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Phytase Activity of Wheat, Barley and Corn Grains during Germination and Effect of Germinated Grain Flour Addition on Bread Quality

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ABSTRACT

Phytase activity (PA) in germinated wheat, barley and corn grains and the effect of germinated grain flour addition (rate of 1% and 3%) on bread quality were determined. Five-day germination resulted in maximum phytase activity (MPA) in wheat (0.226 U/mg) and barley (0.263 U/mg) grains, whereas the lowest in corn kernels (0.108 U/mg). 1% and 3% addition of germinated-wheat flour with MPA (5 day germinated) to dough yielded PA of 0.038 U/mg and 0.106 U/mg, respectively. It was observed that 1% and 3% addition of germinated-wheat flour to dough increased PA at the end of fermentation period, which is the final step in bread making, to 0.218 and 0.315 U/mg, respectively. 3% addition of 5-day germinated-wheat flour into the bread dough significantly influenced the fermentation period, bread hardness and color properties of bread crumb. In conclusion, bread quality can be improved by the addition of 3% 5-day germinated-wheat flour to bread dough without any sacrifice from the organoleptic quality of bread.

Key Words: Phytase, Enzyme activity, Bread quality, Wheat, Barley, Corn

Çimlendirme Sırasında Buğday, Arpa ve Mısır Tanelerinde Fitaz Aktivitesi ve Çimlendirilmiş Tahıl Unu Kullanımının Ekmek Kalitesi Üzerine Etkisi

ÖZET

Çimlendirilen buğday, arpa ve mısırda bulunan fitaz aktivitesi ve çimlenen tanelerden elde edilen unların %1 ve %3 oranlarında kullanılmasının ekmek kalitesine etkileri belirlenmiştir. Maksimum fitaz aktivitesi 5 gün çimlendirme sonucunda buğdayda 0.226 U/mg ve arpada ise 0.263 U/mg'dır. En düşük fitaz aktivitesi mısırda 0.108 U/mg'dır. Maksimum fitaz aktivitesine sahip çimlendirilmiş (5 gün) buğday ununun %1 ve %3 oranlarında ilave edilmesi ile üretilen hamurun fitaz aktiviteleri sırasıyla 0.038 U/mg ve 0.106 U/mg'dır. %1 ve %3 çimlenmiş buğday unu katkılı hamurların ekmek üretiminde son aşama olan son fermantasyon basamağındaki fitaz aktivitelerinin sırasıyla 0.218 U/mg ve 0.315 U/mg'a arttığı görülmüştür. 5 gün çimlendirilen buğday unu ekmek hamuruna %3 oranında eklendiğinde ekmek sertliği ve ekmek içi rengi önemli düzeyde etkilenmiştir. Sonuç olarak çimlenmenin beşinci gününde maksimum fitaz aktivitesi gösteren buğdaydan elde edilen ununun %3 oranında ekmek hamuruna eklenmesi ekmeğin organoleptik kalitesini bozmaksızın ekmeğin kalite kriterlerinden bazılarını arttırabilmektedir.

Anahtar Kelimeler: Fitaz, Enzim aktivitesi, Ekmek kalitesi, Buğday, Arpa, Mısır

INTRODUCTION

Cereals are essential foods in a large part of the world and are a major source of minerals; therefore, it is important that the minerals from these products have a high bioavailability. The intake of large amounts of foods rich in phytate may cause mineral deficiency symptoms. However, the bioavailability of the minerals in cereals

improves if phytate is degraded by phytase [1]. Reduction of phytate by phytase may increase the bioavailability of minerals in bread while maintaining its organoleptic quality [2].

Phytates can chelate minerals such as zinc, iron, calcium and magnesium, exhibiting anti-nutrient activity. Addition of phytase to foods can improve the nutritional value of plant-based foods [3]. It enhances the reduction of phytate during food processing such as soaking, grinding, malting, fermentation, heat treatment and germination [4]. Not only does phytate bind minerals in the food in which it originates, but in a meal it can bind minerals from other foods that do not contain phytate. Moreover, it has the ability to chelate endogenously secreted minerals that circulate in the gut as a part of the digestive process [2] .

