The Importance of Lactic Acid Bacteria for Phytate Degradation during Cereal Dough Fermentation

ANNA REALE,[†] URSULA KONIETZNY,[‡] RAFFAELE COPPOLA,[†]
ELENA SORRENTINO,[†] AND RALF GREINER*[,§]

Distaam, Università degli Studi del Molise, Via De Sanctis, 86100 Campobasso, Italy, Waldstrasse 5c, 76706 Dettenheim, Germany, and Centre for Molecular Biology, Federal Research Centre for Nutrition and Food, Haid-und-Neu-Strasse 9, 76131 Karlsruhe, Germany

Lactic acid fermentation of cereal flours resulted in a 100 (rye), 95−100 (wheat), and 39−47% (oat) reduction in phytate content within 24 h. The extent of phytate degradation was shown to be independent from the lactic acid bacteria strain used for fermentation. However, phytate degradation during cereal dough fermentation was positively correlated with endogenous plant phytase activity (rye, 6750 mU g^{-1}; wheat, 2930 mU g^{-1}; and oat, 23 mU g^{-1}), and heat inactivation of the endogenous cereal phytases prior to lactic acid fermentation resulted in a complete loss of phytate degradation. Phytate degradation was restored after addition of a purified phytase to the liquid dough. Incubation of the cereal flours in buffered solutions resulted in a pH-dependent phytate degradation. The optimum of phytate degradation was shown to be around pH 5.5. Studies on phytase production of 50 lactic acid bacteria strains, previously isolated from sourdoughs, did not result in a significant production of intra- as well as extracellular phytase activity. Therefore, lactic acid bacteria do not participate directly in phytate degradation but provide favorable conditions for the endogenous cereal phytase activity by lowering the pH value.

KEYWORDS: Inositol phosphates; lactic acid bacteria; phytase; phytate

INTRODUCTION

Wholemeal bread is a staple food in many countries, because whole cereal flours provide fiber, complex carbohydrates, proteins, vitamins, and minerals. However, the presence of significant amounts of phytate in the flours and the wholemeal breads produced thereof interferes with mineral absorption. Phytate [*myo*-inositol(1,2,3,4,5,6)hexakisphosphate], the major storage form of phosphorus in the plant seed (*1*), forms insoluble complexes with numerous divalent and trivalent metal cations, particularly at slightly alkaline pH values (*2*), as prevailing in the small intestine, the major site of mineral absorption in the human gastrointestinal tract. The formation of insoluble metal cation−phytate complexes at physiological pH values is regarded as the major reason for poor mineral availability, because these complexes are essentially nonabsorbable from the gastrointestinal tract. Minerals of concern in this regard would include Zn^{2+}, $Fe^{2+/3+}$, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+} (*3*, *4*). Especially, zinc and iron deficiencies were reported as a consequence of high phytate intakes (*5*, *6*).

Because excessive amounts of phytate in the diet can lead to mineral deficiencies, phytate should be avoided among vulnerable groups and eliminated by extraneous processing efforts. Lactic acid fermentation was reported to significantly reduce the phytate content in plant-based foods (*7*−*13*) with a concomitant improvement of mineral solubility (*7*, *8*, *14*). The dephosphorylation of phytate is initiated by a class of enzymes called phytases (*15*), and extracellular phytase activity was suggested to be responsible for the observed reduction in phytate content during lactic acid fermentation (*8*−*11*). However, the majority of studies revealed that lactic acid bacteria did not show significant extracellular phytase activities (*11*, *12*, *16*−*18*) and the very low phytase activities observed were suggested to be due to nonspecific acid phosphatases (*17*). So far, only *Lactobacillus amylovorus* and *Lactobacillus plantarum* were reported to produce significant extracellular phytase activities (*19*). The observed reduction in phytate content during lactic acid fermentation might therefore be due to an activation of endogenous plant phytases or a coprecipitation of phytate and proteins as a consequence of a fall in pH during fermentation. The majority of plant grains and seeds exhibits phytase activity from pH 3 to 10 with maximal activity at pH values between 5 and 5.5 (*20*). The pH of the unfermented plant material is in general close to 7 and reaches a value of about 4 after complete lactic acid fermentation, thus passing through the interval that was shown to be optimal for plant phytases. Coprecipitation of phytate with proteins has been demonstrated to occur in a whey protein system when the pH was lowered simply by addition

* To whom the correspondence should be addressed. Tel: +49(0)721/6625 479. Fax: +49(0)721/6625 457. E-mail: ralf.greiner@bfel.de.
[†] Università degli Studi del Molise.
[‡] Waldstrasse 5c.
[§] Federal Research Centre for Nutrition and Food.

of lactic acid (*21*), and precipitation of complexes formed by phytate and proteins has been reported for several other food systems (*22*−*24*).

