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Nielsen, Anne V. F.; Meyer, Anne S.

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Phytase-mediated mineral solubilization from cereals under *in vitro* gastric conditions

Anne V. F. Nielsen and Anne S. Meyer*

Center for BioProcess Engineering, Department of Chemical and Biochemical Engineering,
Technical University of Denmark, Soeltofts Plads 229, DK-2800 Kgs. Lyngby, Denmark

*Correspondence to: am@kt.dtu.dk

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ABSTRACT

BACKGROUND: Enzymatic dephosphorylation of phytic acid (inositol hexakisphosphate) in cereals may improve mineral bioavailability in humans. This study quantified enzymatic dephosphorylation of phytic acid by measuring inositol tri- to hexakisphosphate (InsP3-6) degradation and iron and zinc release during microbial phytase action on wheat bran, rice bran and sorghum under simulated gastric conditions.

RESULTS: InsP3-6 was depleted within 15-30 min of incubation using an *Aspergillus niger* phytase or *Escherichia coli* phytase under simulated gastric conditions with the two enzymes dephosphorylating cereal phytic acid at similar rates and to similar extents. Microbial phytase-catalysed phytate dephosphorylation was accompanied by increased iron and zinc release from the cereal substrates. For wheat bran at pH 5, the endogenous wheat phytase activity produced mineral release equal to or better than that of the microbial phytases. No increases in soluble cadmium, lead or arsenic were observed with microbial phytase-catalyzed phytate dephosphorylation.

CONCLUSION: Microbial phytase treatment abated phytate chelation hence enhanced the release of iron and zinc from the phytate-rich cereals at the simulated gastric conditions. The data infer that acid stable microbial phytases can help improve iron bioavailability from phytate-rich cereal substrates via post-ingestion activity.

KEYWORDS

Phytate, iron, zinc, wheat, rice, sorghum

INTRODUCTION

Globally, iron deficiency is the most common nutritional deficiency, and it is estimated that around 2 billion people are affected, particularly women of the childbearing age and children.¹ At least half of the cases of anemia may be attributable to iron deficiency.¹ Cereals are a major world staple food known to contain significant amounts of iron but this iron has low bioavailability (2-3 %²) compared with 15-35 % reported for meat.³ Zinc deficiency is prevalent in particularly African and Asian countries, which is also attributed partly to low bioavailability of zinc from cereals.⁴ One of the reasons for this low bioavailability of iron and zinc from cereals is presumed to be chelation of these mineral cations by phytic acid (myo-inositol (1,2,3,4,5,6)-hexakisphosphoric acid). Phytic acid, the primary phosphorous storage compound in seeds,⁵ is negatively charged at physiological pH values, thus capable of acting as a ligand for the chelation of positively charged compounds,⁶ notably mineral cations such as Mg^{2+} , Cu^{2+} , Zn^{2+} , and Fe^{3+} . It is known that the addition of an exogenous microbial phytase (myo-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolase; e.g. EC 3.1.3.8) to a phytate-rich meal can increase iron and zinc absorption from the meal.⁷⁻⁹ The mechanism underlying this effect is the phytase-catalyzed sequential dephosphorylation of phytate resulting in the release of the chelated ions, notably iron and zinc, making them available for absorption.¹⁰ Preferably, this phytase-catalyzed dephosphorylation should take place prior to food ingestion or during passage through the stomach. This is due to the low pH in the stomach that favors the solubilization of the phytate substrate¹¹ and the absorption of iron and zinc taking place in the upper part of the small intestine.^{12,13} It has been reported that at least four phosphates should be cleaved off from the phytic acid in order to significantly improve iron and zinc absorption.^{14,15}

Recently, the World Health Organization (WHO) evaluated the phytase from the mold *Aspergillus niger* as safe for food use.¹⁶ For phytase-catalyzed dephosphorylation to take place

in the human stomach, the phytase to be used must be stable towards low pH and pepsin during the retention period in the gastric ventricle. Selected thermostable microbial phytases have already been used in feed for monogastric animals to increase bioavailability of phosphorous and other minerals.^{5-6,10} Other microbial phytases than the *A. niger*-derived phytase have shown good performance with regard to low pH and presence of proteases.¹⁷⁻²¹ In our previous work, the phytases from *Escherichia coli* and *A. niger* were found to be better with respect to gastric stability at low pH than the thermostable phytases from *Peniophora lycii* and *Citrobacter braakii* developed for animal feed use.¹⁰ The aim of the present study was to assess phytate degradation and iron and zinc release potential of the *E. coli* and *A. niger* derived phytases on natural cereal substrates (rice, wheat and sorghum), in gastric conditions simulating the human stomach. Rice, wheat and sorghum were chosen due to their importance in nutrition worldwide with annual global productions of 825, 650 and 60 million tons, respectively.²² Globally, rice and wheat rank first and second, respectively, as the most important calorie sources for humans.²² Sorghum, in spite of the smaller production volume, is one of the most important crops in Africa and India due to its great resistance to drought.²² All three cereals contain significant amounts of phytate, iron, and zinc.^{23,24}

