Fermentation of Soybean Flour with *Aspergillus usamii* Improves Availabilities of Zinc and Iron in Rats

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Summary Soybean flour was fermented with *Aspergillus usamii* to improve the availabilities of dietary zinc and iron through the degradation of phytate. Three kinds of experimental diets that differed in protein sources were prepared: one consisting of 40% regular soybean flour (RS diet), one consisting of 40% fermented soybean flour (FS diet), and one consisting of 20% regular soybean flour and 20% fermented soybean flour (RF diet). Zinc solubilities in the upper and the lower segments of the 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 the RS group did. 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 the fermentation of soybean flour improved the availabilities of dietary zinc and iron, which may be induced by increasing the 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 lower than in the FS group. No difference was noted in liver and plasma iron concentrations between the RF group and the FS group. Although phytase activity in FS degrades phytate in the RF diet, higher activity may be needed to degrade phytate completely.

Key Words fermentation, soybean flour, zinc, iron, rat

The growth retardation is highly prevalent in young children in many developing countries, which is due to micronutrient deficiencies, especially zinc and iron (1, 2). Soy formulas are commonly fed to infants because soybean products are good protein sources. However, soybean products have large amounts of phytic acid, which suppresses the availabilities of zinc (3–5) and iron (6, 7) by making insoluble complexes with these elements in the digestive tract.

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Tchango (8) reported that fermentation of maize-soybean tempe flour with *Rhizopus oligosporus* reduced phytic acid and increased protein digestibility. *Aspergillus usamii* is used for making mirin (9) and sake (10). The digestibilities of starch and protein in koji prepared with *Aspergillus usamii* were reported to be higher than those prepared with *Aspergillus niger*, from which phytase is frequently used as feed additive, and *Rhizopus oligosporus* (9).

In the previous report, the fermentation of soybean flour with *Aspergillus usamii* degraded phytic acid almost completely into inorganic phosphorus and inositol (11). First, the present study examined the effects of fermented soybean flour on availabilities of zinc and iron.

The addition of microbial phytase (EC 3.1.3.26) to diets containing phytate was reported to improve the bioavailability of zinc (12–14) and iron (15). Zhu et al (16) indicated that incubation of soybean flour with wheat bran partly degraded phytate in soybean flour, which was due to phytase activity (EC 3.1.3.8) from bran. Morris and Ellis (17) observed that the soaking of wheat bran increased zinc concentration in the femur of rats through the degradation of phytate by the activation of endogenous phytase in bran. Although nonfermented soybean products showed little or no phytase activity (18), it was found in soybean flour fermented by *Aspergillus usamii* (11). Second, this study examined the efficacy of phytase activity from fermented soybean flour on bioavailabilities of zinc and iron in a diet consisting of both regular and fermented soybean flours.

**MATERIALS AND METHODS**

*Diet preparation.* Soybean flour was fermented by the method described in the previous report (11). Briefly, commercial soybean flour was steamed and approximately $8 \times 10^7$ spores of *Aspergillus usamii* were added to 100 g of steamed soybean flour. The flour was then fermented for 48 h. Following the first fermentation, water was added to the product until moisture became 50%, and it was fermented again for 12 h. After the second fermentation, the flour was dried at 45°C. Three diets were prepared, one consisting of 40% regular soybean flour (RS diet), one consisting of 40% fermented soybean flour (FS diet), and one consisting of 20% RS and 20% FS (RF diet) (Table 1).