Biological role of phytate in grains was reported as a phosphorous store, an energy store and an activator of dormancy of grains [5] . Phytase can be found in grains such as wheat, barley and oat, and biochemical properties of the phytase can change depending on its original source.

Phytases (myo-inositol hexakiphosphate phosphohydrolases) have been studied intensively in the last few years because of its application in reducing phytate contents of animal feeds and foodstuff [6]. Phytase catalyzes the hydrolysis reaction of phytic acid (myo-inositol 1, 2, 3, 4, 5, 6 hexakisphosphate). There are two types of phytases; 3- phytase (EC 3.1.3.8) from microorganisms and 4- or 6-phytase (EC 3.1.3.26) from plant phytases [7, 8] .

Primary objectives of our study were to purify and characterize phytases from different grains and to determine maximum phytase activity (MPA) in wheat, barley and corn grains during germination. The effects of adding flour with MPA to bread dough, mixing and fermentation on phytase activity (PA) and bread quality characteristics were also evaluated.

MATERIALS and METHODS

Materials

Grains (wheat (*Tr.durum*), barley (beyaz arpa) and corn (pioneer 32K61)) used in this study were purchased from a local store in Denizli, Turkey. Grains were germinated for 5 days and then dried in an incubator at 42°C for four hours. Dried grains were ground with a grinder to obtain flour with MPA. This flour was added to bread formulae at the levels of either 1% or 3%. Wheat flour of Type 650 (Denizli Un A.Ş, Denizli, Turkey) was used as control, and compressed yeast (Pakmaya, İzmit, Turkey) and salt (Horoz Tuz, Denizli, Turkey) were purchased from a local market. All chemicals were of analytical grade, and 0.1% Tween-80, 0.75% H_2O_2, 100 mM Na-acetate pH 5.0, acetone, ammonium sulfate, H_2SO_4 were purchased from Merck, 0.5% NaOCl, ammonium molybdate from Prolabo, phytic acid dodecasodium salt from Aldrich, bovine serum albumin (fraction 5) from Merck (Germany), citric acid from Surechem, Sephadex G-100 from Sigma-Aldrich (Germany) and phytase from Novenzymes (Denmark).

Methods

Seed Germination

Grains (wheat, barley and corn) were soaked in 0.1% Tween-80 for five minutes, followed by 0.5% NaOCl for 2 minutes and 0.75% H_2O_2 for a minute. After soaking, grains were rinsed with sterile water thoroughly. Grains were then allowed to germinate in sterile boxes at 20 ºC under dark. Grains were rinsed with sterile water once a day. At the end of a 5-day period, water was removed completely [9] .

Extraction of Phytase

Enzymatic extracts for flour with MPA were prepared according to a method reported by Konietzny *et al.* [9] centrifugation time was extended with slight modification. Cell debris was removed by centrifugation at 11,000g for 30 min. Acetone at -20 ºC was slowly added to the supernatant and stirred yield a final concentration of 55% (v/v). After solution was stirred for an hour, centrifugation at 11,000*g* for 30 min was carried out. Precipitate was then suspended in buffer by stirring continuously. Any insoluble material was removed by centrifugation. Acetone fraction was used to precipitate ammonium sulfate at 40-80% saturation. Phytase was obtained against 50 mM of the buffer sodium acetate, pH 4.5. Afterwards, phytase-containing fractions were loaded onto a Sephadex G-100 column (3.5x20 cm) equilibrated with 50 mM sodium acetate, pH 5.5. Flow rate in the column was between 150-200 mL/h. All operations were carried out at about -4 ºC. Column was washed with 300mL of the same buffer. Then, proteins bound to column material were eluted with a linear gradient from zero to 0.6 M NaCl (2L) in 50mM sodium acetate, pH 5.5. Absorbances of enzyme fractions were measured at 280 nm by a Shimadzu UV-1601 brand spectrophotometer (Shimadzu Scientific Instruments, Inc., Tokyo, Japan).