Surprisingly few studies have been conducted simulating the action of exogenously added phytase in the gastric environment with regard to mineral release from cereals and none of the existing studies used rice bran or sorghum as the cereal substrate.^{25,26} This type of experiment is relevant for ingestion of phytases along with the phytate-rich meal itself, as opposed to food processing or pretreatment prior to ingestion.²⁷⁻³³ In comparison with *in vivo* studies, where mineral absorption is measured from blood samples, the approach in the present study allows for simultaneous evaluation of InsP3-6 degradation and mineral solubilization. Mineral cation solubility is a suitable parameter for understanding factors affecting mineral absorption and has been shown to correlate well with absorption in the human gastrointestinal tract.

EXPERIMENTAL

Chemicals and Enzymes

Phytic acid sodium salt hydrate from rice, $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, 70% HClO_4 , hydrochloric acid, acetic acid, sodium acetate, pepsin (EC 3.4.23.1) from porcine gastric mucosa (P7000, 561 U/mg solid), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, vanadate-molybdate reagent and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). *E. coli* phytase (EC 3.1.3.8) expressed in *Pichia pastoris* (trade name Granular phytase) was kindly provided by Challenge Group (Beijing, China). *A. niger* phytase (EC 3.1.3.8) expressed in *Pichia pastoris* was produced in house as described previously.¹⁰

Cereal fractions

Wheat bran (Finax, Valsemoellen, Koege, Denmark) and rice bran (Govinda Natur GmbH, Neuhausen, Germany) were purchased from local suppliers. Whole grain sweet white sorghum was purchased from Bob's Red Mill (Milwaukie, OR, USA) and ground using a coffee mill (Krupps, Ballerup, Denmark). All ground cereals and cereal fractions were sieved dry using a sieving system (Retsch, Haan, Germany). The size fraction containing particle sizes between 355 and 710 μm was used for all experiments. Dry matter was assessed gravimetrically by measuring the weight loss after incubation of cereals or cereal fractions at 105 °C for 48 h.

Total phytic acid content was determined by extraction in triplicate using a method adapted from Carlsson et al.³⁴ In brief, 150 mg cereal substrate was weighed, 1.5 mL of 0.5 M HCl was added, and each sample was incubated at 22 °C with shaking at 1000 rpm for 3 h. Subsequently, samples were centrifuged for 10 min at 16,100 $\times g$, supernatants were diluted with water and analyzed for inositol phosphates using ion exchange chromatography as described previously.^{10,34–36}

Total mineral content was measured for each of the three cereal types using a standard method (Dansk Standard 259).³⁷ Cereal samples were digested by incubation with 7 M HNO₃ for 30 min at 120 °C and 200 kPa pressure in an autoclave.³⁷ All samples were completely solubilized. Digestion solutions were analyzed for iron, zinc, aluminum, arsenic, cadmium and lead using inductively coupled plasma optical emission spectrometry (ICP-OES) (on a Varian 720-ES instrument, Agilent Technologies, Santa Clara, CA, USA) at wavelengths of 238.204, 334.502, 396.152, 228.812, 361.051 and 283.305 nm, respectively.

Phytase activity

Phytase activity was evaluated according to a method described by Bohn et al.³⁵ and García-Mantrana et al.³⁸ In brief, 350 µL of 5 mM phytic acid in 200 mM acetate buffer (pH 5.5) was preheated at 37 °C and mixed with 50 µL phytase solution. Reactions were stopped after 0, 15, 30, 45, 60 and 120 seconds of reaction by addition of 50 µL 3.6 M HCl and incubation at 99 °C for 10 min, thus inactivating the phytases. Released phosphate was quantified by mixing 100 µL appropriately diluted reaction sample with 100 µL vanadate-molybdate solution (1 part vanadate-molybdate reagent and 4 parts 200 mM acetate buffer of pH 5.5) followed by incubation for 10 min at 30 °C and absorption measurement at 400 nm in a microplate reader. A phosphate standard curve was prepared from NaH₂PO₄ and the absorbance response was linear up to 1 mM phosphate. One unit phytase activity (U) corresponded to the amount of enzyme required to release 1 µmol phosphate per minute under the conditions specified above. Activity was evaluated from initial rates of phosphate release within the linear range of the curve.

