Use of Fungal Phytase to Improve Breadmaking Performance of Whole Wheat Bread

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The possible use of phytase as a breadmaking improver has been tested in whole wheat breads by adding different amounts of fungal phytase. The effect of phytase addition on the fermentation stage and the final bread quality was analyzed. The phytase addition shortened the fermentation period, without affecting the bread dough pH. Regarding the whole wheat bread, a considerable increase of the specific bread volume, an improvement of the crumb texture, and the width/height ratio of the bread slice were obtained. An in vitro assay revealed that the improving effect of phytase on breadmaking might be associated with the activation of α-amylase, due to the release of calcium ions from calcium−phytate complexes promoted by phytase activity. As a conclusion, phytase offers excellent possibilities as a breadmaking improver, with two main advantages: first, the nutritional improvement produced by decreasing phytate content, and second, all the benefits produced by α-amylase addition can be obtained by adding phytase, which promotes the activation of endogenous α-amylase.

Keywords: *Whole wheat bread; phytase; phytate; breadmaking; whole bread quality; α-amylase*

INTRODUCTION

The growing awareness of the potential benefits of high fiber diets has created interest in the use of legume and grain seeds in human nutrition (*1*). The increasing demand for whole grain bread or bran bread, due to their high content on dietary fiber, has also resulted in interest in phytic acid, because the bran contains a high proportion of phytates (*2*). *Myo*-inositol hexaphosphate, commonly known as phytic acid or phytate, is widely distributed in plant seeds and grains. It is primarily present as a salt of mono- and divalent cations (K^+, Ca^{2+}, and Mg^{2+}) (*3*). It is widely known that phytates have adverse effects on the bioavailability of multivalent cations, especially Zn^{2+}, Ca^{2+}, Mg^{2+}, and Fe^{3+}, due to the formation of insoluble complexes (*4*−*8*). The aleurone layer of cereals constitutes a reservoir of phytates that form a complex with protein, phytase, α-amylase, and protease (*9*). The enzyme phytase, present in seeds as well as bacteria, yeast, and fungi, dephosphorylates phytates in sequential steps ending with the formation of inositol and phosphoric acid (*10*).

During the transformation of flour into bread, phytate content decreases as a consequence of the activity of native phytase, but usually not to such extent to greatly improve mineral bioavailability. Reduction of phytate content during breadmaking depends on phytase action, which in turn is influenced by several factors, such as the degree of flour extraction, the proofing time and temperature, the acidity of the dough, the yeast, and enzymes added to the dough and the presence of calcium salts (*11*).

To increase the nutritional value of bread, certain breadmaking procedures designed to diminish the phytate content have been reported. These include, the addition of commercial phosphoesterases from wheat (phytase, phosphatase) to whole wheat flour (*12*) and the activation of the naturally occurring phytase by soaking and malting the grain. This resulted in the degradation of phytate and thereby produced cereal products with high mineral bioavailability (*13*).

However, these studies focused on the degradation of phytate did not consider the effect on the breadmaking process. In a recent study, Haros et al. (*14*) envisaged the potential use of phytase in fiber-rich breads; however, only one concentration of phytase was tested.

The goal of this study was to optimize the phytase addition for the production of whole wheat breads. The effect of increasing amounts of phytase in the bread formulation on the destruction of phytates during the breadmaking was analyzed. To understand the effects promoted by phytase addition, a model was also proposed considering the possible interactions between phytates, proteins, and divalent cations.

MATERIALS AND METHODS

Materials. A commercial blend of whole wheat flour from the local market was used for the preparation of breads. Flour characteristics are listed in Table 1. Compressed yeast was used as a starter. A commercial phytase (3.1.3.8) from *Aspergillus niger* (320 U/mL) and fungal α-amylase (Fungamyl 1500 BG) (35.3 U/g) were gifts from Novo Nordisk (Bioindustrial, Spain).