*Feeding study.* Eighteen male Wistar rats aged 6 weeks and weighing approximately 100 g were purchased from Japan SLC (Shizuoka, Japan) and were cared for according to the Guide for the Care and Use of Laboratory Animals (Kyoto University Animal Care Committee). The rats were individually housed in stainless steel cages in a room with controlled temperature (22°C), relative humidity (60%), and lighting (light, 19:00–7:00; dark, 7:00–19:00, a 12 h cycle). They were given distilled water and experimental diets ad libitum. All rats were fed the RS diet during a 7-d preliminary period. They were then randomly allotted to three dietary groups of six animals each and fed one of three experimental diets for 4 weeks just before the anesthesia. The rats were exsanguinated under pentobarbital
Table 1. Composition of the experimental diets.

| Ingredient (g/kg)       | RS\(^1\) | RF\(^2\) | FS\(^3\) |
|-------------------------|----------|----------|----------|
| Soybean flour           | 400      | 200      | 0        |
| Fermented soybean flour | 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     |
| DL-Methionine           | 3        | 3        | 3        |

Chemical analysis\(^6\)

| Ingredient (g/kg)       | RS\(^1\) | RF\(^2\) | FS\(^3\) |
|-------------------------|----------|----------|----------|
| Crude protein (g/kg)    | 188      | 200.4    | 212.8    |
| 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 flour.
\(^2\) Diet containing regular and fermented soybean flour.
\(^3\) Diet containing fermented soybean flour.
\(^4\) 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.

Analyses. Phytic acid contents in diets were measured according to Association of Official Analytical Chemists procedure (19). Phytase activities in RS and FS were determined by the method of Han et al. (20) with minimal modification that true inorganic phosphorus concentration was measured by the method of

anesthesia and blood was collected with heparinized tubes from the abdominal aorta at 9:00 o'clock at the end of the trial. The small intestine was removed and divided into two segments of equal length. Digest in the upper and the lower segments of small intestine were collected by flushing 10 mL of ice-cold saline and diluted to 20 mL. The right femur and the liver were also collected.

Analyses. Phytic acid contents in diets were measured according to Association of Official Analytical Chemists procedure (19). Phytase activities in RS and FS were determined by the method of Han et al (20) with minimal modification that true inorganic phosphorus concentration was measured by the method of
Takahashi (21). One unit of enzyme activity was expressed as nmol phosphorus production for 1 min.

After the value of pH in diluted digest was measured, the digest was homogenized at 1,000 rpm for 1 min and 10 mL of the homogenate was centrifuged at 10,000 × 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. 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 digest.

Statistical analysis. The data from digest were tested by three-way ANOVA, which was employed to establish significant parameters between the diets (RS, RF, and FS), the segments of small intestine (the upper and the lower), the animals nested in the diet’s effect, and their interactions. The other data were tested by one-way ANOVA. All data were analyzed by using the general linear models (GLM) procedure of Statistical Analysis Systems (22) at the probability level of p<0.05. Significant differences among the means of each group were determined by using Duncan’s new multiple-range test (23) where the dietary effects were significant.

RESULTS

Body weight gain did not differ among the dietary groups. However, zinc concentrations in the femur and plasma were higher in the FS (femur, p<0.001; plasma, p<0.001) and the RF (femur, p=0.003; plasma, p<0.001) groups than in the RS group (Table 2). Furthermore, the higher zinc concentrations were shown in the femur and plasma of the FS group compared with the RF group (femur, p=0.001; plasma, p=0.004). Iron concentrations in the liver and plasma were higher in the FS (liver, p=0.009; plasma, p<0.001) and the RF (liver, p<0.001; plasma,
Table 3. Concentrations of iron in the liver and plasma in rats fed diets containing regular and/or fermented soybean flour.1

| Dietary treatment | RS2 | RF3 | FS4 | Effect5 |
|-------------------|-----|-----|-----|---------|
| Liver (μg/g)      | 79 ± 4b | 194 ± 28a | 159 ± 16a | ** |
| Plasma (mg/L)     | 3.60 ± 0.19b | 5.75 ± 0.38a | 6.49 ± 0.26a | ** |

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

The value of pH in each segment of the small intestine did not differ among the dietary groups (p>0.05) (Table 4). Zinc solubilities in the upper and lower
small intestine were higher in the FS (upper, \( p < 0.001 \); lower, \( p < 0.001 \)) and the RF (upper, \( p = 0.002 \); lower, \( p < 0.001 \)) groups than in the RS group and were not different between the FS group and the RF group (upper, \( p = 0.47 \); lower, \( p = 0.55 \)). Iron solubility in the upper small intestine did not differ among the dietary groups \( (p > 0.05) \). On the other hand, iron solubility in the lower small intestine was lower in the RS group than in the other groups (FS, \( p < 0.001 \); RF, \( p = 0.001 \)).

