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Effect of Zinc Sulfate Fortification in Germinated Brown Rice on Seed Zinc Concentration, Bioavailability, and Seed Germination

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ABSTRACT: Rice is the staple food for more than half of the world's population and, hence, the main source of a vital micronutrient, zinc (Zn). Unfortunately, the bioavailability of Zn from rice is very low not only due to low content but also due to the presence of some antinutrients such as phytic acid. We investigated the effect of germination and Zn fortification treatment on Zn bioavailability of brown rice from three widely grown cultivars using the Caco-2 cell model to find a suitable fortification level for producing germinated brown rice. The results of this study showed that Zn content in brown rice increased significantly ($p < 0.05$) as the external Zn concentrations increased from 25 to 250 mg/L. In contrast, no significant influence ($p > 0.05$) on germination percentage of rice was observed when the Zn supply was lower than 150 mg/L. Zn fortification during the germination process has a significant impact on the Zn content and finally Zn bioavailability. These findings may result from the lower molar ratio of phytic acid to Zn and higher Zn content in Zn fortified germinated brown rice, leading to more bioavailable Zn. Likewise, a significant difference ($p < 0.05$) was found among cultivars with respect to the capacity for Zn accumulation and Zn bioavailability; these results might be attributed to the difference in the molar ratio of phytic acid to Zn and the concentration of Zn among the cultivars evaluated. Based on global intake of Zn among the world population, we recommend germinated brown rice fortified with 100 mg/L $ZnSO_4$ as a suitable concentration to use in the germination process, which contains high Zn concentration and Zn bioavailability. In the current study, the cultivar Bing91185 fortified with Zn through the germination process contained a high amount as well as bioavailable Zn, which was identified as the most promising cultivar for further evaluation to determine its efficiency as an improved source of Zn for target populations.

KEYWORDS: *rice, zinc, fortification, bioavailability, Caco-2 cell*

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important food crops in the world, providing calories and essential micronutrients for more than three billion people.[1] Rice, however, is inherently a poor source of many essential micronutrients, especially zinc (Zn). Unfortunately, rice is also not a good source of metabolizable Zn, due to the presence of phytic acid, an outstanding absorption inhibitor, which lowers the bioavailability of Zn.[2] As a consequence, at least one-third of world's population is currently affected by Zn deficiency, particular in southeast Asia,[3] where people largely consume rice as a staple food. Therefore, Zn deficiency has been recognized as a global public health concern.[4] Many research groups and consortia all over the world are intensively working on how to increase the amount of Zn and at the same time to decrease the phytic acid content in rice grain. There are several potential approaches to increase the Zn concentration in rice grain, including conventional breeding and genetic engineering; however, conventional breeding is time-consuming and expensive,[5] and farmers are usually not willing to alter the existing cultivar due to socioeconomic reasons; on the other hand, use of genetically modified crops has been meet with considerable consumer resistance amid concerns for its safety.[6] Furthermore, lowering the phytic acid from rice grain is not feasible because phytic acid and its synthetic pathways are important to plant function and productivity.[7] Hence, it is urgent to find an alternative approach to increase more bioavailable Zn in an existing rice cultivar. Histochemical study shows that rice caryopsis is composed of one to several aleurone layers, with a varietal difference on number of aleurone layers also found.[8] During the germination with Zn solution, aleurone layers of rice caryopsis accumulate more Zn, hence, we hypothesized that Zn levels in rice grain also increase. Among several chemical forms used in the Zn fortification program, $ZnSO_4$ is widely accepted due to its low cost and is better absorbed because of its greater solubility at a neutral pH.[9] During rice seed germination, phytic acid content also decreases to a certain level.[10] Therefore, $ZnSO_4$ fortified germinated brown rice might be a quick and cost-effective solution to fight against Zn deficiency in the developing world without altering the existing consumption behavior.

The nutritional components in rice mainly exist in the germ and bran layers, unfortunately, most of which are removed during the polishing process.[11] Among health conscious people, brown rice has become more preferable as it contains more nutritional components than polished rice.[12] Germinated brown rice can be produced in the household, by a simple technique, needing only to be soaked in water until it has a sprout.[13] During germination, hydrolytic enzymes in the rice grain were activated to hydrolyze the high molecular weight polymers, resulting in generation of biofunctional substances

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such as γ-aminobutyric acid (GABA) and improvement of digestive compounds such as free amino acids.[14] After germination, ganoleptic qualities of brown rice were improved due to softening of texture, making it easy to cook.[15,16] For its nutritional value and cooking character, germinated brown rice is commercially used as primary source for various promising food materials. Recently, germinated brown rice has become one of the most interesting rice products and gained great attention, particularly in Asian countries.[17] We therefore selected the brown rice from three cultivars which are commonly consumed and cultivated in southern China.

The Zn nutritional value of fortified germinated brown rice depends not only on the Zn concentration in the rice grain but, to a large extent, on the Zn bioavailability to human after consumption. Therefore, information of Zn bioavailability on Zn fortified germinated brown rice is important to accurately establish the fortification level. The bioavailability of Zn can be defined as the proportion of ingested Zn from food that can be absorbed and utilized for normal metabolic and physiological functions or storage.[18] For assessment of accurate bioavailability of Zn, human feeding trials are essential. However, until now, the cost of human feeding trials has limited its applicability for large scale screening of samples.[19] In vitro methods based on measuring solubility of Zn are convenient screening tools, but often failed to estimate the more complex bioavailability (in vivo),[20] as the solubility method covers only the first phase of the overall Zn absorption process. The development of an in vitro Zn bioavailability model system that stimulants the gastric and intestinal digestion of humans, coupled with culture of human intestinal epithelial cells (Caco-2), offers a more physiological tool for addressing Zn bioavailability issues. The Caco-2 cell model possesses many of the functional and morphological properties of mature human enterocytes, considered to be more rapid and inexpensive methods to addressing Zn bioavailability in the diet.[21,22] This promising technique has been applied to measure the bioavailability of Zn in the present study.