The objective of this work was to elucidate the role of lactic acid bacteria in the degradation of phytate during lactic acid fermentation in general and during sourdough fermentation in particular.

MATERIALS AND METHODS

Materials. *L. plantarum* (DSM20174^T, DSM2601), *L. amylovorus* DSM20531, *Lactobacillus acidophilus* DSM20079, and *Lactobacillus sanfranciscensis* (DSM 1721) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *L. plantarum* (S18, S29), *L. acidophilus* S37, and *Leuconostoc mesenteroides* (S18, S38, and S50) were kindly provided by Lopez (INRA, Clermont-Ferrand, France). All other strains were obtained from the Strain Collection of the Department of Science and Technology of Agro-food, Environment and Microbiology, Università degli Studi del Molise. These strains were previously isolated from southern Italian sourdoughs. Phytic acid dodecasodium salt was purchased from Aldrich (Steinheim, Germany). Ultrasep ES 100 RP18 was obtained from Bischoff (Leonberg, Germany), and AG1 X-8, 100−200 mesh, resin was purchased from Bio-Rad (München, Germany). All reagents were of analytical grade.

Screening for Phytate-Degrading Activity. Phytase production was studied in PSM broth, MRS broth containing 3 mM Na-phytate, and SDB broth containing 3 mM Na-phytate and glucose (20 g/L) or maltose (20 g/L) or both glucose (10 g/L) and maltose (5 g/L) as a carbohydrate source. PSM broth consisted of glucose (2 g/L), sodium citrate (0.1 g/L), Na-phytate (5 g/L), $MgSO_4 \times 7H_2O$ (0.075 g/L), $MnSO_4 \times 1H_2O$ (0.01 g/L), Tween 80 (1 g/L), $FeSO_4$ (0.005 g/L), L-glutamate (0.15 g/L), alanine (0.2 g/L), L-arginine-HCl (0.05 g/L), L-asparagine (0.2 g/L), L-cysteine (0.05 g/L), L-phenylalanine (0.04 g/L), L-histidine (0.04 g/L), L-isoleucine (0.06 g/L), L-leucine (0.06 g/L), l-lysine (0.05 g/L), L-methionine (0.05 g/L), L-proline (0.04 g/L), L-tyrosine (0.002 g/L), L-threonine (0.025 g/L), L-tryptophane (0.025 g/L), thiamine (0.0005 g/L), L-valine (0.015 g/L), vitamin B12 (0.001 g/L), biotin (0.001 g/L), pantothenic acid (0.01 g/L), folic acid (0.001 g/L), niacin (0.01 g/L), riboflavin (0.005 g/L), adenine (0.025 g/L), guanine (0.025 g/L), uracil (0.025 g/L), and thymidine (0.025 g/L). The pH was adjusted to 6.

After cultivation at 30 °C, the cells were collected by centrifugation at 7000*g* and 4 °C for 10 min and resuspended in 0.05 M Tris-HCl, pH 7.5, containing 0.1 M $CaCl_2$. The cells were collected again by centrifugation at 8000*g* and 4 °C for 10 min and resuspended in 0.05 M Tris-HCl, pH 7.5, preheated to 30 °C. The suspension was incubated at 30 °C for 30 min. Thereafter, the cells were collected by centrifugation at 9000*g* and 20 °C for 20 min, resuspended in 0.05 M Tris-HCl, pH 7.5, containing 24% sucrose and 10 mM $MgCl_2$, and incubated for 30 min at 37 °C. A lysozyme solution (5 mg/mL) was added to the cell suspension to give a final of 0.1% (v/v), and the suspension was incubated at 37 °C for 45 min. After centrifugation at 9000*g* and 20 °C for 20 min, the resulting pellets were resuspended in 0.05 M Tris-HCl, pH 7.5, at 4 °C. Sphaeroplasts were disrupted by two cycles of sonication (20 s for each treatment) and incubation for 30 min at 37 °C. The suspension was centrifuged at 14000*g* and 4 °C for 30 min. The clear solutions (cytoplasmatic extracts) were dialyzed against 0.02 M sodium acetate buffer, pH 5, and used for enzyme assays.