Phytate Determination. The extracts of phytates were prepared following the method reported by Latta and Eskin (*15*) as modified slightly by Haros et al. (*14*). Briefly, 10 g of flour, dough, or bread with 50 mL of 0.65 N HCl solution was mixed in a Virtis homogenizer (10 s three times at 20000 rpm).

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Table 1. Characteristics of the Whole Wheat Flour Used

moisture (%)	13.5
protein (%)[a]	14.3
ash (%)[a]	1.66
water absorption (%)[a]	60.5
phytates (mg phytic acid/g)[a]	9.4
phytase activity (μg of P/g min)[a]	46.5

[a] Dry matter.

The homogenate was centrifuged, and supernatant was filtered through glass wool. The supernatant was mixed with 20% trichloroacetic acid (TCA) (6:1, v:v); after cooling, the TCA-insoluble proteins were removed by centrifugation at 12000 rpm for 5 min. The clear supernatant was used for phytate assay.

The phytate content was measured by using the modified Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ and 0.3% sulfosalicylic acid in distilled water) following the method reported by Latta and Eskin (*15*) and adapted to a microplate reader by Haros et al. (*14*). The phytate content was expressed as mg of phytic acid/g of dry matter. In all cases, four replicates were performed for each experimental point.

Determination of Phytase Activity. The enzymatic extracts were prepared following the method reported by Konietzny et al. (*16*) and Haros et al. (*14*). Briefly, 10 g of flour, dough, or bread dispersed in 50 mL of 100 mM sodium acetate buffer, pH 5.0 (containing 5 mM 2-mercaptoethanol and 10 mM EDTA) was homogenized and centrifuged, and the supernatant was filtered through glass wool. The clear solution was stored at 4 °C for further enzymatic assays.

The phytase activity was measured using sodium phytate as a substrate and following the method reported by Kikunaga et al. (*9*). The incubation mixture consisted of 500 μL of 0.1 M sodium acetate buffer, pH 5.0, containing 1.2 mM sodium phytate, with 100 μL of the enzymatic extract; after incubation for 20 min at 50 °C, the phytase reaction was stopped by adding 100 μL of 20% TCA. Samples were centrifuged previously to the quantification. The inorganic phosphate contained in the supernatant was quantified by the ammonium molybdovanadate method (*17*) adapted to a microplate reader by Haros et al. (*14*). The phytase activity was expressed as μg of P/g min in dry matter. In all cases, four replicates were carried out for each experimental point.

Breadmaking Procedure. The bread dough formula consisted of whole wheat flour (100 g), compressed yeast (3.0 g), salt (2.0 g), water (57.3 mL), and variable amounts of phytase (when added). The ingredients were mixed for 7 min, rested for 10 min, divided (50 g), kneaded, and then rested (10 min); doughs were mechanically sheeted and rolled, proofed (up to three times the initial dough volume, at 29 °C, 80% relative humidity), and baked (170 °C, 26 min). After baking, loaves were cooled for 2 h at room temperature.

Technological Evaluation. During the proofing stage, the dough volume increase was followed in a graduated tube, and pH of doughs were potentiometrically determined. Physicochemical characteristics of breads including weight, volume (seed displacement), specific volume index (specific volume of the control was taken as 100, and the specific volume of the samples was relative to the control), width/height ratio of the central slice, moisture content and texture (texture profile analysis, TPA) were determined. TPA was performed using a Texture analyzer TA-XT2i (Stable Micro Systems, Surrey, UK). The sample was compressed twice by using a 1.0-cm diameter plunger, with an interval of 50 s between compressions. The following parameters were evaluated: hardness (firmness), springiness, cohesiveness, chewiness, and resilience (for a detailed information about TPA parameters see ref *18*).

Effect of Phytate and Ca^{2+} on the α-Amylase Activity. The possible effects of phytic acid and Ca^{2+} on α-amylase activity were investigated in model systems by measuring the α-amylase activity in the presence of increased calcium concentrations (0, 5, 10, 15, and 20 mM) and at two levels of phytate concentrations (10 and 20 mM).