The aim of this study was as follows: (i) to confirm a suitable concentration of $ZnSO_4$ for producing Zn fortified germinated brown rice, (ii) to compare the ability of Zn accumulation during germination of different rice cultivars, and (iii) to analyze the effect of germination and Zn fortification treatments on Zn bioavailability of rice assessed by the in vitro digestion/Caco-2 cell model.

MATERIALS AND METHODS

Chemicals and Reagents. Porcine pepsin, pancreatin, bile salts, and sulfosalicylic acid were purchased from Sigma-Aldrich (St Louis, MO). Dulbecco's modified Eagle medium (DMEM with glucose 4.5 g/L), trypsin−EDTA, Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS), glutamine, nonessential amino acids, penicillin, and streptomycin were purchased from Gibco Life Technologies (Grand Island, NY). All other chemicals were of analytical grade commercially obtained from local chemical suppliers. All reagents were prepared with deionized water (≤0.1 μS cm^{-1}) using a Milli-Q system (Millipore, Billerica, MA). All laboratory glassware used in the experiments was soaked for 24 h in 10% nitric acid and subsequently rinsed with deionized water and air-dried.

Zn Fortified Brown Rice Preparation. Three cultivars, namely, LYP9, Bing91185, and XS110, were selected for this study. These cultivars were widely cultivated in southern China. They were cultivated in the same location with standard cultivation practice. After the rice grain was harvested, the brown rice was prepared by removing the husk using a laboratory dehusker (JLGJ4.5, Zhejiang, Taizhou Cereal and Oil Instrument Co. Ltd., China). Brown rice with intact endosperm and embryo were selected for this experiment. For each treatment, brown rice (10 g) was sterilized with 1% (v/v) sodium hypochlorite (NaOCl) for 30 min. Thereafter, it was rinsed three times with deionized water, and then it was put into a 50 mL beaker and soaked in 50 mL portions of different concentrations of $ZnSO_4$ solution (0, 25 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, 250 mg/L, respectively). The control was incubated only with deionized water under the same conditions. Subsequently, the treatments were incubated in the dark at 30 °C for 10 h. After 10 h incubation, the brown rice was removed from the beaker, washed three times with deionized water, and spread in a 15 cm Petri dish with two layers of blotting paper. During 24 h germination, 3 mL of water was added and the water changed every 4 h. At the end of germination period, the water was discarded, the seeds were rinsed twice with deionized water, and the grains were dried at 45 ± 5 °C. The rice samples were powdered to make flour by using a ball mill (Retsch, MM-301, Germany) and then put in a plastic bag and kept at −20 °C until analysis. Part of the rice was cooked for 15 min with 1:2 rice/deionized water (w/v). The cooked rice samples were then homogenized in a Polytron homogenizer, and then the homogenates were frozen and lyophilized before testing via the in vitro digestion/Caco-2 cell model.

Determination of Germination Percentages. Germination percentage is an estimation of the viability of the seeds. A brown rice was considered as germinated when the radical had projected from the embryo. The germination percentage (GP) of brown rice was calculated from the following equation with four replicates: GP = germinated brown rice/total brown rice × 100.

Determination of GABA Content. GABA content was measured by using an automatic amino acid analyzer (L-8900; Hitachi, Tokyo, Japan) attached with a Hitachi ion-exchanging resin (4.6 × 60 mm) column. Sample preparation was according to the manufacturer's protocols, with slight modification. In brief, 1 g of rice sample was deproteinized by thoroughly mixing with 10 mL of 6% sulfosalicylic acid (v/v) for 10 min and centrifuged at 10000*g* for 90 min, the supernatant was poured off and adjusted to pH 2.2, and then the supernatant was filtered through a 0.45 μm nylon disposable syringe filter. Filtrates samples (20 μL) were injected into a Hitachi L-8900 amino acid analyzer. The analytical method was according to the manufacturer's standard protocols.

Zn Determination. The rice flour samples (0.3 g) of each treatment were placed into a digestion tube and digested with nitric acid (4 mL) and hydrogen peroxide (1 mL). After cooling, the digestion solution was transferred to a 25 mL volumetric flask, and the volume was made up with deionized water. The concentrations of Zn in the sample were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500a, Agilent Technologies, California) following our previously described method.[23]

Phytic Acid Determination. Phytic acid from the rice samples was extracted following the procedure described by Dai et al.,[24] with slight modification. Briefly, 0.5 g of rice flour was extracted with 10 mL of 0.2 M HCl for 2 h by a rotary shaker and then centrifuged at 10000*g* for 10 min. The clear supernatant was collected, and 2 mL of 0.2% $FeCl_3$ was added to 2.5 mL of supernatant. The resulting solution was mixed thoroughly, heated in a boiling water bath for 30 min, cooled in room temperature, and centrifuged at 10000*g* for 15 min. Then supernatant was discarded and the residue washed in the tube three times with 5 mL of deionized water. The tube was then centrifuged again at 10000*g* for 10 min after adding 3 mL of 1.5 M NaOH to it. The supernatant was discarded again, and 3 mL of 0.5 M HCl was added to the tube to dissolve the residue. Finally, deionized water was added to the solution made up to the volume of 10 mL. The iron concentration in the solution was measured by ICP-MS (Agilent 7500a, Agilent Technologies, California). The phytic acid content was calculated by multiplying iron content by the factor 4.2.