Fermentation. For fermentation, 10 g of cereal flour was suspended in 100 mL of water. Inactivation of the endogenous cereal phytases was achieved by autoclaving these mixtures for 30 min at 121 °C. Fermentation was started by inoculation with 10^9 cfu of the individual lactic acid bacteria strain. The inocula were collected by centrifugation at 5000*g* for 10 min and washed twice with 0.9% NaCl. Fermentation was performed at 30 °C on a rotary shaker at 200 rpm. Controls were run at different pH values without inoculation.

Assay of Phytase and Acid Phosphatase Activities. Enzyme activities were measured at 37 °C. The phytase activity in the cereal flours was determined by suspending 1 g of dry-milled cereal grains in 20 mL of 100 mM sodium acetate buffer, pH 4.5, containing 100 μmol of sodium phytate preincubated at 37 °C (*25*). After an incubation period of 40 min, 400 μL of the incubation mixtures was removed and 1.5 mL of a freshly prepared solution of acetone/5 N H_2SO_4/10 mM ammonium molybdate (2:1:1 v/v) and thereafter 100 μL of citric acid were added (*26*). Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To calculate the enzyme activity, a calibration curve was produced over the range of 5−600 nmol of phosphate. Activity (U) was expressed as μmol phosphate liberated per minute. Correction for the initial phosphate content of the samples was made. The incubation mixture for the lactic acid bacteria phytase activity determination consisted of 350 μL of 0.1 M sodium acetate buffer, pH 4.5, containing 2 μmol of sodium phytate. The enzymatic reactions were started by adding 50 μL of the enzyme preparations (cytoplasmatic extract, incubation broth) to the assay mixtures. After an incubation period of 30 min, the liberated phosphate was measured by addition of 1.5 mL of a freshly prepared solution of acetone/5 N H_2SO_4/10 mM ammonium molybdate as described above.

Acid phosphatase was determined in 200 μL of 50 mM citrate−NaOH, pH 4.5, containing 1 μmol of *p*-nitrophenyl phosphate. After 15 min, the reaction was stopped by adding 1.0 mL of 1 N NaOH. The acid phosphatase activity was determined by measuring the absorbance of the formed *p*-nitrophenolate at 405 nm. One unit of enzyme was defined as the amount of acid phosphatase releasing 1 μmol *p*-nitrophenolate per minute.

***myo*-Inositol Phosphate Analysis.** *myo*-Inositol phosphates were extracted from the fermentation mixtures or the cultivation broths by addition of HCl to a final concentration of 2.4% and shaking for 3 h at room temperature. Quantification of *myo*-inositol phosphates was performed as described by Sandberg and Ahderinne (*27*). The slurries were centrifuged at 30000*g* for 30 min, and 1 mL of the supernatants was diluted 1:25 with water and applied to a column (0.7 cm × 15 cm) containing AG1-X8, 100−200 mesh resin. The column was washed with 25 mL of water and then with 25 mL of 25 mM HCl. The *myo*-inositol phosphates were eluted with 25 mL of 2 M HCl. The eluates obtained were concentrated in a vacuum evaporator to complete dryness. The residues were dissolved in 500 μL of water, and 20 μL of these solutions was chromatographed on Ultrasep ES 100 RP18 (2 mm × 250 mm, 6.0 μm). The column was run at 45 °C and 0.2 mL min^{-1} of an eluant consisting of formic acid/methanol/water/TBAH (tetrabutylammonium hydroxide) (44:56:5:1.5 v/v), pH 4.25. A mixture of the individual *myo*-inositol phosphate esters (IP_3−IP_6) was used as a standard.

Purification of the Rye Phytase. Purification of the phytase rye was performed as described previously (*28*). The phytase was purified to apparent homogeneity according to denaturing and nondenaturing polyacrylamide gel electrophoresis.