In vitro gastric incubations

Degradation of inositol hexakis- to triphosphates in the cereal substrates was investigated by HPLC analysis after incubation with microbial phytases at 37 °C and 800 rpm shaking in a

thermomixer. The following factors were varied in a full factorial design in triplicates using the same conditions as described by Nielsen et al.:¹⁰ pH (pH 2: 10 mM HCl or pH 5: 200 mM acetate buffer)¹⁰; pepsin (0 or 5000 pepsin activity units/mL)¹⁰, phytase addition (*E. coli* phytase, *A. niger* phytase or none) and incubation time (0, 15, 30, 60 or 120 min). A cereal substrate concentration of 40 mg dry matter per mL reaction mixture was chosen. This corresponds to the dry matter concentration in a 0.5 L stomach after ingestion of 20 g wheat bran. Phytase dosage was 0.4 U/ μ mol phytic acid. This dosage corresponded to the 0.05 % E/S (enzyme protein/substrate mass) (w/w) *E. coli* phytase that was used in a previous study¹⁰ and 0.6 % E/S (w/w) for the *A. niger* phytase. Reactions were stopped after 0, 15, 30, 60 and 120 min by 10 min incubation at 99 °C. Subsequently, samples were centrifuged for 10 min at 16,100 $\times$ g and supernatants were diluted to keep concentrations of each inositol phosphate below 1 mM. Samples were filtered through 0.22 μ m syringe tip filters before HPLC analysis of InsP3-6 content as described previously.^{10,34-35}

Inositol phosphates remaining in the solid cereal residue after gastric incubations were extracted³⁴ as described above with 0.5 M HCl for 3 h. Subsequently, extracted inositol phosphates were separated from the solid cereal residue by centrifugation and filtration and analyzed for inositol tris-, tetra-, penta-, and hexa-phosphates (InsP3, InsP4, InsP5, InsP6) using HPLC as described above. InsP3, InsP4, and InsP5 were quantified as InsP6 equiv using the InsP6 quantification factor (area under the curve divided by molar concentration) as described previously.¹⁰

Soluble minerals analysis

To assess the amount of minerals released due to phytate dephosphorylation, the *in vitro* gastric incubations with and without addition of microbial phytase described above were repeated at a larger scale to allow enough sample for mineral analysis. Included time points were initial samples (“0 min”) and 120 min. Incubations were carried out with 5000 pepsin

activity units/mL at both pH 2 and pH 5. InsP3-6 was measured in the supernatants using HPLC as described above. Subsequently, pH in the supernatants was adjusted to pH 6.5 using 1 M NaHCO₃ and supernatants were centrifuged at 5,000 × g for 30 min to collect compounds precipitated as a result of the pH increase at the bottom. Supernatants were decanted and diluted 1.4-2.7 times with 0.1 M NaHCO₃ (pH 6.5) for mineral analysis using ICP-OES as described above for the total mineral analysis. Mineral solubility was calculated as the fraction of soluble mineral (measured in the supernatant) of total mineral content for the type of cereal or cereal fraction.

Statistical analyses

For comparison of cereal dry matter, total cereal inositol phosphate contents, total cereal mineral contents as well as cereal InsP3-6 contents over time in the gastric incubations, one-way analyses of variance (ANOVA) with Tukey's test for pairwise comparisons were conducted using Minitab 14 (State College, PA, USA). Significance was concluded at a confidence level of 95 %. For analysis of soluble minerals after gastric incubations, one-way ANOVA with Tukey's test for pairwise comparisons were conducted using Minitab 14. Contents of minerals after 120 min gastric incubation (calculated as mg soluble minerals per 100 g dry cereal or cereal fraction) for each individual cereal type were entered as dependent variables (tested individually with other factors constant) and independent factors were phytase (*A. niger*, *E. coli* or no added phytase) and pH (pH 2 or 5).

RESULTS AND DISCUSSION

Cereal fractions

Phytic acid contents in the cereals or cereal fractions, measured as InsP6, were highest in rice bran at 102 g/kg dry weight, followed by wheat bran at 53 g/kg dry weight and sorghum at 20 g/kg dry weight (Table 1). Wheat and rice bran were higher than milled sorghum in phytic acid.

Phytic acid is located primarily in the bran fraction of wheat and rice grains,^{23,39} and in the germ and pericarp fractions of the sorghum grains.⁴⁰ The germ and pericarp fractions could not be separated from the remaining parts of the sorghum grain by milling, resulting in lower concentration of phytic acid in the available sorghum cereal substrate compared to the concentrated bran fractions obtained from rice and wheat (Table 1). On a whole grain basis, all three cereals contain similar amounts of phytic acid,²³ making them equally relevant as substrates for phytate dephosphorylation. Phytic acid levels measured in the present study were generally slightly higher than values reported in the literature.²³ Ranges for Fe and Zn contents were in accordance with values from the National Nutrient Database (Table 1).²⁴