Zn Bioavailability Using an in Vitro Digestion/Caco-2 Cell Model. *In Vitro Digestion of Samples.* The in vitro digestion method was according to Cámara et al.,[21] with slight modification. Briefly, 5 g of cooked rice powder sample was added to 15 mL of mixed solution containing 140 mM NaCl

and 5 mM KCl, and pH was adjusted to 2.0 with 6 M HCl. Then, 0.5 mL of pepsin (0.2 g of pepsin in 5 mL of 0.1 M HCl) was added and incubated at 37 °C in a shaking water bath for 2 h. For the intestinal digestion, the pH of the digest was adjusted to 5.0 with 1 M $NaHCO_3$, and 2.5 mL of pancreatin−bile solution (0.45 g of bile salts and 0.075 g of pancreatin in 37.5 mL of 0.1 M $NaHCO_3$) was added, and the samples were then incubated in 37 °C in a shaking water bath for 2 h. To stop the intestinal digestion, the sample was cooled in ice for 10 min. Then the pH was adjusted to 7.2 by addition of 0.5 M NaOH. The intestinal digest was heated for 4 min in a water bath at 100 °C to inhibit the protease, and then the gastrointestinal digest was cooled in ice and centifuged at 3500*g* for 1 h at 4 °C. Supernatants were transferred and pooled. Glucose (5 mM final concentration) and HEPES (50 mM final concentration) were added into the soluble fraction, and deionized water was used to adjust the osmolarity to 310 ± 10 mOsm/kg (freezing point osmometer, Osmomat 030, Berlin, Germany). The supernatants (soluble fraction) were analyzed for Zn content and used in cell uptake assays.

Caco-2 Cell Culture. Caco-2 cells were obtained from the Institute of Biochemistry and Cell Biology (SIBS, CAS, Shanghai, China) and used in assays at passage 30−46. The Caco-2 cells were normally cultured in 75 cm^2 flasks (Corning Inc., NY) and maintained in high glucose (4.5 g/L) DMEM, supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) nonessential amino acids, 4 mM L-glutamine, and 1% (v/v) antibiotic solution (penicillin−streptomycin). The cells were maintained at 37 °C in an incubator (Heraeus, BB15, Germany) with 5% CO_2, and 95% relative humidity. After reaching 80% confluence, cells were digested by using 0.25% trypsin−EDTA. For Zn bioavailability experiments, 50000 cells/cm^2 in 1.5 mL of complete DMEM was seeded in permeable polycarbonate filters (24 mm diameter, 0.4 μm pore size, Costar Corp., NY), and the basal compartment contained 2.5 mL of complete DMEM. The culture medium was changed every 48 h, and cells were differentiated on the filters after 21 days initial seeding. Subsequently, the development of functional tight junctions in the monolayer of Caco-2 cells was monitored by determining transepithelial electrical resistance (TEER). A Millicell-ERS meter (Millipore Corporation, Bedford, MA) connected to a pair of electrodes was used to measure TEER according to a method described by Ferruzza et al.[25] Only those filters that had TEER values >250 Ω cm^2 at the beginning and the end of the experiment were included. The monolayer used in this study exhibited adequate TEER values 400−500 Ω cm^2.

Zn Uptake Experiment in Caco-2 Cell Model. Prior to the experiments, the growth medium was removed from each well, and the cell monolayer was washed three times with Ca^{2+} and Mg^{2+} free HBSS at 37 °C. Hereafter, the bottom chamber was filled with 2.5 mL of the transport solution (130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM HEPES, pH 7.4) and the upper chamber was filled with 1.5 mL of rice soluble fraction. Cell cultures were incubated at 37 °C under 5% CO_2 with 95% relative humidity for 2 h. The basolateral compartments were collected for the determination of Zn transport across the monolayer. The cell monolayer was washed twice with ice cold HBSS to remove nonspecifically bound mineral and residual medium. The cells on filters were lysed by the addition of 1 mL of deionized water in the well, and then harvested. Cell viability after 2 h of exposure to the uptake solution was assessed by trypan blue exclusion and typically 80−95%.

The concentration of Zn in the gastrointestinal digestion solution (Zn solution fraction) from rice sample, cell retention (mineral fraction in the cell monolayer), and the solution of transport (minerals collected from basolateral compartment) were assessed by ICP-MS (Agilent 7500a, Agilent Technologies, California). Solubility percentages were calculated by using following equation: solubility % = soluble fraction (μg of Zn/g of sample) 100%/*C*, where *C* = total Zn content of sample. The following equation was used for Zn retention percentages: Zn retention % = Zn retention (μg/well) × 100%/*C*, where *C* = mineral soluble added (μg). The following equation was used for Zn transport percentages: Zn transport % = Zn transport (μg/well) × 100%/*C*, where *C* = mineral soluble added (μg). The following equation was used for Zn uptake percentages: Zn uptake % = (retention + transport μg/well) × 100%/*C*, where *C* = mineral soluble added (μg). Due to the differences among samples in terms of solubility of Zn after in vitro digestion, Zn uptake availability was expressed as Zn uptake efficiency, Zn uptake efficiency % = (% solubility × % uptake)/100. Bioavailable Zn (μg/g rice grain) = Zn concentration (mg/kg) × Zn uptake efficiency %.

Quality Control of Zinc and Iron Analysis. Standard reference material rice flour (SRM 1568a) from National Institute of Standards and Technology (Gaithersburg, MD) was used to check the accuracy of Zn and Fe analysis. The measured value was 19.7 ± 0.2 mg/kg for Zn and 6.9 ± 0.3 mg/kg for Fe, which values were in accordance with the certified range of 19.4 ± 0.5 mg/kg for Zn and 7.4 ± 0.9 mg/kg for Fe.

Statistical Analysis. Data were analyzed using the one-way ANOVA model. The least significant difference (LSD) was tested to evaluate significant difference among means at $p < 0.05$ using SPSS 12.0 (SPSS, Inc., Chicago).