RESULTS

Screening for Phytase Activities. Fifty strains of lactic acid bacteria were screened for intra- as well as extracellular phytate-degrading activity using different growth media. Neither phosphate reduction (PSM) nor addition of sodium phytate (MRS and SDB) or modification of the carbon source (SDB) resulted in a significant production of intra- as well as extracellular phytase activities in the studied strains. The intracellular phytase activities determined ranged between 0.3 and 5.7 mU/mL, whereas the extracellular phytase activity could not be observed. However, using *p*-nitrophenyl phosphate as a substrate, significant intra- as well as extracellular acid phosphatase activities were detectable (data not shown). Evaluation of phytate degradation during cultivation of the lactic acid bacteria strains by high-performance liquid chromatography confirmed the absence of significant extracellular phytase activity. No phytate degradation could be established even after 10 days of cultivation at 30 °C in all of the growth broths used (data not shown).

Lactic Acid Fermentation of Cereal Flours. Four lactic acid bacteria strains [*L. plantarum* (DSM20174^T, DSM2601), *L.*

Table 1. *myo*-Inositol Phosphate Content of Cereal Flours during Lactic Acid Fermentation[a]

cereal	time (h)	μmol g^{-1} IP$_6$	IP$_5$	IP$_4$	IP$_3$	IP$_{6--}$	pH
rye	0	11.6 ± 0.4	0.4 ± 0.03	ND	ND	12.0 ± 0.43	7.0
	4	9.2 ± 0.7	1.5 ± 0.2	0.8 ± 0.1	0.3 ± 0.04	11.8 ± 1.05	6.4
	8	5.8 ± 0.3	1.9 ± 0.4	2.5 ± 0.2	1.7 ± 0.2	11.9 ± 1.1	4.8
	12	0.2 ± 0.05	0.3 ± 0.02	3.9 ± 0.4	5.1 ± 0.4	9.5 ± 0.87	4.4
	24	ND	ND	1.7 ± 0.1	3.2 ± 0.3	4.9 ± 0.4	4.0
wheat	0	14.7 ± 0.7	0.5 ± 0.1	ND	ND	15.2 ± 0.8	7.0
	4	12.4 ± 0.4	1.7 ± 0.15	0.8 ± 0.05	0.4 ± 0.1	15.3 ± 0.7	6.5
	8	8.3 ± 0.3	3.4 ± 0.2	3.1 ± 0.3	0.9 ± 0.1	15.7 ± 0.9	5.0
	12	0.7 ± 0.1	2.1 ± 0.2	7.1 ± 0.4	5.0 ± 0.4	14.9 ± 1.1	4.5
	24	0.1 ± 0.02	0.2 ± 0.05	5.3 ± 0.3	6.1 ± 0.5	11.7 ± 0.87	4.2
oat	0	12.9 ± 0.5	0.4 ± 0.1	ND	ND	13.3 ± 0.6	7.0
	4	12.5 ± 0.4	0.6 ± 0.1	0.1 ± 0.03	ND	13.2 ± 0.53	6.7
	8	11.4 ± 0.7	0.9 ± 0.1	0.6 ± 0.1	0.1 ± 0.01	13.0 ± 0.91	5.1
	12	9.6 ± 0.3	1.2 ± 0.2	1.9 ± 0.2	0.3 ± 0.1	13.0 ± 0.8	4.5
	24	7.2 ± 0.2	1.4 ± 0.3	3.4 ± 0.3	0.9 ± 0.1	12.9 ± 0.9	4.3

[a] ND, not detectable. The data are mean values ± standard deviation of three independent experiments with each lactic acid bacteria strain used.

Table 2. *myo*-Inositol Phosphate Content of Heat-Treated Rye Flour during Lactic Acid Fermentation after Addition of Purified Rye Phytase[a]

time (h)	μmol g^{-1} IP$_6$	IP$_5$	IP$_4$	IP$_3$	IP$_{6-3}$	pH
0	11.7 ± 0.6	0.4 ± 0.05	ND	ND	12.1 ± 0.65	7.0
4	9.5 ± 0.4	1.4 ± 0.2	0.9 ± 0.11	0.3 ± 0.07	12.1 ± 0.78	6.2
8	6.1 ± 0.2	1.7 ± 0.3	2.2 ± 0.2	1.6 ± 0.2	11.6 ± 0.9	4.7
12	0.4 ± 0.15	0.2 ± 0.04	3.7 ± 0.3	5.0 ± 0.4	9.3 ± 0.89	4.2
24	ND	ND	1.6 ± 0.15	3.0 ± 0.3	4.6 ± 0.45	3.9