In vitro gastric incubations

All cereals or cereal fractions were treated with phytase in a simulated gastric environment with/without pepsin at pH 2 or 5 for up to 120 min. The *A. niger* phytase and the *E. coli* phytase were evaluated individually and a control without added phytase was included in the study. InsP3-6 contents in the rice bran samples with added phytases decreased significantly with time and InsP3-6 was completely degraded within the first 30 min at both pH 2 and pH 5 (Figure 1a and 1b), whereas no InsP3-6 degradation was observed in the control samples. At pH 5, InsP3-6 degradation occurred almost instantly with the *A. niger* phytase treatment, and faster than with the *E. coli* phytase (Figure 1b). Data for phytase-treatment of wheat bran and sorghum samples (Figure 1c-f) were similar to those obtained on rice bran. An exception were control wheat bran samples without added phytase at pH 5, where a significant decrease in InsP3-6 content with time (Figure 1d), confirmed the presence of endogenous wheat phytase activity as reported elsewhere.^{41,42} Endogenous phytase activity did not appear to be affected by the presence of pepsin, which is in accordance with previous findings (Figure 1d).⁴¹ On the other hand, rice and sorghum appeared to have no significant intrinsic phytase activity (Figure 1a-b and 1e-f), which

is also in agreement with previously published data showing that endogenous phytase activities for rice and sorghum are only ~4 and ~6 % relative to wheat (100 %).⁴²

The data affirmed that both the *E. coli* and the *A. niger* phytase can catalyze almost complete degradation of InsP3-6 under simulated gastric conditions. For milled sorghum, wheat bran and rice bran, 89-99 %, 91-97 % and 98 % of the total InsP3-6 was degraded with the addition of microbial phytases within the two hours at simulated gastric conditions. This should be compared with virtually no degradation of InsP3-6 without phytase addition except from wheat at pH 5, where 89-94 % of the InsP3-6 was degraded after two hours due to endogenous phytase activity alone (Figure 1). Regarding performance of the two phytases, the *E. coli* phytase had higher relative activity at pH 5 compared with the *A. niger* phytase according to previously published pH profiles for these phytases.¹⁰ Therefore, it was a surprise that when dosed equally based on activity measured at pH 5.5, the *A. niger* phytase depleted the InsP3-6 in especially the rice and wheat bran substrates faster than the *E. coli* phytase at pH 5. As a final note on these data, solubility of InsP3-6 was consistently increased by the presence of pepsin, especially at pH 2, which is also found in other studies (data not shown).^{11,43} In addition, no pepsin-catalyzed phytase inactivation was observed (Figure 1, compare full and dashed lines).

Mineral release

Microbial phytase-catalyzed phytate degradation during gastric incubations significantly increased soluble Fe and Zn at duodenal pH. With microbial phytase catalysis of phytate dephosphorylation, Fe solubility was significantly increased from rice bran at pH 2 and 5, for wheat bran at pH 2 and for sorghum at pH 5 compared with the control samples without added microbial phytase. Zn solubility was significantly increased from rice bran at pH 2 and 5 and from wheat bran and sorghum at pH 2. Mineral solubilities from sorghum were already considerable without microbial phytase-catalysed phytate degradation at both pH values (Figure

2). For wheat bran at pH 5, the Fe and Zn solubilities with microbial phytase-catalysed phytate degradation were 22-41 % and 22-57 %, respectively (highest with the *A. niger* phytase), but even without microbial phytase, phytate degradation catalyzed by the endogenous wheat phytase resulted in 38 % Fe solubility and 36 % Zn solubility (Figure 2, see also Figure 1d). In rice bran, Fe solubilities were significantly ($P < 0.002$) increased with catalysis of phytate degradation by any of the microbial phytases from ~0 % to 8-10 % at pH 2 and to 25 % at pH 5. Similarly, rice bran Zn solubility was significantly ($P < 0.01$) increased from ~0 % to 37% at pH 2 and up to ~40-50 % at pH 5 with microbial phytase catalysis (highest with the *A. niger* enzyme treatment) (Figure 2). This is in correspondence with InsP6/Fe ratios decreasing from ~46 without microbial phytase-catalysed phytate degradation to <0.1 with microbial phytase-catalysed phytate degradation (Table 2). InsP6/Zn ratios were similarly decreased to <0.5 from ~190 (Table 2). Iron and zinc absorption are reported to be inhibited by molar InsP6/mineral ratios of 0.4 and 6, respectively.⁴⁴⁻⁴⁵ The InsP6/mineral ratios reported above are thus in agreement with the observation of increased solubility. For the wheat bran substrate at pH 2, Fe solubility was significantly ($P < 0.001$) increased from ~0 % to 8-12 % with any microbial phytase-catalyzed phytate degradation and Zn solubility from 0 % to 14-39 % ($P < 0.001$). Both mineral solubilities from wheat bran at pH 2 were highest with the *E. coli* enzyme treatment, in agreement with the InsP6/Fe ratios being lower for the *E. coli* phytase-treated samples than with the *A. niger* phytase treated samples (Table 2). For wheat bran at pH 5, phytate to mineral ratios were similar with and without microbial phytase-catalysed phytate degradation, thus explaining why no significant increase of mineral solubility was observed with addition of microbial phytase. For sorghum at pH 2, Fe solubility was ~13 % regardless of microbial phytase-catalyzed phytate degradation, whereas Zn solubility was increased ($P < 0.005$) from 5 % to 8-13 % with the microbial phytase-catalyzed phytate degradation. This agrees with the observation that the InsP6/Fe ratios even with phytase treatment are still higher than 0.4, whereas InsP6/Zn