RESULTS

Effect of Zn on Germination Percentage. Brown rice was germinated in a wide range, viz., 0, 25 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, 250 mg/L of $ZnSO_4$ solutions, to find a suitable fortification level without any toxic effect on sprout growth (Table 1). Zinc sulfate had no significant influence ($p > 0.05$) on the germination percentage when the concentration was lower than 150 mg/L. However, the germination percentage was significantly decreased ($p < 0.05$) when the concentration of $ZnSO_4$ was more than 150 mg/L. This indicated that, when producing Zn fortified germinated brown rice, the concentration of $ZnSO_4$ should be lower than 150 mg/L.

Table 1. Effect of $ZnSO_4$ Supply Level on the Germination Rates (%) and GABA Content (mg/100 g) of Brown Rice[a]

$ZnSO_4$ treatment (mg/L)	germination (%)	GABA content (mg/100 g)
BR-CK		8.7 ± 1.2 d
0	87.6 ± 1.0 a	31.1 ± 4.4 a
25	86.5 ± 1.2 a	30.8 ± 4.7 a
50	87.5 ± 1.9 a	30.1 ± 6.3 a
100	86.0 ± 0.8 a	29.7 ± 5.6 a
150	85.4 ± 2.4 a	29.4 ± 6.9 a
200	77.6 ± 1.5 b	17.7 ± 3.2 b
250	74.2 ± 1.1 c	15.9 ± 2.7 c

[a]The results are expressed as mean ± SD with four replications. Values within a column followed by a different letter are significantly different ($p < 0.05$).

Effect of Zn Fortification on GABA Content. GABA is a major substance for germinated brown rice consumers, therefore, we also analyzed how GABA was changed after a different range (0−250 mg/L) of $ZnSO_4$ fortification process (Table 1). The results showed a similar trend with germination percentage. Zinc sulfate had no significant influence ($p > 0.05$) on GABA content when the concentration was lower than 150 mg/L. However, the GABA was significantly decreased ($p < 0.05$) when the concentration of $ZnSO_4$ was more than 150 mg/L. Compared to the nongerminated brown rice, the GABA content in germinated brown rice with or without Zn fortification was increased by 82.8 to 257%.

Effect of Zn Concentration on Zn Fortification. Increasing the concentration of $ZnSO_4$ fortification rates (0−250 mg/L) during the germination process significantly

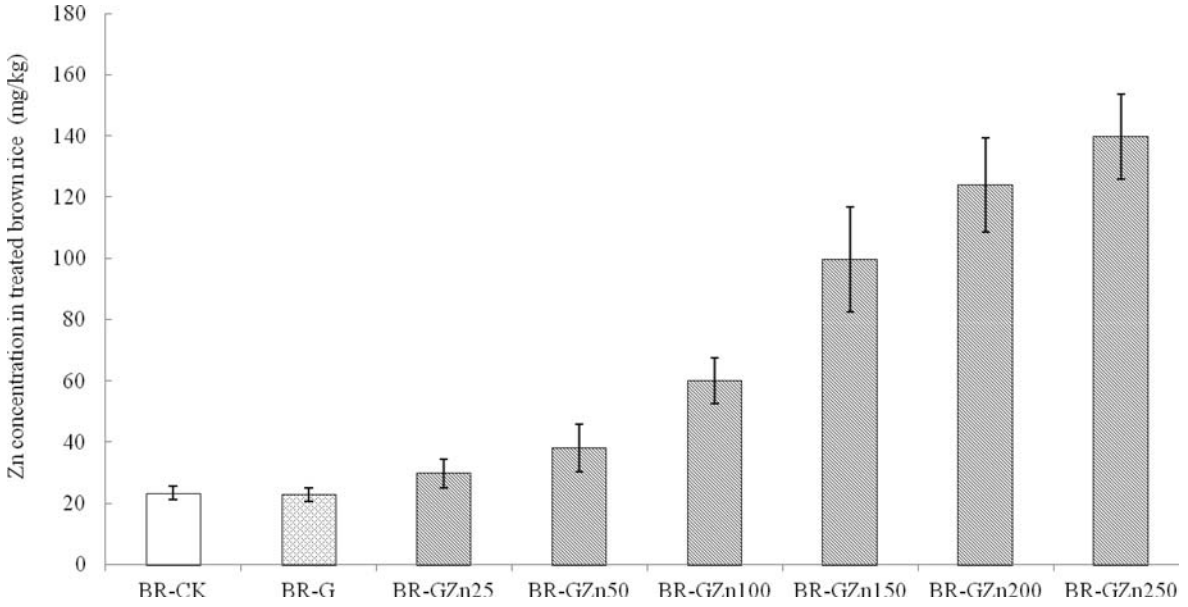

Figure 1. Zinc concentration in the brown rice treated with different rates of $ZnSO_4$: BR-CK refers to nongerminated brown rice; BR-G refers to germinated brown rice at 30 °C, soaking for 10 h, and germinating for 24 h; BR-GZn refers to germinated brown rice with different rates of $ZnSO_4$ at 30 °C, soaking in $ZnSO_4$ for 10 h, and germinating in water for 24 h. Error bars are standard error of means, $n = 4$.