[a] ND, not detectable. The data are mean values ± standard deviation of three independent experiments.

amylovorus (DSM20531), and *L. acidophilus* (DSM20079)], which have been previously reported as phytase producers (*19*), were used for fermentation experiments. Lactic acid fermentation of cereal flours resulted in a significant reduction in phytate content with a concomitant increase in the concentrations of the lower *myo*-inositol phosphates within 24 h (**Table 1**). All strains showed similar pH and growth patterns. After 24 h, the phytate reduction was determined to be 100% in rye, 95−100% in wheat, and 39−47% in oat flour.

No significant phytate degradation was observed during fermentation of heat-treated cereal flours. The heat treatment resulted in a complete inactivation of the endogenous cereal acid phosphatases including phytases. During fermentation, a significant decrease in the content of the partially phosphorylated lower *myo*-inositol phosphate esters (IP$_5$ and IP$_4$) occurred in the nonheat-treated inoculated samples but not in the noninoculated controls. After 24 h of fermentation, IP$_5$ reduction was determined to be 40−63% in rye, 48−61% in wheat, and 52−79% in oat flour, whereas the reduction in IP$_4$ was found to be 78−91% in rye, 87−93% in wheat, and 82−91% in oat flour within 24 h.

Phytate degradation during lactic acid fermentation after addition of 6.75 U of purified rye phytase per gram of heat-treated rye flour (**Table 2**) was not significantly different to that observed with the native rye flour (contains 6.75 U/g of endogenous phytase activity) (**Table 1**).

Incubation of cereal flours in buffered solutions resulted in a pH-dependent phytate degradation (**Table 3**). The optimum of phytate degradation by the endogenous cereal phytases was shown to be within the pH range of the broth during lactic acid fermentation.

Table 3. *myo*-Inositol Phosphate Content of Cereal Flours during Incubation at Different pH Values without Inoculation[a]

cereal	pH	μmol g^{-1} IP$_6$	IP$_5$	IP$_4$	IP$_3$	IP$_{6-3}$
wheat	7.0	10.9 ± 0.6	2.8 ± 0.3	1.2 ± 0.15	0.3 ± 0.06	15.2 ± 1.11
	5.5	ND	ND	ND	0.7 ± 0.1	0.7 ± 0.1
	4.0	2.1 ± 0.2	1.9 ± 0.2	4.2 ± 0.3	5.7 ± 0.2	13.9 ± 0.9
rye	7.0	7.1 ± 0.5	2.9 ± 0.4	1.4 ± 0.12	0.5 ± 0.04	11.9 ± 1.06
	5.5	ND	ND	ND	ND	ND
	4.0	ND	ND	3.3 ± 0.2	4.6 ± 0.5	7.9 ± 0.7
oat	7.0	12.7 ± 0.7	0.5 ± 0.08	ND	ND	13.2 ± 0.78
	5.5	5.1 ± 0.4	1.5 ± 0.11	2.1 ± 0.4	3.7 ± 0.2	12.4 ± 1.11
	4.0	6.9 ± 0.5	1.2 ± 0.1	1.8 ± 0.2	3.1 ± 0.3	13.0 ± 1.1

[a] Incubation was performed for 24 h at 30 °C on a rotary shaker at 200 rpm. ND, not detectable. The data are mean values ± standard deviation of three independent experiments.