**DISCUSSION**

The RS diet contained 50 mg zinc/kg diet, which might be adequate because the dietary zinc requirement was estimated to be a 12 mg/kg diet in rats fed egg white- or casein-based diets (24). However, Sandstrom et al (25) 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 et al (26) showed that 50 mg/kg zinc was insufficient for the 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 (27). 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 intestines of the RS group compared with the FS group. Phytate-zinc complex was reported to precipitate at pH above 6.3 (28). The value of pH was 6.84 in the upper small intestine of the RS group. Thus phytate in RS probably made an insoluble complex with zinc and decreased zinc solubility in the upper small intestine of the RS group. Because the fermentation with *Aspergillus usamii* almost completely degraded phytate into inorganic phosphorus and inositol in soybean flour (11), zinc did not form an 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 (29). Shinoda and Yoshida (4) reported that dietary phytic acid decreased zinc solubility in the small intestine of rats, which resulted in decreasing bone zinc concentration. The fermentation of soybean flour improved zinc bioavailability; this 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 (24). However, the amount and type of protein were suggested to play an important role in iron absorption (30, 31), and iron requirement has not been well defined when protein sources differed. 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 contains phytate (32). Kasaoka et al (33) reported that iron concentration in the liver was 127 µg/g in iron-deficient anemic rats.
Because liver iron concentration was 79 µg/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 than in the RS group. Salz et al (34) reported that iron was absorbed in the whole small intestine, and the main absorption site of iron was duodenum. Monsen and Cook (35) 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 (28) reported that phytate-iron complex increased to precipitate at pH above 7.33. Because the value of pH in digest 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 (36) reported that phytate formed soluble complexes with iron. Kim and Atallah (37) indicated that the solubility of iron did not completely reflect its absorbability and suggested that iron bioavailability depended on the solubility of iron complex and the binding intensity of that complex. Phytate in the RS diet possibly decreased iron absorption by making a 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 flour 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 than in the RS group may be a result of the increase of iron absorption in the lower segment of the small intestine because of the higher iron solubility in this segment of the FS group.

The 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 flour degraded phytate of the regular soybean flour 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 1,200 U/g phytase activity was reported to need to maximally degrade phytate in the digestive tract (12, 38). Phytic acid, i.e., inositol hexaphosphate (IP₆), in wheat bran, was reported to be hydrolyzed to lesser phosphorylated derivatives of inositol, such as inositol tri- (IP₃), tetra- (IP₄), and penta- (IP₅) phosphates, during digestion in the gut (39–41). However, phytic acid in extrusion cooked-bran was not hydrolyzed, and IP₃, IP₄ and IP₅ did not increase in the digestive tract, suggested to be due to the degraded endogenous phytase activity by extrusion cooking (40, 41). The digest of the RF group was considered to contain these lesser phosphorylated derivatives of inositol.
(42) indicated that zinc bound lesser-phosphorylated derivatives of inositol became more soluble as the number of phosphate groups per molecule decreased. Han et al (43) showed that solubilized IP$_3$, IP$_4$, IP$_5$, and IP$_6$ inhibited zinc transport from extracellular fluid into cytoplasm but the inhibitory effects of IP$_3$ and IP$_4$ were weaker than those of IP$_5$ and IP$_6$ in the 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 that resulted from the partial degradation of phytate by phytase that originated in 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. There was no difference 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 a complex with zinc than iron (5); thus lesser phosphorylated inositols are considered to more easily form a complex with zinc than with iron. Han et al (43) 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 that was originated from fermented soybean flour may degrade a measure of intrinsic phytate in regular soybean flour, but the activity appeared to be insufficient for complete degradation.

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