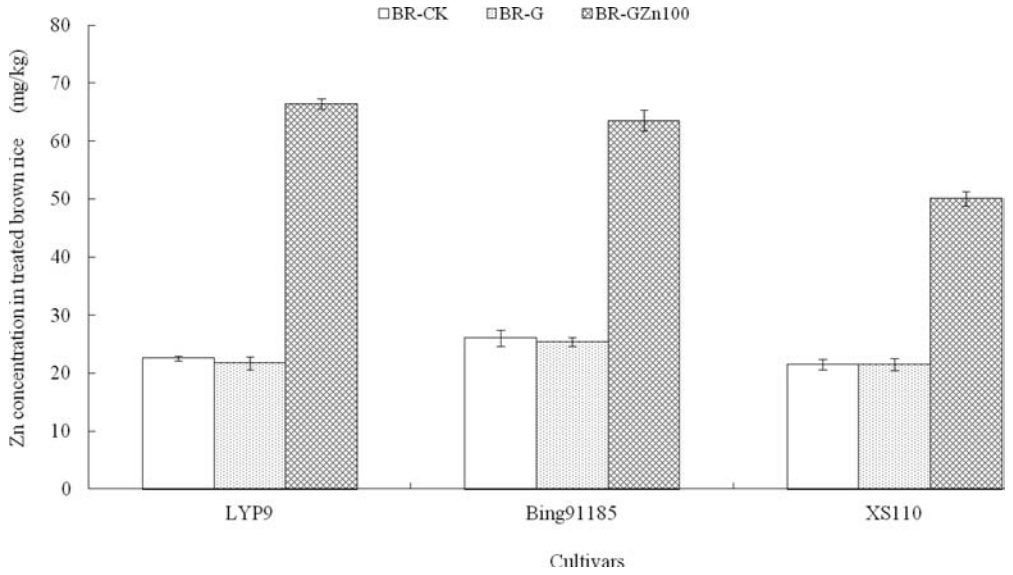

Figure 2. Zinc concentration in different Zn treated brown rice among three cultivars: BR-CK refers to nongerminated brown rice; BR-G refers to germinated brown rice at 30 °C, soaking for 10 h, and germinating for 24 h; BR-GZn100 refers to germinated brown rice with 100 mg/L $ZnSO_4$ at 30 °C, soaking in $ZnSO_4$ for 10 h, and germinating in water for 24 h. Error bars are standard error of means, $n = 4$.

increased ($p < 0.05$) the Zn concentration in the rice grain, compared with germinated or nongerminated brown rice, regardless of the three cultivars used in this study (Figure 1). No significant difference in Zn concentration was observed between nongerminated and normal germinated brown rice. The results showed that Zn concentration in germinated brown rice fortified with $ZnSO_4$ ranged from 29.7 to 139.8 mg/kg, with decreasing order as follows: Zn250 > Zn200 > Zn150 > Zn100 > Zn50 > Zn25. Recommended dietary allowance (RDA) of Zn in the human diet has been estimated to range from 10 to 15 mg/day.[26] In several Asian countries, rice provides 50 to 80% of the energy, thus, they would intake ~225 g of rice/day. Germinated brown rice fortified with 25, 50, 100, 150, 200, and 250 mg/L of $ZnSO_4$ could provide 6.7, 8.6, 13.5, 22.5, 27.9, and 31.5 mg of Zn/day, respectively. As the RDA of Zn among the world population ranges from 10 to 15 mg/day, therefore, we selected Zn fortification level at 100 mg/L, which could provide 13.5 mg of Zn/day, as estimated RDA of Zn in the human diet.

Effect of Cultivars on Zn Fortification. Cultivars had a significant impact on Zn accumulation (Figure 2) when brown rice was germinated with 100 mg/L $ZnSO_4$ solution. In this experiment, we used 100 mg/L $ZnSO_4$ as the suitable concentration for Zn fortification in three brown rice cultivars. Statistical analysis showed that there were significant differences ($p < 0.05$) in Zn levels among three cultivars under nongermination, germination, and fortified germination conditions. Zn concentrations in nongerminated brown rice were

21.5, 22.5, and 26.1 mg/kg in cultivars XS110, LYP9, and Bing91185, respectively. The concentration of Zn in normal germinated brown rice was similar to that of unfortified raw, namely, 21.5, 21.8, and 25.4 mg/kg in cultivars XS110, LYP 9, and Bing91185, respectively. After fortification with 100 mg/L $ZnSO_4$ through the germination process, the Zn concentration in germinated brown rice was 50 mg/kg in XS110, 63.5 mg/kg in Bing91185, and 66.4 mg/kg in LYP9, representing increases of 132%, 154%, and 195%, respectively, compared to unfortified control. However, with respect to Zn content, no significant correlation was found between nongerminated brown rice and fortified germinated brown rice ($p > 0.05$), and no significant correlation was found between normal germinated brown rice and fortified germinated brown rice ($p > 0.05$); on the other hand, significant correlation was found between nongerminated brown rice and normal germinated brown rice ($r = 0.729$, $p < 0.01$).

Effect of Germination and Zn Fortification on Phytic Acid Content. Germination with or without $ZnSO_4$ significantly ($p < 0.05$) reduced the phytic acid content in brown rice (Table 2). Regardless of cultivar, phytic acid content among brown rice ranged from 7.4 to 11.1 mg/g, that after germination ranged from 5.3 to 7.8 mg/g, and that for fortified germinated brown rice ranged from 4.9 to 8.2 mg/g. Generally, the germination process decreases the phytic acid level in brown rice by 31.2%. No significant difference was observed in phytic acid level between the fortified germinated brown rice and normal germinated brown rice. Cultivar XS110 had the lowest and cultivar LYP9 had the highest phytic acid content in the all treatments.

Effect of Germination and Zn Fortification on Molar Ratio of Phytic Acid to Zn. Molar ratio of phytic acid to Zn is also a critical factor in determining the Zn bioavailability. Both germination and the Zn fortification process significantly reduced ($p < 0.05$) the molar ratio of phytic acid to Zn (Table 2). The molar ratios of phytic acid to Zn in the brown rice of three cultivars ranged from 33.8 to 48.6, the molar ratios of phytic acid to Zn in the normal germinated brown rice ranged from 24.5 to 35.5, and the molar ratios of phytic acid to Zn in the fortified germinated brown rice ranged from 9.7 to 12.3. Generally, the molar ratio of phytic acid to Zn was lowest in the fortified germinated brown rice, intermediate in germinated brown rice, and the highest in nongerminated brown rice.