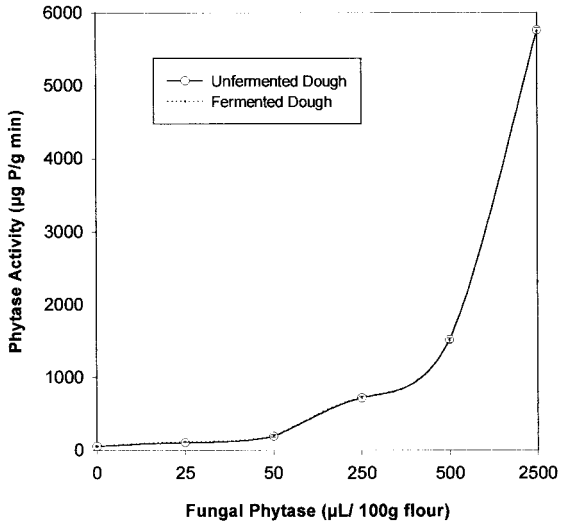

Figure 1. Phytase activities in doughs containing exogenous phytase at different stages of the breadmaking process. Unfermented dough is referred to as dough after mixing and 10 min resting. Fermented dough is taken just before baking.

The α-amylase activity was measured by using a blocked *p*-nitrophenyl maltoheptaoside (BPNPG7, Megazyme International Ireland Ltd., Wicklow, Ireland) as substrate following the method reported by McCleary and Sheenan (*19*) and further adapted to a microplate reader by Sirou et al. (*20*) and Rosell et al. (*21*). Briefly, 15 μL of the substrate reagent (BPNPG7) with 15 μL of calcium (0, 20, 40, 60, or 80 mM) and phytates (40 or 80 mM) solutions and 30 μL of the α-amylase solution (1%, w/v) were pipetted into individual wells of a 96-well microplate. The enzyme reaction proceeded for 15 min at 30 °C, at that time 150 μL of 1% Trizma base solution was added to stop the reaction. The phenoxide colored form of *p*-nitrophenol was measured at 405 nm by using a microplate reader. One unit of α-amylase activity was defined as the amount of enzyme which releases 1 μmol of *p*-nitrophenol/min and milliliter under the defined assay conditions. In all cases, four replicates were assayed for each experimental point.

RESULTS AND DISCUSSION

Influence of Phytase Addition on the Fermentative Properties of Whole Wheat Dough. Different amounts of phytase from *Aspergillus niger* were added to the dough, and the phytase activity was tested at different stages of the proofing time. The phytase activity in unfermented doughs (dough taken after mixing and with 10 min resting) and fermented dough (after the proofing time, before baking) is shown in Figure 1. In the whole wheat flour, the endogenous phytase activity was 46.5 μg of P/g min (Table 1), and this steadily increased with the addition of increased amounts of fungal phytase. The enzyme activity in unfermented doughs ranged from 50 to 5800 μg of P/g min in doughs containing from 0 to 2500 μL/100 g of flour fungal phytase, respectively.

The phytase activity was the same in unfermented and fermented dough; therefore, the fermentation stage did not modified the enzyme activity.

The addition of phytase decreased the proofing time (Figure 2). The proofing time for dough containing 2500 μL of phytase/100 g of flour decreased up to 24% as compared to the control.

Dough pH was also determined during the proofing since the phytase activity is highly dependent on pH. The changes in pH values during this stage was approximately 0.20 pH units in all cases. The pH values