values have been decreased to less than 2 (Table 2). At pH 5, Fe solubility from sorghum was increased ($P < 0.002$) from 21 % to 31-36 % with microbial phytase-catalyzed phytate degradation, whereas microbial phytase treatment had no significant influence on the Zn solubility with values of 23-29 % regardless of phytase treatment (Figure 2). As InsP_6/Fe was lower than 0.5, the observed increase in Fe solubility is supported by the phytate/mineral ratios, whereas the lack of increase in Zn solubility cannot be explained by phytate/mineral ratios, as these were <1 , well below the ratio reported to inhibit Zn absorption (Table 2). At present, no explanation can thus be found for this phenomenon.

In summary, both of the microbial phytases were able to significantly increase solubility of Zn and Fe in these experiments for almost all combinations of cereal/cereal fraction and pH, except for wheat bran at pH 5, where the endogenous phytase was apparently sufficiently active to reach the same degree of dephosphorylation and almost the same mineral solubility as that achieved with the added *A. niger* phytase. In fact, the mineral solubility obtained after incubation of wheat bran at pH 5 without microbial phytase-catalysed phytate degradation was higher than that achieved with the *E. coli* phytase-catalysed phytate degradation (Figure 2), which could not be explained by $\text{InsP}_6/\text{mineral}$ ratios that were all equally low. At present, no explanation has been found. For all cereals or cereal fractions, Fe and Zn solubilities were higher after gastric incubations at pH 5 than at pH 2 (Figure 2), which was attributed to higher solubility of inositol phosphates at pH 5 compared with pH 2 (data not shown), meaning that the phytate substrate is more accessible to the phytase and chelated minerals thus released to a higher extent. This was supported partially by the $\text{InsP}_6/\text{mineral}$ ratios (Table 2). Choice of pH in a simulated gastric environment is thus very important parameter to be considered in the design of *in vitro* experiments.

Other studies on wheat phytic acid dephosphorylation using exogenous phytases in simulated gastric conditions observed similar iron and zinc solubilities as the present study.^{25,26} Concerning rice bran and sorghum, no other studies investigating the phytase catalysis in the actual simulated gastric conditions were found. However, phytase pretreatment of rice bran increased Fe and Zn solubility in an *in vitro* digestion model⁴⁶ to a magnitude similar to what was found in the present study. For sorghum, activation of endogenous phytases during germination (although not being directly comparable with the simulated digestion of the present study) resulted in 25-35 % phytate removal, in turn increasing Zn dialyzabilities to values similar to the solubilities reported in the present study and Fe dialyzabilities to a lower extent than the solubilities reported in the present study.⁴⁷ This difference is probably because a more extensive phytate degradation is required to release Fe compared to Zn. Another study, although not directly comparable with the simulated digestion in the present study, reported near complete removal of white sorghum phytic acid by lactic fermentation with added phytase.⁴⁸ This phytate dephosphorylation⁴⁸ resulted in much higher Fe solubilities than reported in the present study, which may be attributed to other components in the lactic fermentation, e.g. organic acids, which are also known to influence solubility of Fe. This organic acid effect was confirmed by another study, where addition of organic acids increased phytate degradation in whole-wheat bread from ~57 % to ~85 %.²⁷ It was observed that 57 % phytate degradation did not increase Fe dialyzability, but at 85 % phytate degradation with the organic acids, Fe dialyzability was increased to a level similar to the solubility reported in the present study. Finally, a study testing the effect of activating endogenous wheat phytase increased dialyzable Fe and Zn from wheat flour,²⁸ although to a lower level than solubilities reported in the present study, probably due to less extensive degradation of inositol phosphates.

As a final note, no significant effects of phytase treatment on soluble As, Cd and Pb were observed, thus providing no basis for believing that exposure to As, Cd and Pb will be increased

by co-consumption of phytases with phytate-rich cereal meals. This has also been backed up by *in vivo* studies.^{49–51} Soluble Al was detected ($P < 0.001$) only for rice bran at pH 2 with added phytases (*E. coli* and *A. niger*) at pH 2, but not in any other incubations. This observed solubilization of Al from rice bran should be subjected to further research to evaluate the potential risk of increased Al bioavailability *in vivo*.

