphorus, g/kg	5.5	7.7	5.6
Available phosphorus, g/kg	2.0	4.2	3.6

¹ Diet containing regular soybean meal. ² Diet containing regular soybean meal supplemented with inorganic phosphorus. ³ Diet containing fermented soybean meal. ⁴ Supplied per kg of diet: vitamin A (retinyl acetate), 3920 IU; vitamin D₃, 1540 IU; vitamin E (dl- α -tocophenyl acetate), 12IU; riboflavin, 12mg; pantothenic acid, 8mg; niacin, 40mg; choline chloride, 2800mg; vitamin B₁₂ 8 μ g; vitamin K, 8mg; folic acid, 200 μ g; copper, 20mg; iodine, 1200 μ g; manganese, 160mg; zinc, 160mg. ⁵ Analytical values.

Table 8-2. Weight gain, feed intake and feed/gain ratio in chicks fed diets based on regular or fermented soybean meal ¹

	Dietary treatment			
	RS ²	RS+Pi ³	FS ⁴	Effect ⁵
Weight gain ⁶ , g/day	9.6±0.8 ^b	12.3±0.8 ^a	11.9±1.0 ^a	***
Feed intake, g/day	24.7	32.1	27.8	
Feed/gain ratio	2.57	2.34	2.61	

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular soybean meal supplemented with inorganic phosphorus. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, ***; P<0.001. ⁶ Gain is calculated from initial and final body weight.

Table 8-3. Femoral dry weight, ash content, ash density, and contents of phosphorus and calcium in chicks fed diets based on regular or fermented soybean meal ¹

	Dietary treatment			
	RS ²	RS+Pi ³	FS ⁴	Effect ⁵
Dry weight, g	0.81±0.09 ^b	1.19±0.06 ^a	1.17±0.10 ^a	***
Ash content, g	0.27±0.02 ^b	0.46±0.03 ^a	0.46±0.04 ^a	***
Ash density, g/cm ³	0.18±0.02 ^c	0.23±0.02 ^b	0.24±0.02 ^a	***
Phosphorus, mg	50.8±5.0 ^b	89.9±5.0 ^a	85.9±6.9 ^a	***
Calcium, mg	108±23 ^b	178±9 ^a	173±14 ^a	***

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular soybean meal supplemented with inorganic phosphorus. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, ***; P<0.001.

Chapter 9

Fermentation of Soybean Meal with *Aspergillus Usamii* Reduces Phosphorus Excretion in Chicks

Soybean meal is commonly used for poultry feeds. However, more than half of phosphorus in soybean meal exists as phytate phosphorus (Earle and Miner, 1938). The digestibility of phytate phosphorus is low for poultry and thus inorganic phosphorus is commonly supplied to soybean meal-based diets. Biehl et al. (1995) has suggested that the poor digestibility of phytate phosphorus induces an environmental pollutant through increasing phosphorus excreta, phosphorus in animal waste spread on the soil can reach into ground water and into ponds, streams, lakes, and oceans. Moreover, supplementation with inorganic phosphorus increases animal feed costs. Many researchers have tried to improve the digestibility of phytate phosphorus by such means as the addition of microbial phytase (Nasi, 1990; Nelson et al., 1971) or 1,25-dihydroxycholecalciferol (Edwards, 1993) to feeds. In previous experiments, fermentation of soybean meal with *Aspergillus usamii* almost completely degraded phytate phosphorus into inorganic phosphorus (Ilyas et al., 1995) and improved phosphorus availability in chicks (Chapter 8; Matsui et al., 1996). The present study examined phosphorus excretion in chicks fed a fermented soybean meal-based diet.

MATERIALS AND METHODS

Diet Preparation. Fermented soybean meal (FS) was prepared by the

method described in a previous report (Ilyas et al., 1995). Briefly, commercial soybean meal was steamed and approximately 8×10^7 spores of *Aspergillus usarii* were added to 100 g of steamed soybean meal. Then the soybean meal was fermented for 48 h. Following the first fermentation, the product was added water until moisture became 50 % and fermented again for 12 h. After the second fermentation, FS was dried at 45 °C. The following three diets were prepared; a regular soybean meal (RS)-based diet, a RS-based diet supplemented with inorganic phosphorus (RS+Pi), and a FS-based diet (Table 9-1).