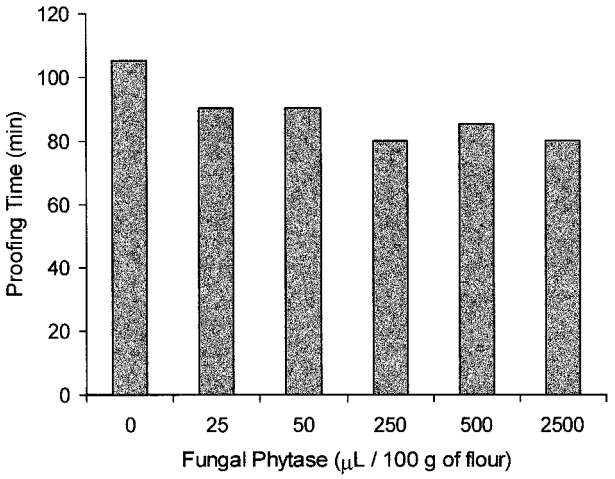

Figure 2. Effect of adding fungal phytase on the dough proofing time to reach an optimum dough increase.

Table 2. Effect of Adding Fungal Phytase on Quality Parameters of Fresh Bread[a]

phytase conc (μL/100 g of flour)	moisture (%, d.m.)	width/height ratio	specific vol index	firmness (g)
0	31.8 ± 0.1	1.346 ± 0.026	100.0 ± 1.3	590 ± 46
25	30.3 ± 0.1	1.350 ± 0.009	105.5 ± 1.5	559 ± 10
50	31.9 ± 0.1	1.347 ± 0.006	103.1 ± 1.7	542 ± 32
250	31.8 ± 0.1	1.336 ± 0.026	106.3 ± 0.4	503 ± 17
500	33.5 ± 0.1	1.316 ± 0.025	118.0 ± 2.6	474 ± 17
2500	31.7 ± 0.1	1.288 ± 0.009	121.1 ± 1.8	423 ± 20

[a] Mean ± SD, $n = 4$.

ranged between 5.63 and 5.43 in all doughs. Wheat phytase has an optimal pH of 5.15 (*22*), and its activity diminishes markedly as the pH is moved from the optimum. Conversely, *Aspergillus niger* phytase has two pH optima, one at 5.0 and the other at 2.5−3.0 (*11*). Therefore, as the pH decreases along the proofing, higher phytase activity is observed.

Quality Characteristics of Breads Containing Exogenous Phytase. The addition of fungal phytase practically did not modify the moisture content of the loaves but did decrease the width/height ratio of the bread slices (Table 2) by about 5%. Therefore, an improvement of the loaf shape was obtained in the presence of phytase. In addition, the specific volume index was increased by up to 21% by adding 2500 μL of phytase/100 g of flour as compared to the control (without phytase addition).

The crumb firmness decreased by 28.3% as fungal phytase concentration was increased. Small differences were found when analyzing the other texture parameters (results not showed).

Effect of Adding Fungal Phytase in the Evolution of Phytates during the Whole Wheat Breadmaking Process. The change in phytic acid content through the breadmaking process is shown in Figure 3. Phytate content in the control dough (without exogenous phytase) decreased during breadmaking, showing a reduction of 4.7% after mixing and resting (unfermented dough), and a further drop up to 22.4% during the proofing (fermented dough). Baking hardly affected the decomposition of phytate, 7.2 mg phytic acid/g of dry matter remained in fresh bread, which means a reduction of 23.8% with regard to the initial value of phytic acid in the flour. The results are in agreement with previous ones of Reinhold et al. (*23*), who found that the phytate levels decreased to 15−25%

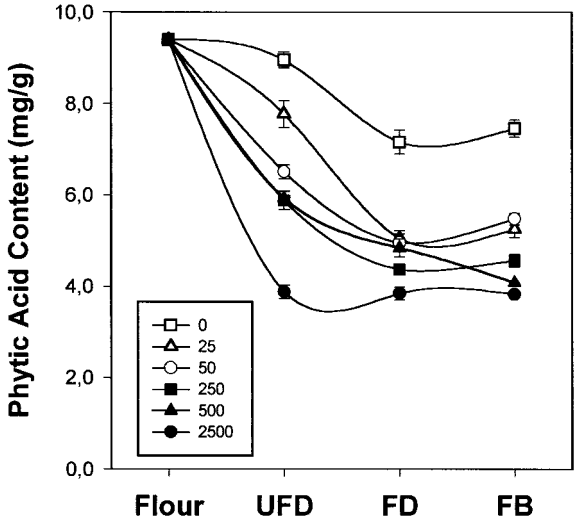

Figure 3. Effects of adding different amounts (μL/100 g of flour) of fungal phytase on phytate degradation through different stages of the breadmaking process. UFD: unfermented dough; FD: fermented dough; FB: fresh bread. Symbols and vertical bars represent the mean of four replicates ± SEM.