CONCLUSIONS

The present study has shown the efficacy of microbial phytase catalysis in a simulated gastric environment for complete phytate dephosphorylation resulting in improved Fe and Zn solubility at pH conditions corresponding to the duodenum. The results obtained in this study infer that addition of acid stable microbial phytases, such as the *A. niger* or *E. coli*, principally has the potential to increase iron and zinc bioavailability via action in the gastric ventricle even post ingestion, e.g. if the phytases are added to the food vehicle or used as a sprinkle over a meal. Provided that cost-efficient solutions for promoting phytase catalysis *in vivo* can be designed the newly WHO safety-evaluated concept encompassing post-digestion phytase action may thus prospectively assist in combatting iron deficiency and iron deficiency anemia.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

InsPX, inositol phosphate (X indicates the number of phosphate groups)

REFERENCES

1. World Health Organization, *Essential Nutrition Actions*. WHO Press, Geneva (2013).
2. Hurrell RF, Influence of vegetable protein sources on trace element and mineral bioavailability. *J Nutr* **133**: 2973S – 2977S (2003).
3. Hurrell R and Egli I, Iron bioavailability and dietary reference values. *Am J Clin Nutr* **91**: 1461S – 1467S (2010).
4. Wessells KR and Brown KH, Estimating the global prevalence of zinc deficiency: Results based on zinc availability in national food supplies and the prevalence of stunting. *PLoS One* **7**: (2012).
5. Bohn L, Meyer AS and Rasmussen SK, Phytate: Impact on environment and human nutrition. A challenge for molecular breeding. *J Zhejiang Univ Sci B* **9**: 165–191 (2008).
6. Nielsen AVF, Tetens I and Meyer AS, Potential of phytase-mediated iron release from cereal-based foods: A quantitative view. *Nutrients* **5**: 3074–3098 (2013).
7. Troesch B, Egli I, Zeder C, Hurrell RF, de Pee S and Zimmermann MB, Optimization of a phytase-containing micronutrient powder with low amounts of highly bioavailable iron for in-home fortification of complementary foods. *Am J Clin Nutr* **89**: 539–544 (2009).
8. Sandberg A-S, Hulthén LR and Türk M, Dietary *Aspergillus niger* Phytase Increases Iron Absorption in Humans. *J Nutr* **126**: 476–480 (1996).
9. Egli I, Davidsson L, Zeder C, Walczyk T and Hurrell R, Dephytinization of a complementary food based on wheat and soy increases zinc, but not copper, apparent absorption in adults. *J Nutr* **134**: 1077–1080 (2004).
10. Nielsen AVF, Nyffenegger C and Meyer AS, Performance of microbial phytases for gastric inositol phosphate degradation. *J Agric Food Chem* **63**: 943–950 (2015).
11. Pontoppidan K, Pettersson D and Sandberg A-S, Interaction of phytate with protein and minerals in a soybean–maize meal blend depends on pH and calcium addition. *J Sci Food Agric* **87**: 1886–1892 (2007).
12. Andrews, NC, Iron Metabolism, in *Principles of Molecular Medicine*, ed. by Runge, MS and Patterson, C. Humana Press, Totowa, NJ, pp. 848–853 (2006).

13. Krebs NF, Westcott JE, Huffer JW and Miller LV, Absorption of exogenous zinc (Zn) and secretion of endogenous Zn in the human small intestine. *FASEB J* **12**: A345–A345 (1998).
14. Sandberg A-S, Brune M, Carlsson N-G, Hallberg L, Skoglund E and Rossander-Hulthén L, Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *Am J Clin Nutr* **70**: 240–246 (1999).
15. Han O, Failla ML, Hill AD, Morris ER and Smith JC, Inositol phosphates inhibit uptake and transport of iron and zinc by a human intestinal cell line. *J Nutr* **124**: 580–587 (1994).
16. World Health Organization, *Safety evaluation of certain food additives*, WHO Food Additives Series 67. WHO Press, Geneva (2012).
17. Xiang T, Liu Q, Deacon AM, Koshy M, Kriksunov IA, Lei XG, et al., Crystal structure of a heat-resilient phytase from *Aspergillus fumigatus*, carrying a phosphorylated histidine. *J Mol Biol* **339**: 437–445 (2004).
18. Kim H-W, Kim Y-O, Lee J-H, Kim K-K and Kim Y-J, Isolation and characterization of a phytase with improved properties from *Citrobacter braakii*. *Biotechnol Lett* **25**: 1231–1234 (2003).
19. Lassen, SF, Bech, L, Fuglsang, CC, Ohmann, A, Breinholt, J and Østergaard, PR, Peniophora phytase. US Patent 6054306 (2000).
20. Greiner R, Konietzny U and Jany K-D, Purification and characterization of two phytases from *Eschericia coli*. *Arch Biochem Biophys* **303**: 107–113 (1993).
21. Ariza A, Moroz O V, Blagova E V, Turkenburg JP, Waterman J, Roberts SM, et al., Degradation of phytate by the 6-phytase from *Hafnia alvei*: A combined structural and solution study. *PLoS One* **8**: e65062 (2013).
22. Awika JM, Major cereal grains production and use around the world, in *Advances in cereal science: Implications to food processing and health promotion*, ed. by Awika JM, Piironen V and Bean S. American Chemical Society, Washington DC, pp 1–13 (2011).
23. Reddy NR, Occurrence, Distribution, Content, and Dietary Intake of Phytate, in *Food Phytates*, ed. by Reddy NR and Sathe SK. CRC Press, Boca Raton, FL (2002).
24. U.S. Department of Agriculture, Agricultural Research Service. USDA National Nutrient Database for Standard Reference, Release 27. Nutrient Data Laboratory Home Page <http://www.ars.usda.gov/nutrientdata> (2015).
25. Shockravi S, Almgren A, Carlsson N-G and Sandberg A-S, Dephytinisation of Sangak and Barbari bread made from different extraction rate flours increases iron and zinc bioaccessibility in Caco-2 cells. *Int J Food Sci Technol* **47**: 2252–2258 (2012).