DISCUSSION

Phytase production by lactic acid bacteria is still controversially discussed within the scientific community. Particularly regarding the importance of lactic acid bacteria phytase for phytate degradation during sourdough fermentation, the scientific data are interpreted as supporting the hypothesis that either lactic acid bacteria phytase is significantly involved in phytate degradation during sourdough fermentation (*8*, *10*, *11*) or the intrinsic cereal phytases are responsible for phytate degradation after being activated by a fall in pH due to lactic acid production by the lactic acid bacteria (*7*, *12*, *16*, *29*). To act on phytate, phytates must have access to the phytate in the dough. Our studies revealed that none of the 50 lactic acid bacteria strains included produced measurable extracellular phytase activity in MRS and SDB medium as well as in the phosphate-reduced media PSM. A phosphate-reduced medium was included, because a tight regulatory inhibition of the formation of phytases by phosphate levels was generally observed in all microorganisms (*30*). Phosphate was shown to exert its effect on the synthesis of phytases at the level of transcription. The efficient derepression of phytase formation by phosphate starvation in most microorganisms suggests a possible role for these enzymes in providing the cell with phosphate. The assumption could also explain why, with the exception of sourdough bacteria, there is no clear evidence for lactic acid bacteria with the ability to degrade phytate. Lactic acid bacteria are adapted to environments rich in nutrients and energy where evolutionary selection pressure would not favor the capability to produce a phytase. In addition, our findings are in agreement with the majority of studies on phytase production by lactic acid bacteria (*11*, *12*, *16*−*18*). So far, only *L. amylovorus* and *L. plantarum* were reported to produce significant extracellular phytase activity (*19*), but we have been unable to reproduce this finding even when using the same *L. amylovorus* strain (DSM20531) under identical growth conditions.

A low intracellular phytase activity was determined in all lactic acid bacteria included in the study. This is in accordance with the results reported by De Angelis et al. (*11*). The intracellular phytase activity might be found in almost every cell, since phytate is a common cellular constituent with a significant turnover (*31*). However, it is very unlikely that intracellular phytases are involved in extracellular phytate dephosphorylation even if it could not be ruled out that phytate is taken up by bacterial cells. It is not known, for example,

how bacteria with an apparent lack of extracellular phytase activity, such as some *Pseudomonas* strains, either grow in the absence of a readily utilizable phosphate source or acquire phosphate. Because no phosphate was detected in the growth medium either initially or throughout the growth period (*32*), phytate might be transported into the bacterial cells. Furthermore, Wang et al. (*33*) suggested that in *Klebsiella pneumoniae*, the gene encoding the phytase is cotranscribed from a polycistronic mRNA, which also acts as a template for an inositol phosphate transporter.

Because of the apparent lack of lactic acid bacterial extracellular phytase activity, the observed reduction in phytate content during lactic acid fermentation might be due to an activation of intrinsic cereal phytases as a consequence of a fall in pH during fermentation. This suggestion was supported by the findings that with all lactic acid bacteria used in this study a similar decrease in phytate content was observed during sourdough fermentation and the extent of phytate degradation correlates very well with the intrinsic phytase activity of the cereal flours. In addition, no significant phytate degradation was observed during fermentation after heat treatment of the cereal flours prior to fermentation. The heat treatment (autoclaving at 121 °C for 30 min) resulted in a complete inactivation of the endogenous cereal acid phosphatases including phytases. Autoclaving was chosen for enzyme inactivation, because treatment of the flours by microwave as described by Lopez et al. (*8*) and de Angelis et al. (*11*), respectively, did not result in a complete loss of the intrinsic phytase activity. Because a significant decrease in the content of the partially phosphorylated *myo*-inositol phosphate esters still occurred in the inoculated samples, but not in the noninoculated controls, lactic acid bacteria produced extracellular phosphatases capable of acting on partially phosphorlyated *myo*-inositol phosphates. The presence of extracellular phosphatases, which do not act on phytate, is in agreement with results obtained from screening studies. The lack of phytate degradation could be overcome by addition of a purified phytase to the fermentation broth. A further confirmation for the importance of the intrinsic cereal phytases came from the incubation of the cereal flours in buffered solutions, which resulted in a pH-dependent phytate degradation. The optimum of phytate degradation was shown to be close to a pH value of 5.5. It was already reported that cereal phytases exhibit maximal activity at pH values between 5 and 5.5 (*20*).

Lactic acid fermentation significantly reduces phytate content in plant-based foods. This report clearly demonstrates that phytate reduction is mainly due to the activity of the intrinsic plant phytases. The importance of lactic acid bacteria for phytate dephosphorylation is limited to providing favorable conditions for the endogenous cereal phytases by lowering pH value. All lactic acid bacteria strains used in this study lacked extracellular phytase activity. However, even if a wild-type lactic acid bacterium produces extracellular phytase activity, it is very unlikely that its production will be sufficient to allow significant phytate dephosphorylation during fermentation.

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