Determination of Phytase Activity

Total protein concentration was determined in duplicates by the Coomassie blue G-250 dye binding assay with bovine serum albumin as a standard [20]. Ammonium molybdate method was used to determine PA. Any cloudiness in solutions was removed by centrifugation prior to absorbance measurement at 355 nm. In order to calculate the enzyme activity, a calibration curve was produced over a range of 5 to 550 nmol phosphate. One unit (U) of activity was equivalent to 1µmol phosphate liberated per min [9] .

Bread Making Experiments

Breads were prepared according to AACC-10/10 method with slight modification [10] [11] in a pilot plant facility of the Department of Food Engineering, Pamukkale University, Denizli, Turkey. Bread making formulas are shown in Table 1.

All of these ingredients were mixed in a blender (Arzum Ev Aletleri, Istanbul, Turkey) with its dough preparation utility for 10 minutes at maximum speed. Fermentation was carried out in an apparatus attached to an oven with vapor injection (PFS 9, Ozkoseoglu, Istanbul, Turkey). In this apparatus, temperature and relative humidity knobs were set to 30ºC and 95% in the final proofing, respectively. Fermentation ended when the height of geometrical central of the dough reached to 1.5 cm (proof time). Then, dough samples were baked for 30 min at 200±5ºC inside the oven with vapor injection. Then, bread samples were cooled down to room temperature. Bread weight, volume [12], specific volume [determined by the ratio of bread volume to weight (cc/g)], crumb cell structure, texture and hardness were measured after 24 h and 72 h of storage [13]. Colors of bread crust and breadcrumbs were measured by Miniscan XE Type Hunter-Lab (Reston, Virginia, USA) according to a method described by Aurand et al. [14]. Color values were recorded as L (darkness/lightness), a (greenness to redness), and b (blueness to yellowness). Duplicates of experiments with two measurements were run for each of bread. Bread crumb cell structures and textures were evaluated by panelists manually. A panel of 20 subjects in the Department of Food Engineering (Pamukkale University, Denizli, Turkey) evaluated the sensory properties of breads, and gave scores for color, crumb cell structure and textures of bread samples on a hedonic scale from 1 (dislike extremely) to 9 (like extremely). The panel consisted of students, staff and faculty members (12 males, 8 females). Duplicates of experiments with two measurements were run for each treatment.

Table 1. Formulas for bread making experiments[1]

Ingredient	Control	1% Wheat	3% Wheat	1% Barley	3% Barley	1% Corn	3% Corn
Flour	300	300	300	300	300	300	300
Water	159	159	159	159	159	159	159
Salt	6	6	6	6	6	6	6
Yeast	6	6	6	6	6	6	6
1% Grains[2]	-	3	-	3	-	3	3
3% Grains	-	-	3	-	3	-	3

[1]all ingredients were added in flour basis (g)
[2]flours were obtained from grains germinated for 5 days

Statistical Analysis

Data were analyzed using MSTAT-C [15] . The choice of experimental design was a complete randomized design with two factors, germinated grain type and additional levels of germinated grain flour to dough samples. Post-ANOVA analysis was performed using Duncan's multiple comparison test to determine significant differences among means at α levels either 0.10, 0.05 or 0.01.

RESULTS and DISCUSSION

Grains that are germinated for MPA and purified are summarized in Table 2. PA, detected germinated wheat grains , increased linearly until 5 day and reached a MPA on 5 days of germination (0.226 U/mg), afterwards slightly decreased. PA in wheat, barley and corn during germination is shown in Figure 1. Konientzy et al. [9] also reported PA of 0.02 U/g grain in ungerminated-spelt grains and MPA of 1.10 U/g grain in five-day-germinated-grains. In our study, we observed MPA 0.263 U/mg in wheat grains germinated for five days, which is similar to that reported by Konientzy et al, [9]. We found that PA in five-day germinated all grains was higher than those germinated for 3 or 7 days. Greiner et al. [16] reported MPA in barley grains at the end of the 4th day of germination and Chang [17] reported MPA in corn kernels after 4 days or germination.