Feeding Study. Thirty 1-week-old male White Leghorn chicks with an average body weight of 88.4 g were used. Chicks were individually housed in electrically heated brooder battery cages for collection of excreta. The chicks were allowed free access to distilled water and experimental diets. The chicks were divided into three groups of ten chicks each and were fed one of three experimental diets for 4 weeks. Individual body weight was measured at the initiation and the end of feeding trial. Feed intake was recorded and excreta was collected in the last 5 days of feeding trial. The chicks were exsanguinated under diethyl ether anesthesia and the left femur was collected at the end of feeding trial.

Analyses. Femora were cleaned of adhering tissues, dried at 100 °C for 24 h, and weighed. The volume of the dried femur was measured using an Archimedean theorem (Whiting and Draper, 1981). Specific gravity of the femur was calculated from the dry weight and the volume. The diets and excreta were

dried at 135 °C for 2 h. All samples were digested by nitric acid and perchloric acid for mineral analysis. Phosphorus contents in the femur, diets, and excreta were measured by Gomori's method (Gomori, 1942). Calcium and magnesium contents in the femur and diets were measured using an atomic absorption spectrophotometry (AA-782, Nippon Jarrel Ash, Kyoto, Japan). Dietary phytate phosphorus content was measured by AOAC procedure (1990). Nonphytate phosphorus content was calculated from the contents of total phosphorus and phytate phosphorus. Phosphorus retention was calculated from phosphorus intake and phosphorus content in excreta.

Statistical Analysis. All data were tested by one-way ANOVA, which were analyzed using the GLM procedure of SAS (1985) at the probability level of $P < 0.05$. Significant differences among the means of each group were determined using Duncan's new multiple range test (Duncan, 1955), where the dietary effects were significant.

RESULTS

Daily intake and the amount of excreta did not differ among the dietary groups (Table 9-2). Body weight gain was less in the RS group than in the RS+Pi and the FS groups. Dry weight, specific gravity, and phosphorus content of the femur were higher in the RS+Pi and the FS groups than in the RS group and did not differ between the RS+Pi group and the FS group (Table 9-3). Contents of calcium and magnesium in the femur were higher in the RS+Pi and the FS groups than in the RS group. Femoral calcium content did not differ between the FS group and the RS+Pi group. On the other hand, femoral magnesium content was higher in the FS group than in the RS+Pi group.

The amount of retained phosphorus (g/day) was higher in the RS+Pi and the FS groups than in the RS group and did not differ between the RS+Pi group and the FS group (Table 9-4). The amount of excreted phosphorus was much higher in the RS+Pi group than in the FS group and the RS group. Thus phosphorus retention (% of intake) in the RS+Pi group was lower than in the FS group and was similar to that in the RS group.

DISCUSSION

The RS group gained less weight than did the RS+Pi group although daily feed intake did not differ between these groups. Dry weight, specific gravity and phosphorus content of the femur were higher in the RS+Pi group than in the RS group. Additionally, the amount of retained phosphorus (g/day) was also higher in the RS+Pi group than in the RS group. The RS diet contained only 2.3 g nonphytate phosphorus per kg of diet because RS used in the present study contained 5.6 g/kg phytate phosphorus. Since the estimated requirement of nonphytate phosphorus was 4.0 g/kg in Leghorn-type chicks of 0 to 6 weeks of age (NRC, 1994), the RS group was considered to be deficient in phosphorus. Thus the RS group showed less body weight gain, lower femoral parameters and less amount of retained phosphorus than did the RS+Pi group which was fed an enough amount of nonphytate phosphorus.

Total phosphorus content in the FS diet was as low as that in the RS diet. However, the FS diet contained 3.9 g/kg of nonphytate phosphorus because phytate phosphorus in RS was almost completely degraded into inorganic phosphorus by the fermentation. The content of nonphytate phosphorus did not differ between the FS diet and the RS+Pi diet which was supplied 0.2 % of

inorganic phosphorus. Body weight gain, dry weight, specific gravity, and femoral phosphorus content, and the amount of retained phosphorus were larger in the FS group than in the RS group, and these parameters were not different between the FS group and the RS+Pi group. These results indicated that fermentation improved phosphorus availability in RS through the degradation of phytate phosphorus and chicks fed the FS diet without supplemental inorganic phosphorus did not exhibit phosphorus deficiency, as supported by data in the previous experiment (Chapter 8; Matsui et al., 1996). Additionally, the substitution of FS for RS is equivalent to the addition of 0.2 % inorganic phosphorus.