Effect of Germination and Zn Fortification on Zn Solubility. The amount of Zn solubilized after in vitro digestion is an indicator for bioavailability. Zn fortification had a significant ($p < 0.05$) effect on Zn solubility (Table 3). Zn solubility percentages ranged from 22.9 to 26.7% in nongerminated brown rice, from 25 to 28.8% in normal germinated brown rice, and from 27.3 to 31.5% in Zn fortified germinated brown rice. Generally, no significant difference ($p > 0.05$) was found between nongerminated brown rice and normal germinated brown rice in Zn solubility level; however, they were lower than Zn fortified germinated brown rice by 15.4%. After germination, the cultivar Bing91185 with or without Zn fortification had higher Zn solubility than other cultivars.

Effect of Germination and Zn Fortification on Zn Bioavailability. The soluble fraction obtained from in vitro digestion was used to carry out the uptake, retention, and transport experiments with Caco-2 (Table 3). The percentages of Zn retention, transport, and uptake efficiency in Zn fortified germinated brown rice of three cultivars were significantly ($p < 0.05$) higher than that of normal germinated brown rice. No significant difference ($p > 0.05$) was observed between

Table 2. Phytic Acid (PA) in Different Zn Treated Brown Rice (BR) and Molar Ratios of [PA]/[Zn] among the Three Cultivars[a]

cultivars	treatments	PA (mg/g)	[PA]/[Zn]
LYP9	BR-CK	11.1 ± 0.5 a	48.6 ± 2.4 a
	BR-G	7.8 ± 0.4 b	35.5 ± 2.9 b
	BR-GZn100	8.2 ± 0.4 b	12.3 ± 0.7 c
Bing91185	BR-CK	9.5 ± 0.2 a	37.1 ± 2.0 a
	BR-G	7.2 ± 0.5 b	28.1 ± 2.1 b
	BR-GZn100	6.3 ± 0.4 c	9.8 ± 0.7 c
XS110	BR-CK	7.4 ± 0.6 a	33.8 ± 1.8 a
	BR-G	5.3 ± 0.5 b	24.5 ± 2.9 b
	BR-GZn100	4.9 ± 0.3 b	9.7 ± 0.6 c

[a]BR-CK refers to nongerminated brown rice. BR-G refers to germinated brown rice at 30 °C, soaking in water for 10 h, and germinating for 24 h. BR-GZn100 refers to germinated brown rice with Zn ($ZnSO_4$ rate = 100 mg/L) at 30 °C, soaking in $ZnSO_4$ for 10 h, and germinating in water for 24 h. The results are expressed as mean ± SD with four replications. Values for each cultivar within a column followed by a different letter are significantly different ($p < 0.05$).

Table 3. Zinc Bioavailability in Brown Rice with Different Zn Pretreatments among the Three Cultivars[a]

			efficiency (%)		
cultivars	treatments	Zn solubility (%)	retention	transport	Zn uptake
LYP9	BR-CK	25.1 ± 2.1 b	3.9 ± 1.2 b	9.0 ± 1.0 a	3.2 ± 0.5 b
	BR-G	25.3 ± 2.6 b	5.5 ± 1.3 ab	8.9 ± 3.3 a	3.6 ± 0.9 b
	BR-GZn100	29.3 ± 1.1 a	6.5 ± 0.9 a	10.6 ± 0.9 a	5.0 ± 0.4 a
Bing91185	BR-CK	26.7 ± 0.5 b	7.6 ± 1.1 b	12.5 ± 1.9 b	5.2 ± 0.2 b
	BR-G	29.0 ± 0.9 b	7.6 ± 0.6 b	12.5 ± 3.7 b	5.8 ± 1.1 b
	BR-GZn100	31.5 ± 0.8 a	9.2 ± 1.0 a	17.7 ± 0.6 a	8.5 ± 0.3 a
XS110	BR-CK	22.9 ± 2.5 b	6.6 ± 1.2 b	11.4 ± 2.1 b	4.1 ± 0.6 b
	BR-G	23.7 ± 2.2 b	8.1 ± 0.5 b	11.7 ± 2.4 b	4.6 ± 0.7 b
	BR-GZn100	27.3 ± 0.8 a	10.2 ± 1.2 a	15.9 ± 1.1 a	7.1 ± 0.4 a

[a]BR-CK refers to nongerminated brown rice. BR-G refers to germinated brown rice at 30 °C, soaking for 10 h, and germinating for 24 h. BR-GZn100 refers to germinated brown rice with Zn ($ZnSO_4$ rate = 100 mg/L) at 30 °C, soaking in $ZnSO_4$ for 10 h, and germinating in water for 24 h. The results are expressed as mean ± SD with four replications. Values for each cultivar within a column followed by a different letter are significantly different ($p < 0.05$).