After grains were sprout for five days, phytase was purified through four steps; centrifugation at 11,000*g*, precipitation in acetone, precipitation in ammonium sulfate and elution through a column of Sephadex G-100. In every purification step, optical density (OD), total protein content, specific activity, recovery and purification coefficients were determined (Table 3). As it can be seen from Table 3, PA of wheat and barley samples at the end of the purification step was higher than that of corn samples. This means that liberated phosphate amount of corn seeds higher than that of the wheat and barley. PA in wheat and barley samples at the final stage of purification is similar to that reported by Greiner et al. [16] and Konietzny et al. [9].

Five-day-germinated wheat, barley or corn samples were ground and added into bread dough at either 1% or 3% on the weight bases of flour in the formula. PA in dough samples after the mixing stage is shown in Table 3. According to this table, PA is higher in dough samples with 1% germinated-grain flour than control. In addition, MPA was observed in dough samples with 3% germinated-wheat flour.

Phytase in dough samples of the final stage of proof time was purified, and its activity at the stages of both mixing and final proof time was determined. Therefore, changes in PA from mixing to final proof time could be compared. Table 4 shows PA after final proof fermentation in dough samples with either 1% or 3% germinated-grain flour. PA of dough samples with 1% germinated-grain flour was higher than that of control samples based on its specific activity shown in Table 4. We observed MPA evolution in dough sample with 3% germinated-wheat flour.

Table 2. Purification scheme for germinated grains with maximum phytase activity.

Type of Grain	Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Recovery (%)	Purification Coefficient
Wheat	Centrifugation at 11,000*g*	14.40	1.89	0.13	100.0	1.00
	Acetone precipitation	23.71	3.42	0.14	180.0	1.09
	$(NH_4)_2SO_4$ precipitation	0.79	0.02	0.03	1.1	0.19
	Elution through Sephadex G-100	0.03	0.01	0.23	0.4	1.72
Barley	Centrifugation at 11,000*g*	16.52	1.76	0.11	100.0	1.00
	Acetone precipitation	17.81	2.32	0.13	131.8	1.20
	$(NH_4)_2SO_4$ precipitation	0.68	0.07	0.10	4.0	0.96
	Elution through Sephadex G-100	0.21	0.05	0.26	3.1	2.48
Corn	Centrifugation at 11,000*g*	14.30	1.47	0.10	100	1.00
	Acetone precipitation	11.52	0.68	0.06	44.6	0.55
	$(NH_4)_2SO_4$ precipitation	1.81	0.04	0.02	2.4	0.18
	Elution through Sephadex G-100	0.25	0.03	0.11	1.8	1.05

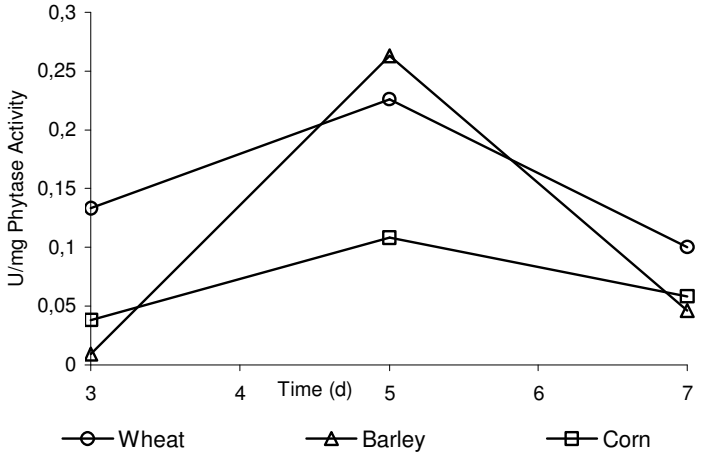

Figure 1. Phytase activity during germination of wheat, barley and corn.