Although the nutritional status of phosphorus did not differ between the FS group and the RS+Pi group, the amount of excreted phosphorus was much higher in the RS+Pi group than in the FS group. Thus, phosphorus retention (% of intake) in the RS+Pi group was lower than in the FS group. The increase of phosphorus in animal wastes is one of the most serious problems in animal waste management and the supplementation of inorganic phosphorus to diets increases the animal feeding cost (Biehl et al., 1995). The substitution of FS for RS reduced phosphorus excretion without adversely affecting body weight gain and the femoral parameters.

Femoral contents of calcium and magnesium were higher in the RS+Pi group and the FS group than in the RS group, and calcium content did not differ between the FS group and the RS+Pi group. Phytic acid has been suggested to inhibit availabilities of cations such as calcium (Morris and Ellis, 1985; Nelson and Kirby, 1987) and magnesium (Brink et al., 1992; Miyazawa et al., 1996). On

the other hand, calcium absorption was reported to be inhibited by phytic acid when the molar ratio of phytic acid to calcium was more than 0.2 in chicks (Nelson and Kirby, 1987). Because the molar ratio of phytic acid to calcium was approximately 0.017 in the RS diet, phytic acid might not inhibit calcium absorption in the RS diet. Jongbloed (1987) suggested that the availabilities of calcium and magnesium were depressed in a RS-based diet when dietary calcium and magnesium were adequate and dietary phosphorus was inadequate. Availabilities of calcium and magnesium were probably improved by increasing nonphytate phosphorus contents in the RS+Pi and the FS diets, i.e., phosphorus was deficient in the RS group and thus mineralization of bone was suppressed, which resulted in the lower contents of calcium and magnesium in the femur of this group. In contrast to the RS group, phosphorus was adequate in the RS+Pi and the FS groups and thus more calcium and magnesium could be deposited as bone minerals in these groups than in the RS group. On the other hand, femoral magnesium content was higher in the FS group than in the RS+Pi group. The difference of femoral magnesium content between the RS+Pi group and the FS group may be due to phytic acid in the RS+Pi diet. In a previous report, FS-based diet showed higher magnesium availability than RS-based diet in rats when the diets contained adequate level of phosphorus (Chapter 4; Hirabayashi et al., 1995).

In conclusion, fermentation of soybean meal increased phosphorus availability and reduced phosphorus excretion without affecting growth of chicks. Using the fermented soybean meal as substitute for regular soybean meal appeared to save 0.2 % of dietary inorganic phosphorus.

Table 9-1. Composition of the experimental diets

	RS ¹	RS+Pi ²	FS ³
Ingredient, g/kg			
Yellow maize	575.5	571.5	575.5
Soybean meal	300	300	0
Fermented soybean meal	0	0	300
Rice bran	70	70	70
Soybean oil	20	20	20
Calcium carbonate	16.5	10.5	16.5
Dicalcium phosphate	0	10	0
Sodium chloride	3	3	3
Vitamin-trace mineral pre-mix ⁴	14	14	14
DL-methionine	1	1	1
Chemical analysis ⁵			
Calcium, g/kg	12.0	11.7	10.1
Phosphorus, g/kg	5.2	7.1	5.8
Available phosphorus, g/kg	2.3	4.0	3.9
Magnesium, g/kg	2.4	2.6	2.3

¹ Diet containing regular soybean meal. ² Diet containing regular soybean meal supplemented with inorganic phosphorus. ³ Diet containing fermented soybean meal. ⁴ Supplied per kg of diet: vitamin A (retinyl acetate), 3920 IU; vitamin D₃, 1540 IU; vitamin E (dl- α -tocophenyl acetate), 12IU; riboflavin, 12mg; pantothenic acid, 8mg; niacin, 40mg; choline chloride, 2800mg; vitamin B₁₂ 8 μ g; vitamin K, 8mg; folic acid, 200 μ g; copper, 20mg; iodine, 1200 μ g; manganese, 160mg; zinc, 160mg. ⁵ Analytical values.