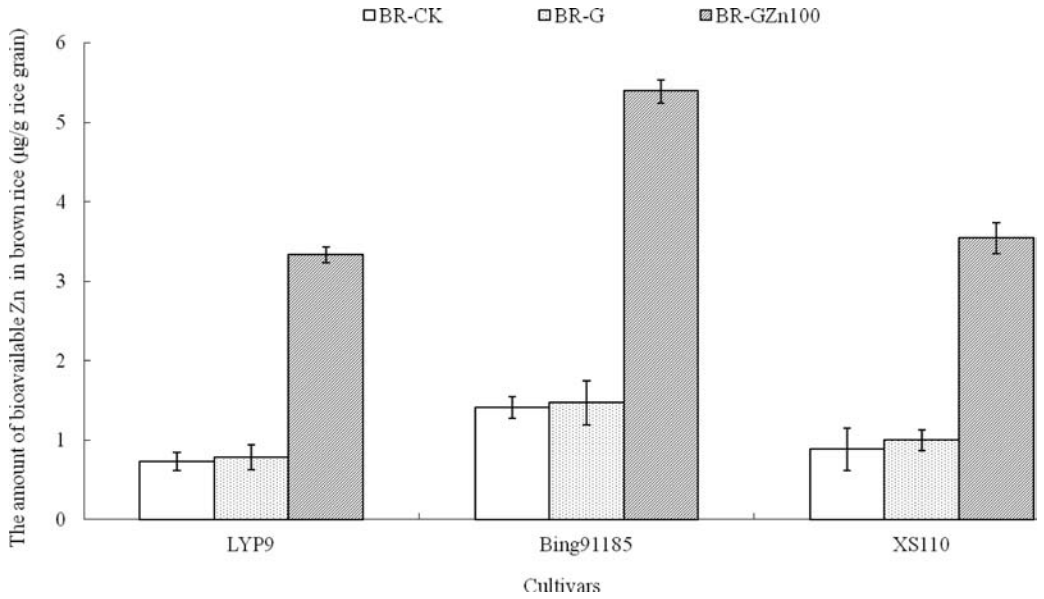

Figure 3. The amount of bioavailable zinc in different zinc treated brown rice among the three cultivars: BR-CK refers to nongerminated brown rice; BR-G refers to germinated brown rice at 30 °C, soaking for 10 h, and germinating for 24 h; BR-GZn100 refers to germinated brown rice with 100 mg/L $ZnSO_4$ at 30 °C, soaking in $ZnSO_4$ for 10 h, and germinating in water for 24 h. Error bars are standard error of means, $n = 4$.

nongerminated brown rice and the normal germinated brown rice. Comparing nongerminated brown rice with Zn fortified germinated brown rice, the percentages of Zn retention, transport, and uptake efficiency significantly increased by 43.3%, 33.9%, and 60.8%, respectively. The amount of bioavailable Zn from rice grain has the same trend as Zn uptake efficiency, comparing normal germinated brown rice with fortified germinated brown rice, the amount of bioavailable Zn in the Zn fortified brown rice was significantly increased 3-fold (Figure 3). Genotypic variation on percentage of Zn solubility, retention, transport, uptake, and amount of bioavailable Zn is illustrated in Table 3 and Figure 3. Bing91185 contains the highest percentages of Zn retention, transport, and uptake efficiency as well as bioavailable Zn in all treatments.

DISCUSSION

In most resource poor developing countries, it has become clear that Zn deficiency is attributed only to insufficient amounts bioavailable Zn in the rice based diets. Zn fortified germinated brown rice might be a quick and cost-effective solution to achieving adequate bioavailable Zn intake in resource poor Zn deficient developing countries. We reported here the optimum concentration for producing Zn fortified germinated brown rice in relation to its bioavailability. The results are presented in Table 1, showing that the germination percentage of rice was affected by different levels of $ZnSO_4$. No visible detrimental impacts on germination of sprouts were observed when concentration of $ZnSO_4$ was lower than 150 mg/L. In contrast, toxic effects reduced the germination percentage when $ZnSO_4$ concentration was more than 150 mg/L. This could be explained by the fact that Zn functions as a cofactor of many enzymes. The low concentration of $ZnSO_4$ increased respiratory intensity of cereal seeds and the activity of α-amylase, protease, peroxidase, and polyphenoloxidase of cereal seeds. Consequently, the low concentration of $ZnSO_4$ enhanced germination of cereal seeds.[27] Similar results were also reported when barley and buckwheat were fortified with $ZnSO_4$.[27,28] Therefore, results from our study suggested that $ZnSO_4$ concentration should be lower than 150 mg/L during the fortified germinated brown rice production. The germination process increased GABA content; however, the Zn fortification process reduced GABA content to a certain level, which shows a similar trend with germination percentages (Table 1). Compared to the normal germination process, no significant differences were found in GABA content when the Zn fortification level was less than 150 mg/L. This can be explain by the fact that the germination process increased GABA content due to activation of glutamate decarboxylase that calalyzes the decarboxylation of L-glutamic acid to carbon dioxide and GABA.[13] The reduction of GABA content during Zn fortification occurred because GABA is also related to seed growth.[29]

Zn fortification in the germination progress could dramatically increase Zn concentration in the brown rice. Moreover, the Zn concentration in the brown rice was greatly correlated with the concentration of $ZnSO_4$ (Figure 1). The Zn accumulation during the germination process might be due to penetration of Zn solution across the aleurone layers of rice endosperm, possibly via the dorsal vascular bundle present in the endosperm.[30] Regardless of the cultivar, the Zn concentration of Zn fortified germinated brown rice ranged from 29.7 to 139.8 mg/kg among the different $ZnSO_4$ fortification levels (25−250 mg/L). As the RDA of Zn among the world population ranged from 10 to 15 mg/day, therefore, we selected the Zn fortification level of 100 mg/L, which could provide an estimated RDA of 13.5 mg of Zn/day in the human diet. Fortification rates of more than 100 mg/L $ZnSO_4$ exceed the RDA and also increase the fortification cost. Furthermore, germination percentages and GABA content were higher in the case of fortification rate of 100 mg/L compared to 150 mg/L $ZnSO_4$. Thus, results from the current study recommended that brown rice should be fortified with 100 mg/L $ZnSO_4$ to provide the RDA of Zn in the human diet. The Zn concentration of germinated brown rice fortified with 100 mg/L $ZnSO_4$ was 1.6 times higher than that of brown rice with

germination or without germination (Figure 2). The results indicated that fortification of Zn in brown rice through the germination process would be a promising approach for boosting Zn content in the rice products. Moreover, Zn accumulation varied significantly between cultivars. As the cultivars were grown and harvested from the same field, the variation might be come from the genetic control, agreeing with a previous report.[31] After fortification with $ZnSO_4$ 100 mg/L, Zn concentration in germinated brown rice ranged from 50 (in XS110) to 66.4 mg/kg (in LYP9). Clearly, the cultivars LYP9 and Bing91185 were more effective than XS110 in accumulation of Zn in rice grain through the germination process. Similar results were also found in a previous report where it was reported that the amount of Zn absorbed depended on the cultivars.[23] Thus, in future studies, more cultivars should be screened to select a suitable cultivar that can accumulate more Zn in the germination process.