Table 3. Phytase activity and protein amount of dough samples after mixing bread dough

Treatment		Total Protein (mg)*	Total Activity (U)*	Specific Activity (U/mg)*
Control		0.772 ± 0.047	0.001 ± 0.000	0.001 ± 0.000
Wheat	1%	0.232 ± 0.020	0.009 ± 0.002	0.038 ± 0.005
Barley		0.728 ± 0.021	0.007 ± 0.036	0.010 ± 0.003
Corn		0.761 ± 0.032	0.002 ± 0.001	0.002 ± 0.001
Wheat	3%	0.088 ± 0.010	0.009 ± 0.002	0.106 ± 0.022
Barley		0.417 ± 0.003	0.003 ± 0.001	0.007 ± 0.020
Corn		0.514 ± 0.056	0.004 ± 0.002	0.008 ± 0.002

*Values are average ± SD for 2 replications

Table 5 exhibits the effect of variation sources on final proof time, specific volumes of breads, crumb cell structure, crumb texture, crust color, crumb color or crumb hardness. Differences according to the analysis of variance results for in the final proof times of bread samples were found significant ($P<0.05$). The effect of germinated-grain types and their amounts added to dough samples on specific volumes, crumb cell structures, crumb texture and crust colors of bread samples was found statistically insignificant ($P>0.05$). On the other hand, crumb colors and crumb hardness after 72-hour-storage period were effected by the addition of flours from germinated-grains to dough samples ($P<0.05$).

Duncan's multiple comparison test was performed as a mean separation technique. The results are shown Table 6 and Table 7. Flour of barley grains yielded shorter proof time than corn flour. There was insignificant difference between the proof times for bread samples of wheat and corn. Hunter-Lab Color *b* value of corn was the highest among the samples, meaning that bread crumb from germinated-corn kernels was the yellowest in color (Table 7).

Table 4. Phytase activity and protein amount of dough samples after final proof time

Sample	Level	Total Protein (mg)*	Total Activity (U)*	Specific Activity (U/mg)*
Control		0.224 ± 0.055	0.001 ± 0.005	0.004 ± 0.002
Wheat	1%	0.125 ± 0.024	0.016 ± 0.002	0.128 ± 0.073
Barley	1%	0.267 ± 0.049	0.005 ± 0.003	0.018 ± 0.008
Corn	1%	1.523 ± 0.300	0.019 ± 0.004	0.012 ± 0.008
Wheat	3%	0.019 ± 0.007	0.006 ± 0.003	0.315 ± 0.137
Barley	3%	0.185 ± 0.026	0.011 ± 0.003	0.059 ± 0.029
Corn	3%	0.391 ± 0.010	0.014 ± 0.004	0.035 ± 0.007

*Values are average ± SD for 2 replications

Table 5. Analysis of variance results for bread making experiments

Source	Variables											
	Final Proof Time	Specific Volume	Crumb Cell	Crumb Texture	Crust Color			Crumb Color			Crumb Hardness	
					L	a	b	L	a	b	24h	72h
Grain type (A)	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Addition levels (B)	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns
A x B	ns	ns	ns	ns	ns	ns	ns	**	***	**	ns	**

*, ** and *** mean significantly differ at P< 0.10, P< 0.05 and P< 0.01 respectively; ns: not significant.

Table 6. Duncan's multiple comparison test for germinated grains*

Type of Grain	Proof Time	Crumb Color *b* Value
Control	58.02 ^{ab}	19.32 ^{b}
Wheat	56.33^{ab}	19.33^{b}
Barley	53.50^{b}	19.65^{b}
Corn	60.33^{a}	20.43^{a}

*Same letters within a column express statistical insignificance

Addition of germinated grain flour to bread dough decreased final proof time (P<0.05). While addition of 3% germinated-grain flour decreased crumb color *L* value by two units, it increased *b* value of crumb color by a unit (P<0.05, Table 7).