Table 9-2. Daily intake, fecal excretion, and body weight in chicks fed diets based on regular or fermented soybean meal ¹

	Dietary treatment			
	RS ²	RS+Pi ³	FS ⁴	Effect ⁵
Daily intake, g/day	59.5±1.3	61.8±1.4	60.5±1.8	NS
Fecal excretion, g/day	16.4±0.5	16.4±0.5	16.5±0.6	NS
Body weight				
Initial, g	88.0±0.5	88.9±0.4	88.5±0.4	NS
Final, g	436±6 ^b	487±7 ^a	472±9 ^a	***
Gain ⁶ , g	348±5 ^b	399±7 ^a	383±9 ^a	***

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular soybean meal supplemented with inorganic phosphorus. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, ***; P<0.001, NS; not significant (P>0.05). ⁶ Gain is calculated from initial and final body weight.

Table 9-3. Femoral dry weight, specific gravity, and contents of phosphorus, calcium, and magnesium in chicks fed diets based on regular or fermented soybean meal ¹

	Dietary treatment			
	RS ²	RS+Pi ³	FS ⁴	Effect ⁵
Dry weight, g	2.05±0.04 ^b	2.52±0.05 ^a	2.44±0.05 ^a	***
Specific gravity, g/cm ³	0.839±0.007 ^b	0.880±0.006 ^a	0.887±0.004 ^a	***
Phosphorus, mg	163±6 ^b	217±7 ^a	214±11 ^a	***
Calcium, mg	318±12 ^b	452±23 ^a	472±25 ^a	***
Magnesium, mg	5.73±0.27 ^c	9.60±0.29 ^b	10.70±0.44 ^a	***

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular soybean meal supplemented with inorganic phosphorus. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, ***; P<0.001.

Table 9-4. Excretion and retention of phosphorus in chicks fed diets based on regular or fermented soybean meal ¹

	Dietary treatment			Effect ⁵
	RS ²	RS+Pi ³	FS ⁴	
Phosphorus intake, mg/day	307±7 ^c	439±10 ^a	349±10 ^b	***
Excreted phosphorus, mg/day	240±7 ^b	337±11 ^a	234±14 ^b	***
Retained phosphorus, mg/day	66±6 ^b	95±6 ^a	116±11 ^a	***
Phosphorus retention, % of intake	21.5±2.0 ^b	21.8±1.3 ^b	33.2±3.2 ^a	**

¹ Values are means ± SEM. Means within a row not sharing the same superscript are significantly different (P<0.05). ² Diet containing regular soybean meal. ³ Diet containing regular soybean meal supplemented with inorganic phosphorus. ⁴ Diet containing fermented soybean meal. ⁵ Statistical effect, **, P<0.01, ***, P<0.001.

Chapter 10

Summary

Chapter 3. Fermentation of Soybean Meal with *Aspergillus****Usamii* Improves Phosphorus Absorption in Rats**

Fermentation of soybean meal with *Aspergillus usamii* almost completely degraded phytate-phosphorus into inorganic phosphorus. Effects of fermented soybean meal were investigated on absorption of phosphorus and calcium. Phosphorus solubility in the lower small intestine was higher in rats fed a fermented soybean meal-based diet than rats fed a regular soybean meal-based diet. Apparent phosphorus absorption was also higher in the fermented soybean meal group than in the regular soybean meal group. However, phosphorus content in the femur and phosphorus concentration in plasma did not differ between these groups. Solubility of calcium, apparent calcium absorption, femoral calcium content, and plasma calcium concentration were not different between these groups. These results suggested that fermentation with *Aspergillus usamii* improved absorption of phosphorus in regular soybean meal through increasing phosphorus solubility in the small intestine by the degradation of phytate-phosphorus.

Chapter 4. Fermentation of Soybean Meal with *Aspergillus*

Usamii Improves Magnesium Availability in Rats

Fermentation of soybean meal with *Aspergillus usamii* almost completely degraded phytic acid into inorganic phosphorus and inositol. Effects of fermented soybean meal on the availability of magnesium were investigated. Magnesium solubility in the lower small intestine was higher in rats fed a fermented soybean meal-based diet than in rats fed a regular soybean meal-based diet. The fermented soybean meal group showed higher apparent magnesium absorption than did the regular soybean meal group. Magnesium concentration in the femur was also higher in the fermented soybean meal group than in the regular soybean meal group. These results suggested that the dephytinization of soybean meal by the fermentation with *Aspergillus usamii* improved magnesium availability through increasing magnesium solubility in the lower small intestine.