of their initial value during proofing of whole wheat bread, and McKenzie-Parnell and Davies (*24*), who found a reduction of 30−48% during breadmaking and bread contained 5.2−7.1 mg of phytic acid/g of dry matter. Phytates have been reported to decrease through the breadmaking process as a consequence of the phytase activities from both the flour and the yeast (*25*). However, in the present study no phytase activity was detected in the compressed yeast; therefore, the phytate decrease should be attributed to the endogenous flour phytase. It should be remarked that controversial results have been reported about phytase activity in yeasts; for instance, Tangkongchitr et al. (*26*) and Harland and Frölich (*27*) did not find phytase activity in yeast, whereas Nayini and Markakis (*28*) reported activities in baker's yeasts.

The addition of fungal phytase to the whole wheat doughs promoted a significant decrease in phytate content (Figure 3). The largest degradation occurred after mixing and resting (unfermented dough), resulting in a phytate content drop from 17.3 to 51.9%, when adding 25 and 2500 μL of fungal phytase/100 g of flour, respectively. During fermentation, the phytates were reduced up to 59.0% in dough containing 2500 μL of fungal phytase/100 g of flour.

The rate of phytate hydrolysis during proofing was slower with the increased exogenous phytase concentration. In the dough containing 2500 μL of fungal phytase/100 g of flour, only 5.8% of phytate was hydrolyzed during proofing. This result could be explained by product inhibition (*29*) or that an excessive accumulation of the inorganic phosphorus may lead a rephosphorylation of partially hydrolyzed phytic acid (*30*). The residual values of phytates in fresh bread containing fungal phytase ranged from 5.2 to 3.8 mg of phytic acid/g of dry matter. Practically, baking affected slightly the phytate hydrolysis; similar phytate content was obtained in fermented dough and fresh bread.

In Vitro Studies To Explain the Phytase Effect on Breadmaking. The breadmaking improvements resulting from phytase addition (short fermentation times, bread volume increase, and soft bread crumbs) are usually associated with the addition of α-amylase.

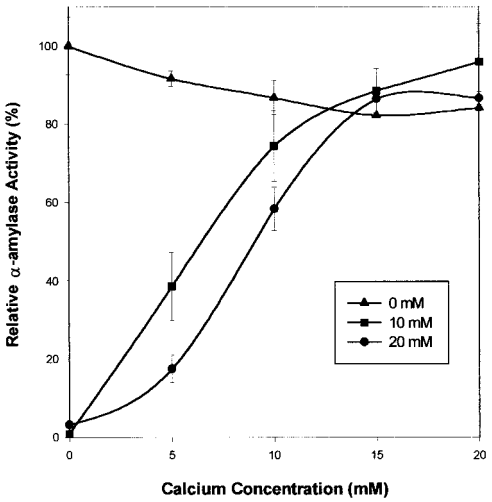

Figure 4. Calcium influence on the activity of α-amylase in the presence of different concentrations of phytic acid. Symbols and vertical bars represent the mean of four replicates ± SEM. Symbols represent different concentrations of phytic acid.

Therefore, the addition of phytase may be related in some way to the α-amylase activity. No α-amylase activity was detected in the commercial fungal phytase preparation (results not showed), and consequently, the observed effects appear to be due to the action of the phytase on the α-amylase naturally present in flour. It is known that phytates form complexes with divalent ions, particularly with calcium cations, which in turn are necessary for α-amylase activity (*31*).