26. Larsson M, Minekus M and Havenaar R, Estimation of the bioavailability of iron and phosphorus in cereals using a dynamic *in vitro* gastrointestinal model. *J Sci Food Agric* **74**: 99–106 (1997).
27. Porres JM, Etcheverry P, Miller DD and Lei XG, phytase and citric acid supplementation in whole-wheat bread improves phytate-phosphorus release and iron dialyzability. *J Food Sci* **66**: 614–619 (2001).
28. Shilpa KS and Lakshmi AJ, Comparison of enhancement in bioaccessible iron and zinc in native and fortified high-phytate oilseed and cereal composites by activating endogenous phytase. *Int J Food Sci Technol* **47**: 1613–1619 (2012).
29. Sanz-Penella JM, Laparra JM, Sanz Y and Haros M, Assessment of iron bioavailability in whole wheat bread by addition of phytase-producing bifidobacteria. *J Agric Food Chem* **60**: 3190–3195 (2012).
30. Sanz-Penella JM, Frontela C, Ros G, Martinez C, Monedero V and Haros M, Application of bifidobacterial phytases in infant cereals: Effect on phytate contents and mineral dialyzability. *J Agric Food Chem* **60**: 11787–11792 (2012).
31. Akhter S, Saeed A, Irfan M and Malik KA, *In vitro* dephytinization and bioavailability of essential minerals in several wheat varieties. *J Cereal Sci* **56**: 741–746 (2012).
32. Frontela C, Haro JF, Ros G and Martínez C, Effect of dephytinization and follow-on formula addition on *in vitro* iron, calcium, and zinc availability from infant cereals. *J Agric Food Chem* **56**: 3805–3811 (2008).
33. Luo Y, Xie W and Cui Q, Effects of phytases and dehulling treatments on *in vitro* iron and zinc bioavailability in faba bean (*Vicia faba* L.) flour and legume fractions. *J Food Sci* **75**: (2010).
34. Carlsson N-G, Bergman E-L, Skoglund E, Hasselblad K and Sandberg A-S, Rapid analysis of inositol phosphates. *J Agric Food Chem* **49**: 1695–1701 (2001).
35. Bohn L, Josefsen L, Meyer AS and Rasmussen SK, Quantitative analysis of phytate globoids isolated from wheat bran and characterization of their sequential dephosphorylation by wheat phytase. *J Agric Food Chem* **55**: 7547–7552 (2007).
36. Skoglund E, Carlsson N-G and Sandberg A-S, High-performance chromatographic separation of inositol phosphate isomers on strong anion exchange columns. *J Agric Food Chem* **46**: 1877–1882 (1998).
37. Dansk Standard, DS 259:2003: Determination of metals in water, sludge and sediments - general guidelines for determination by atomic spectrophotometry in flame. *Dansk Standard* (2003).
38. García-Mantrana I, Monedero V and Haros M, Application of phytases from bifidobacteria in the development of cereal-based products with amaranth. *Eur Food Res Technol* (2014).