Chapter 5. Fermentation of Soybean Meal with *Aspergillus*

Usamii Improves Zinc Availability in Rats

Soybean meal was fermented with *Aspergillus usamii* to improve zinc availability through the degradation of phytic acid. Rats fed a diet containing fermented soybean meal showed greater femoral zinc than did animals fed a diet containing regular soybean meal. Zinc solubility in the small intestine was higher in the rats fed fermented soybean meal than in the rats fed regular soybean meal. These results suggested that fermentation with *Aspergillus usamii* improved zinc availability in dietary soybean meal, which was induced by the increase of zinc solubility in the small intestine. Adding the same amount of phytate that was contained in the regular soybean meal-based diet did not affect the amount of zinc present in rats fed a fermented soybean meal-based diet with sodium phytate. Phytase activity was found in fermented soybean meal and this activity may degrade added phytate in fermented soybean meal-based diet.

Chapter 6. Degradation of Intrinsic Phytate Using Fermented Soybean Meal Improves Magnesium Availability in Rats

Phytase activity was found in the soybean meal fermented with *Aspergillus usamii*. To investigate the possibility to degrade intrinsic phytic acid derived from regular soybean meal by phytase activity from fermented soybean meal, three diets were prepared; a diet consisting of 40% regular soybean meal (RS diet), a diet consisting of 40% fermented soybean meal (FS diet), and a diet consisting of 20% regular soybean meal and 20% fermented soybean meal (RF diet). Fecal phosphorus excretion (% of intake) in the RF group was lower in the RS group but tended to be higher than in the FS group ($P=0.07$). Thus, apparent phosphorus absorption (% of intake) was lower in the RF group than in the FS group. Fecal magnesium excretion (% of intake) was lower in the RF and the FS groups than in the RS group and did not differ between the RF group and the FS group. Apparent magnesium absorption (% of intake) was higher in the RF and the FS groups than in the RS group, which were not different between the RF group and the FS group. Magnesium concentrations in the femur and plasma were higher in the RF and the FS groups than in the RS group. As a result, magnesium availability in the RF group was as high as in the FS group which may be the result from phytase activity originated from FS degrading intrinsic phytate from RS.

Chapter 7. Fermentation of Soybean Meal with *Aspergillus*

***Usamii* Improves Availabilities of Zinc and Iron in Rats**

Soybean meal was fermented with *Aspergillus usamii* to improve availabilities of dietary zinc and iron through the degradation of phytate. Three kinds of experimental diets which differed in protein sources were prepared; a diet consisting of 40% regular soybean meal (RS diet), a diet consisting of 40% fermented soybean meal (FS diet), or a diet consisting of 20% regular soybean meal and 20% fermented soybean meal (RF diet). Zinc solubility in the upper and the lower segments of small intestine were higher in rats fed the FS diet than in rats fed the RS diet. The FS group showed higher solubility of iron in the lower small intestine than did the RS group. Zinc concentrations in the femur and plasma, and iron concentrations in the liver and plasma were higher in the FS group than in the RS group. These results suggested that fermentation of soybean meal improved availabilities of dietary zinc and iron, which may be induced by increasing solubilities of these minerals in the small intestine through the reduction of phytate content. Femoral and plasma zinc concentrations in the RF group were higher than in the RS group but were lower than in the FS group. Liver and plasma iron concentrations were not different between the RF group and the FS group. Although phytase activity in FS degrades phytate in the RF diet, higher activity may need to degrade phytate completely.