The addition of phytase leads to hydrolysis of phytates, and consequently a breakdown of the phytate−calcium complexes; hence, free calcium ions become available for the α-amylase activity. To confirm the main role of phytate−calcium ion complex in controlling the α-amylase activity, an in vitro study was performed. The effect of increasing calcium concentrations on the α-amylase activity in the presence of different concentrations of phytic acid is shown in Figure 4.

α-Amylase activity slightly decreased as the concentrations of Ca^{2+} were increased in absence of phytic acid. Calcium ions, as well as other cations, strongly influenced α-amylase activity, particularly at high concentrations; cations may interact with negatively charged groups of proteins, affecting their conformation (*3*). These authors found that 64% of α-amylase activity remains at 24 mM calcium concentration. However, calcium ions are required for the activity and stability of α-amylases. When α-amylase was incubated in the presence of phytic acid (10 and 20 mM) and absence of calcium, no enzyme activity was detected. In the presence of both phytic and calcium, the enzyme activity increased gradually with the calcium concentration. Calcium concentrations of 20 mM were needed to recover the total α-amylase activity. Therefore, it appears that the inhibitory effect of phytates is due to the sequestration of calcium ions.

Thus, phytase is a good breadmaking improver for preparation of whole wheat breads. It improves the nutritional value of these breads and, in addition, breadmaking performance by promoting the activation of endogenous α-amylase.

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LITERATURE CITED

(1) Snider, M.; Liebman, M. Calcium additives and sprouted wheat effects on phytate hydrolysis in whole wheat bread. *J. Food Sci.* **1992**, *57* (1), 118−120.

(2) Barber, S. Chemical changes during bread dough fermentation and their relation to the quality of bread. In *Chemical Changes during Food Processing*; S. B., Poveda, Ed.; Instituto Agroquimica y Tecnologia de Alimentos: Valencia, Spain, 1984; pp 145−178.

(3) Deshpande, S. S.; Cheryan, M. Effects of phytic acid, divalent cations, and their interactions on α-amylase activity. *J. Food Sci.* **1984**, *49*, 516−519, 524.

(4) Guenter, W.; Sell, J. L. A method for determining true availability of magnesium from food stuff using chicken. *J. Nutr.* **1974**, *104*, 1446−1457.

(5) Davies, N. T.; Nightingale, R. The effect of phytate on intestinal absorption and secretion of zinc and whole-body retention of zinc, copper, iron, and manganese in rats. *J. Nutr.* **1975**, *34*, 243−247.

(6) Erdman, J. W. Oilseed phytates: Nutritional implications. *J. Am. Oil Chem. Soc.* **1979**, *56*, 736−741.

(7) Zemel, M. B.; Shelef, L. A. Phytic acid hydrolysis and zinc and iron in whole wheat bread as affected by calcium containing additives. *J. Food Sci.* **1982**, *47*, 535−537.

(8) Munoz, J. M. Overview of the Effects of Dietary Fiber on the Utilization of Minerals and Trace Elements. In *Handbook of Dietary Fiber in Human Nutrition*, Spiller, G. A., Ed.; CRC Press: Boca Raton, FL, 1985; pp 193−200.

(9) Kikunaga, S.; Katoh, Y.; Takahashi, M. Biochemical Changes in Phosphorus Compounds and in the Activity of Phytase and α-Amylase in the Rice (*Oryza sativa*) Grain during Germination. *J. Sci. Food Agric.* **1991**, *56*, 335−343.

(10) Nayini, N. R.; Markakis, P. Effect of fermentation time on the inositol phosphates of bread. *J. Food Sci.* **1983**, *48*, 262−263.

(11) Türk, M.; Sandberg, A.-S. Phytate degradation during breadmaking: effect of phytase addition. *J. Cereal Sci.* **1992**, *15*, 281−294.

(12) Knorr, D.; Watkins, T. R.; Carlson, B. L. Enzymatic reduction of phytate in whole wheat breads. *J. Food Sci.* **1981**, *46*,1866−1869.