Table 7. Duncan's multiple comparison tests for percent addition of germinated grain to bread dough*

Percent Addition	Crumb Color *L* Value	Crumb Color *a* Value	Crumb Color *b* Value	Hardness after 72h
0	79.42^{a}	0.96^{b}	19.12^{b}	10.50^{b}
1	77.05^{ab}	1.10^{b}	20.01^{ab}	12.17^{ab}
3	76.09^{b}	1.36^{a}	20.29^{a}	14.50^{a}

*Same letters within a column express statistical insignificance

During the experiments of bread making, we also observed small but visually visible color differences among bread samples with added germinated-grain flour. Addition of 3% germinated-grain flour increased color *a* value of crumb, and breads of this flour were high in yellow color intensity compared to control or breads of addition of 1% germinated-grain. Crumb hardness was also effected by the addition of germinated-grain flour to bread dough (P<0.05). Higher the hardness values, softer the bread samples. Bread samples with 3% germinated-grain flour were softer compared with control. However, the difference in hardness values of bread samples with 1% and 3% germinated-grain flour was insignificant. Structures of bread samples prepared with three types of grain flour and three addition levels of germinated-grain flour are shown in Figure 2.

CONCLUSIONS

In this study, we found that PA on the fifth day of germination for wheat and barley grains is higher than corn kernels, and differences in PA between wheat and barley grains are statistically insignificant. The addition of germinated wheat grains to bread dough shortened proof time significantly, in comparison to germinated-corn kernel addition. Corn bread samples had higher yellowness in crumb color (*b*) than wheat or barley bread samples. Addition of 3% germinated-grain flour increased color *a* and *b* values of crumb color while reducing color *L* value compared with samples without germinated-grain flour.

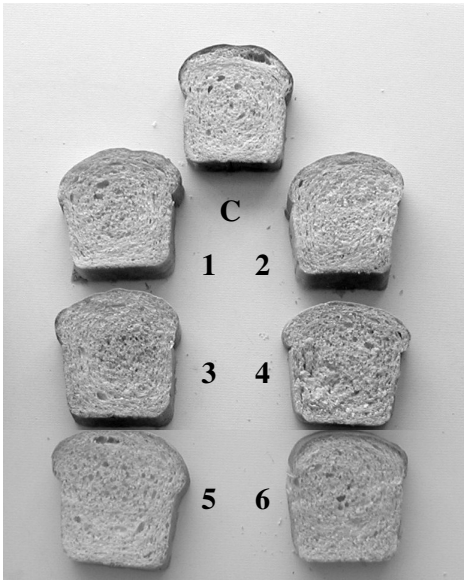

Figure 2. Breads with 1% or 3% germinated wheat, barley or corn flour. C: control, 1: with 1% germinated wheat flour, 2: with 3% germinated wheat flour, 3: with 1% germinated barley flour, 4: with 3% germinated barley flour, 5: with 1% germinated corn flour, 6: with 3% germinated corn flour.

Due to several health and demographic problems such as elevated cholesterol levels in plasma and aging population, common cereal products sold in markets are enriched with fiber. However, too much consumption of high fiber foods may lead to several problems such as anemia because of the presence of phytate, a metal chelator. Phytate has the ability to make minerals unavailable for absorption in the body. However, much of the phytate in dough is hydrolyzed during bread baking due to PA [18] [19]. Minerals, vitamins and dietary fiber are present in flours together with phytate, and mechanical removal of phytate lowers the nutritive values of grains as well as organoleptic quality of bread. In order to retain organoleptic and nutritive quality of bread, enzymatic removal of phytate can serve as an alternative method. This study appears that the supplementation of wheat and barley phytases to breads may improve the nutritional value of some cereals such as brandy breads in particular. The results of our study suggest that germinating grains for five days yields MPA, and addition of germinated-grain flour to bread dough may improve both nutritional and organoleptic quality of breads.

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