39. Canan C, Cruz FTL, Delaroza F, Casagrande R, Sarmento CPM, Shimokomaki M, et al., Studies on the extraction and purification of phytic acid from rice bran. *J Food Compos Anal* **24**: 1057–1063 (2011).
40. Hulse JH, Laing EM and Pearson OE, *Sorghum and the millets: Their composition and nutritive value*. Academic Press Inc., New York (1980).
41. Esmaeilipour O, Van Krimpen MM, Jongbloed AW, De Jonge LH and Bikker P, Effects of temperature, pH, incubation time and pepsin concentration on the *in vitro* stability of intrinsic phytase of wheat, barley and rye. *Anim Feed Sci Technol* **175**: 168–174 (2012).
42. Egli I, Davidsson L, Juillerat MA, Barclay D and Hurrell RF, The influence of soaking and germination on the phytase activity and phytic acid content of grains and seeds potentially useful for complementary feeding. *J Food Sci* **67**: 3484–3488 (2002).
43. Kies AK, De Jonge LH, Kemme PA and Jongbloed AW, Interaction between protein, phytate, and microbial phytase. *In vitro* studies. *J Agric Food Chem* **54**: 1753–1758 (2006).
44. Hurrell RF, Phytic acid degradation as a means of improving iron absorption. *Int J Vitam Nutr Res* **74**: 445–452 (2004).
45. Fredlund K, Isaksson M, Rossander-Hulthén L, Almgren A and Sandberg A-S, Absorption of zinc and retention of calcium: Dose-dependent inhibition by phytate. *J Trace Elem Med Biol* **20**: 49–57 (2006).
46. Wang Y, Cheng Y, Ou K, Lin L and Liang J, *In vitro* solubility of calcium, iron, and zinc in rice bran treated with phytase, cellulase, and protease. *J Agric Food Chem* **56**: 11868–11874 (2008).
47. Afify AE-MMR, El-Beltagi HS, El-Salam SMA and Omran AA, Bioavailability of iron, zinc, phytate and phytase activity during soaking and germination of white sorghum varieties. *PLoS One* **6**: 1–7 (2011).
48. Svanberg U, Lorri W and Sandberg A-S, Lactic fermentation of non-tannin and high-tannin cereals: Effects on *in vitro* estimation of iron availability and phytate hydrolysis. *J Food Sci* **58**: 408–412 (1993).
49. Rimbach G, Walter A, Most E and Pallauf J, Effect of microbial phytase on zinc bioavailability and cadmium and lead accumulation in growing rats. *Food Chem Toxicol* **36**: 7–12 (1998).
50. Troesch B, Jing H, Laillou A and Fowler A, Absorption studies show that phytase from *Aspergillus niger* significantly increases iron and zinc bioavailability from phytate-rich foods. *Food Nutr Bull* **34**: S90–S101 (2013).
51. Rimbach G, Brandt K, Most E, Pallauf J and Pallaup J, Supplemental phytic acid and microbial phytase change zinc bioavailability and cadmium accumulation in growing rats. *J Trace Elem Med Biol* **9**: 117–122 (1995).

Table 1. Details of cereals/cereal fractions. Dry matter, inositol phosphate and mineral contents.

For each line, significant ($P < 0.05$) differences are indicated with different letters. Molar

InsP6/mineral ratios were calculated by dividing the molar amounts of InsP6 in the cereals and

dividing by the mineral contents converted to moles.

	Wheat bran	Rice bran	Milled sorghum
Dry matter (g/kg)	923.1 $\pm$ 0.4a	930.1 $\pm$ 0.02b	875.8 $\pm$ 0.2c
InsP6 (g/kg DM)	52.7 $\pm$ 1.4a	102.1 $\pm$ 0.9b	19.5 $\pm$ 0.8c
InsP6 (mol/kg DM)	0.0798 $\pm$ 0.002a	0.155 $\pm$ 0.001b	0.0295 $\pm$ 0.001c
InsP3-6 (mol/kg DM)	0.0887 $\pm$ 0.003a	0.164 $\pm$ 0.001b	0.0300 $\pm$ 0.001c
Fe (g/kg DM)	0.159 $\pm$ 0.04a	0.169 $\pm$ 0.07a	0.0403 $\pm$ 0.0122b
Zn (g/kg DM)	0.0775 $\pm$ 0.0049a	0.0477 $\pm$ 0.0172b	0.0235 $\pm$ 0.0071b
Molar InsP6/Fe ratio	28	51	41
Molar InsP6/Zn ratio	67	212	82

Results are given as averages of triplicate determinations $\pm$ standard deviations.

DM: Dry matter.

Table 2. Molar ratios of soluble inositol hexakisphosphate (InsP6) to iron and zinc after two hours at simulated gastric conditions. Values indicated as below a given value corresponds the detection limit, as no InsP6 was detected in these samples.

Cereal	pH	<i>A. niger</i> phytase		<i>E. coli</i> phytase		No added phytase	
		InsP6/Fe	InsP6/Zn	InsP6/Fe	InsP6/Zn	InsP6/Fe	InsP6/Zn
Rice bran	2	<0.1	<0.5	<0.1	<0.5	45	187
	5	<0.1	<0.5	<0.1	<0.5	46	193
Wheat bran	2	0.43	1.0	<0.1	<0.3	21	49
	5	<0.1	<0.3	<0.1	<0.3	<0.1	<0.3
Milled sorghum	2	0.76	1.5	0.69	1.4	24	48
	5	<0.5	<1	<0.5	<1	30	61