(13) Bergman, C. J.; Gualberto, D. G.; Weber, C. W. Mineral binding capacity of dephytinized insoluble fiber from extruded wheat, oat and rice brans. *Plant Food Hum. Nutr.* **1997**, *51*, 295−310.

(14) Haros, M.; Rosell, C. M.; Benedito, C. Fungal phytase as a potential breadmaking additive. *Eur. Food Res. Technol.* **2001**, DOI 10.1007/S002170100396.

(15) Latta, M.; Eskin, M. A simple and Rapid Colorimetric Method for Phytate Determination. *J. Agric. Food Chem.* **1980**, *28*, 1313−1315.

(16) Konietzny, U.; Greiner, R.; Jany, K.-D. Purification and characterization of a phytase from spelt. *J. Food Biochem.* **1995**, *18*, 165−183.

(17) Tanner, J. T.; Barnett, S. A. Methods of Analysis for Infant Formula: Food and Drug Administration and Infant Formula Council Collaborative Study, Phase III. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 777−785.

(18) Collar, C.; Armero, E. Texture properties of formulated wheat doughs. Relationship with dough and bread technological quality. *Eur. Food Res. Technol.* **1997**, *204*, 136−145.

(19) McCleary, B. V.; Sheenan, H. Measurement of cereal α-amylase: a new assay procedure. *J. Cereal Sci.* **1987**, *6*, 237−251.

(20) Sirou, Y.; Lecommandeur, D.; Lauriere, C. Specific enzymatic micro-assays of α-amylase and *β*-amylase in cereals. *J. Agr. Food Chem*. **1990**, *38*, 171−174.
(21) Rosell, M. C.; Haros, M.; Escrivá, C.; Benedito de Barber, C. Experimental approach to optimize the use of α-amylases in breadmaking. *J. Agric. Food Chem*. **2001**, *49*, 2973−2977.
(22) Kruger, J. E.; Reed, G., Enzymes and Color. In *Wheat Chemistry and Technology*, Pomeranz, Y., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1988; pp 465−466.
(23) Reinhold: J. G.; Parsa, A.; Karimian, N.; Hammick, J. W.; Ismail-Beigi, F. Availability of zinc in leavened and uptake by rat intestine in vitro. *J. Nutr*. **1974**, *104*, 976−982.
(24) McKenzie-Parnell, J. M.; Davies, N. T. Destruction of Phytic Acid During Home Breadmaking. *Food Chem*. **1986**, *22*, 181−192.
(25) Türk, M.; Carlsson, N.-G.; Sandberg, A.-S. Reduction in the levels of phytate during wholemeal breadmaking; effect of yeast and wheat phytases. *J. Cereal Sci*. **1996**, *23*, 257−264.
(26) Tangkongchitr, U.; Seib, P. A.; Hoseney, R. C. Phytic Acid. III. Two barriers to the loss of phytate during breadmaking. *Cereal Chem*. **1982**, *59*, 216−221.
(27) Harland, B. F.; Frölich, W. Effects of phytase from three yeasts on phytate reduction in Norwegian whole wheat flour. *Cereal Chem*. **1989**, *66*, 357−358.
(28) Nayini, N. R.; Markakis, P. The phytase yeast. *Lebensm.-Wiss. Technol.* **1984**, *17*, 24−26.
(29) Schwimmer, S. Enzymes and Their Action in Foods as Health and Safety Benefits. In *Source Book of Food Enzymology*, AVI Publishing Company, Inc.: Westport, CT, 1980; pp 650−666.
(30) Cheryan, M. Phytic acid interactions in foods systems. *CRC, Crit. Rev. Food Sci. Nutr*. **1980**, *13*, 297−335.
(31) Cawley, R. W.; Mitchell, T. A. Myoinositol hexaphosphate as a potential inhibitor of α-amylase. *Phytochemistry* **1968**, 17, 201−204.

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