Although the amount of Zn is important for Zn bioavailability, information about changes of antinutrient during the germination and fortification process is crucial because this determines how much Zn is absorbed in the human gut. Within cereal foods, Zn bioavailability is considerably reduced by phytic acid. In the current study, we documented that phytic acid could be decreased by germination but not by the Zn fortification process. With or without Zn fortification, germinated brown rice contains lower phytic acid and molar ratio of phytic acid to Zn than nongerminated brown rice (Table 2). Furthermore, differences were observed between cultivars with phytic acid content (Table 2). The possible explanation was that, during germination, endogenous phytase activity in cereal grain increases as a result of de novo synthesis and/or activation, resulting in reductions in phytic acid content which varied among the species and variety.[10]

In the in vivo situation, Zn needs to be soluble before it can be taken up by the enterocytes. In the current study, we also determined the soluble Zn in the nongerminated brown rice, normal germinated brown rice, and Zn fortified germinated brown rice by in vitro digestion. The results showed that, regardless of cultivars, in the digestion process the solubility of Zn in Zn fortified brown rice was higher than that of unfortified brown rice with germination or without germination. No significant difference was observed between the results for normal germinated brown rice and nongerminated brown rice (Table 3). These results agreed with previous studies, observing that germination in brown rice might not be increasing the level of soluble Zn after in vitro digestion,[32,33] but after Zn fortification, the amount of soluble Zn in rice grain was increased.[30] The solubility method involves a simulation of the gastrointestinal digestion followed by a measurement of soluble Zn in the digest and thus covers only the first phase of the overall Zn absorption process. The soluble fraction obtained from the gastrointestinal digestion was used to carry out uptake, retention, and transport experiment in Caco-2 cell models which offer a more physiological tool for screening Zn bioavailability in food matrices, particularly when combined with a simulated digestion step. The Caco-2 model has been used to evaluate bioavailability of Zn in the school meal[21] and different bean cultivars[34] and infant foods,[22,35] concluding that assays in Caco-2 cells offer a better indicator of bioavailability than solubility. In this current study, regardless of cultivar and treatment, the mean value of Zn bioavailability (uptake efficiency) from brown rice was 5.2%, falling within the recently reported Zn bioavailability of cereal foods (ranging from 4.1% to 48.1%).[22] Regardless of cultivar, Zn fortified germinated brown rice showed a higher percentage of Zn retention, transport, and uptake efficiency by Caco-2 cell than those of brown rice with germination or without germination. No significant difference was observed between those of germinated brown rice and nongerminated brown rice. The amount of bioavailable Zn has the same trend with Zn uptake efficiency (Figure 3). The results indicated that the germination process has little effect on Zn bioavailability, but after germination with $ZnSO_4$ solution, an inhered increase of Zn bioavailability from rice grain was observed. The results were well in line with the previous study.[36] The possible explanation was that, despite the expected reductions in the phytate and the gain in the molar ratio of phytic acid to Zn in rice by germination processing, the molar ratio of phytic acid to Zn of normal germinated brown rice was still above 15, which was the critical value of Zn bioavailability,[37] and the residual phytate might still interfere with Zn bioavailability in rice grain. Normal germinated brown rice was unable to provide enough Zn, so the bioavailability of Zn in brown rice is unlikely to be increased by germination alone. However, brown rice fortified with Zn in the germination process could decreased the value of the molar ratio of phytic acid to Zn to lower than 15 and increase the total amount of Zn and, as a result, increase the Zn bioavailability. Furthermore, cultivar difference on Zn uptake efficiency and the amount of bioavailable Zn in Zn-fortified germinated brown rice was found (Table 3 and Figure 3). The differences in Zn bioavailability observed among the cultivars in this study might be attributed to the differences in level of phytic acid, the molar ratio of phytic acid to Zn, or Zn accumulated in the rice grain.[38] The cultivar Bing91185 contains higher Zn bioavailability than the other cultivars (Table 3 and Figure 3). Thus, in the current study, the cultivar Bing91185 was identified as the most promising cultivar for Zn fortification in the germination process.

In conclusion, the level of Zn fortified in germinated brown rice is linearly correlated with concentration of $ZnSO_4$, regardless of cultivar. From the results of this current study, we recommend that germinated brown rice be fortified with 100 mg/L $ZnSO_4$, which leads to a higher amount of total Zn as well as bioavailable Zn than brown rice with or without germination. Germination alone could not increase Zn content as well as Zn bioavailability from rice grain. These could result from the higher level of molar ratio of phytic acid to Zn and lower amount of Zn in germinated or nongerminated brown rice than Zn fortified germinated brown rice, leading to little Zn bioavailability. The cultivar Bing91185 was identified as the most promising cultivar for germinated brown rice and Zn fortification program. The effectiveness of Zn fortification in the germination process suggests that consuming Zn fortified germinated brown rice might be a rapid method to improve the amount of Zn intake and Zn bioavailability.

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Notes

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