Chapter 8. Fermentation of Soybean Meal with *Aspergillus*

Usamii Improves Phosphorus Availability in Chicks

The present study examined effects of fermentation of soybean meal with *Aspergillus usamii* on phosphorus availability in chicks. Thirty 1-week-old male Leghorn type chicks were divided into three groups and fed following each diet for 4 week: a diet consisting of regular soybean meal (RS diet; total phosphorus, 5.5 g/kg; nonphytate phosphorus, 2.0 g/kg), a diet consisting of regular soybean meal supplying inorganic phosphorus (RS+Pi diet; total phosphorus, 7.7 g/kg; nonphytate phosphorus, 4.2 g/kg) or a diet consisting of fermented soybean (FS diet; total phosphorus, 5.5 g/kg; nonphytate phosphorus, 2.0 g/kg). Body weight gain was significantly higher in the chicks fed the FS diet and the RS+Pi diet than in birds fed the RS diet during the 4 week feeding trial. The 5-week-old chicks were killed and the left femur was collected at the end of the feeding period. Femoral dry weight, ash, calcium and phosphorus contents, as well as ash density, were significantly greater in chicks fed the FS and the RS+Pi diets than in birds fed the RS diet. Body weight gain and femoral parameters did not differ between the chicks fed the FS diet and birds fed the RS+Pi diet. It was concluded that fermentation improved phosphorus availability of soybean meal in chicks and dietary supplementation of inorganic phosphorus was not necessary for chicks fed FS.

Chapter 9. Fermentation of Soybean Meal with *Aspergillus*

Usamii Reduces Phosphorus Excretion in Chicks

Fermentation with *Aspergillus usamii* almost completely degrades phytate phosphorus in soybean meal. Phosphorus excretion was investigated in chicks fed a fermented soybean meal-based diet. Thirty chicks were fed one of three experimental diets; a regular soybean meal-based diet (total phosphorus, 5.2 g/kg; non-phytate phosphorus, 2.3 g/kg), a regular soybean meal-based diet added inorganic phosphorus (total phosphorus, 7.1 g/kg; non-phytate phosphorus, 4.0 g/kg), or a fermented soybean meal-based diet without supplemental inorganic phosphorus (total phosphorus, 5.8 g/kg; non-phytate phosphorus, 3.9 g/kg) for 4 weeks. Body weight gain, the amount of retained phosphorus (g/day) and femoral phosphorus content were lower in the regular soybean meal group than in the phosphorus-supplied group and the fermented soybean meal group. The latter two groups showed similar body weight gain and femoral phosphorus content. On the other hand, phosphorus excretion was markedly more in the phosphorus-supplied group than in the other groups. As the result, phosphorus retention (% of intake) was lower in the phosphorus-supplied group than in the fermented soybean meal group. In conclusion, fermentation improved phosphorus bioavailability in soybean meal and supplemental inorganic phosphorus was not necessary for adding to the fermented soybean meal-based diet, which remarkably reduced phosphorus excretion.

Conclusion

Fermented soybean dishes known as tempe (*Rhizopus oligosporus* fermented product), miso (*Aspergillus oryzae* fermented product) and natto (*Bacillus natto* fermented product) are popular dietary items in the Orient. Fermentation of defatted soybean meal with *Aspergillus usamii* almost completely degraded phytate into inorganic phosphorus and inositol without changing amino acid composition. Furthermore, Aflatoxin B1 was not detected in the fermented soybean meal and the contents of crude protein, crude fiber, ether extract and crude ash did not significantly differ between the regular soybean meal and the fermented soybean meal (Ilyas et al., 1995). This study showed the effects of fermented soybean meal on mineral availability. Fermented soybean meal improved bioavailabilities of magnesium, zinc and iron through converting phytate-phosphorus to available phosphorus. Furthermore, both extrinsically added phytate and intrinsic phytate did not affect on availabilities of minerals in diets containing fermented soybean meal, which was due to the phytase activity in the fermented soybean meal.

The environmental pollution by phosphorus and heavy metals such as zinc and iron from animal waste have been increasing (Baars et al., 1988; Biehl et al., 1995; Brennan, 1991; Hart et al., 1985; Picket et al., 1992; Sterrett et al., 1982, 1983). Biehl et al. (1995) has also suggested that supplementation with inorganic phosphorus increases animal feed costs. Dietary phosphorus and heavy metals thus can be reduced by feeding fermented soybean meal as substitute for regular soybean meal through improving availabilities of minerals, which may result in decreasing excreted minerals and saving costs. Furthermore,

mineral availability of another ingredients containing phytate might be improved because of the phytase activity in fermented soybean meal. In conclusion, fermented soybean meal becomes superior feed materials as a protein source.

The use of soybean protein has been increasing for many commercial processed foods such as analogs of chicken, ham and beef because it is good and economically cheap source of protein. Thus the modifications and improvements in nutritional characteristics of soybean become more important. Using fermented soybean protein as substitute for unfermented regular soybean protein appeared to greatly improve nutritional quality of soybean products. Therefore, fermented soybean protein could become a useful food materials.

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