J. Agr. Sci. Tech. (2011) Vol. 13: 1065-1076

Evaluation of the Effect of Fermentation, Hydrothermal Treatment, Soda, and Table Salt on Phytase Activity and Phytate Content of Three Iranian Wheat Cultivars

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ABSTRACT

This study was carried out to determine the effect of fermentation, hydrothermal treatment, soda (Na_2CO_3), and table salt (NaCl) addition on the extent of phytase activity and phytate degradation in three Iranian wheat cultivars, namely, Mahdavi, Ghods, and Roshan. The samples were milled to three different extraction rates, i.e. whole, 85%, and 75% flours and three kinds of leavening procedure (fermented, soda, and control), and four NaCl percentages (0.0, 0.5, 1.0, 1.5%) were used for preparing dough in three replications. To evaluate the effect of heat treatment on phytic acid breakdown, baking was also done. The results indicated that among the wheat varieties, Mahdavi had the highest level of phytase activity and phytic acid content followed by Ghods and Roshan; in which, most of the phytate was concentrated in bran fractions. Fermentation (1% yeast at 37ºC for 3 hours), hydrothermal treatment (pH 4.8 at 55ºC for 12 hours) and salt addition (0 to 1.5%) to the dough samples resulted in an increased phytase activity, whereas soda addition (1%) decreased the enzyme activity. Heat treatment reduced phytic acid content significantly.

Keywords: Fermentation, Hydrothermal, Phytase, Phytate, Soda, Table salt**.**

INTRODUCTION

Phytate is typically found in the outer (aleuron) layers of cereal grains and in the endosperm of legumes and oil seeds constituting approximately 1-3% of their dry weight (Graf, 1983; Bohn *et al.,* 2004). Although there are reports on phytate beneficial effects such as anticancer properties (Shamsuddin and Vucenik, 1999), its typical negative effect is to form a complex with multivalent metal ions, especially zinc, calcium, and iron. This binding can result in the formation of very insoluble salts with poor bioavailability of the minerals. (Reddy, 1982; Wu *et al.,* 2009). Phytic acid is hydrolyzed enzymatically by phytases, or chemically, to lower inositol phosphates during storage, fermentation, germination and food processing. Various food processing methods such as soaking, malting, and fermentation activate the endogenous phytase that catalyze the stepwise hydrolysis of phytic acid, while processing methods such as heat treatments i.e. blanching, baking, autoclaving, and frying, cause autolysis of phytic acid (Servi *et al.*, 2008; Garcia-Estepa *et al*., 1999). Phytase enzymes, widely present in organisms such as plants, microorganisms, and animal cells enhance minerals availability (Lonnerdal, 2002; Konietzny *et al.,* 2002) and release of phosphorus. Phytate is susceptible to degradation during the fermentation of bread dough (Giovanelli *et al*., 1994) that involves phytase activity of endogenous enzymes, naturally present in cereals, or microbial

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[DOR: 20.1001.1.16807073.2011.13.7.7.2]

enzymes (yeasts and/or lactic acid bacteria naturally present in flour or added as starter). (Reale *et al.,* 2004; Fretzdorff *et al*., 1992). Phytase enzyme from various origin have different optimal pH and temperature; for example, an optimum pH of 5.5 at 55°C has been reported for wheat phytase (Leenhardt, *et al.*, 2005); whereas optimum temperature and pH for rye phytase is 45°C and 6.0, respectively (Greiner *et al.,* 1998). Microbial phytases have lower pH-optima between 4.0 and 5.0 (Vohra and Satyanarayana, 2003).

Up to now, the effect of different food processing in reducing phytic acid content and, thereby, increasing mineral availability has been evaluated. Leenhardt *et al.* (2005) and Harland and Harland (1980) reported that a fermentation time of 4 hr or more would be necessary to reduce the phytate content of whole wheat sponges to a nutritionally acceptable level. Bahrami and Shahedi (2004) used 4 h fermentation with 2.5% yeast for preparation of Sangak and Lavash breads and indicated considerable reduction in phytic acid content. On the other hand, application of soda has negative effects on phytase activity. Soda (sodium bicarbonate) is a white crystal powder that begins to lose CO_2 at about 50ºC and it is converted into Na_2CO_3 at 100ºC. Although the process would be fast, it bears detrimental effects on bread taste and human health. Faridi *et al.* (1983) reported that supplementing 0.2-0.4 % soda invariably decreased the rate of phytate hydrolysis. Baking is another process used during bread making and its influence on phytic acid content was studied by Jamalian and Sheikhol-Eslam (2004). Hydrothermal treatment of whole grain was historically practiced to facilitate dehulling and it has been reported to have a potentially positive influence on the nutritional value of cereals by reducing hexa and penta inositol phosphates to inorganic phosphates and lower inositol phosphates (Fredlund *et al.*, 1997; Urbano *et al*., 2003). Mosharraf *et al.* (2009) indicated significant reduction in phytic acid content of Sangak bread after hydrothermal treatment of bran. Consumption of bread made from flours with high extraction rates has recently become popular in Iran. Such breads contain relatively high levels of phytic acid. As a consequence, if the consumer eats a marginal diet in essential minerals, the phytate may lead to a nutritional deficiency. Therefore, the aim of this study is to peruse the effect of different processes and compounds in phytase activity during bread making while the possibility of using hydrothermal process for reduction of phytic acid in bran will be also studied.

MATERIALS AND METHODS

Dough Preparation

The experimental material consisted of three important common fall white wheat cultivars (*Aestivum triticum)*, namely, Mahdavi, Ghods, and Roshan selected based on their highest production in Isfahan Province. Isfahan Wheat Research Station provided the cultivars that they claimed had been grown under identical conditions in 2005-6 crop season. Grains after harvest were conditioned to 14% moisture content and milled to flour using an experimental Brabender Quadrumat Sr Mill (Duisburg, Germany) to obtain bran and wheat flour with extraction rates of 100%, 85%, and 75%.

The corresponding dough including the control (flour, water), fermented (flour, water, 1% yeast) and chemically leavened dough (flour, water, 1% soda) were prepared by adding 68, 60, and 55 ml water to 100 g flours with 100%, 85%, and 75% extraction percentage, respectively, based on water absorption of these flours as determined by Farinograph E (Duisburg, Germany) standard method of 54-21, (AACC, 2003). For the evaluation of salt effect on phytase activity, four levels of table salt (0, 0.5, 1, and 1.5% based on flour weight)were added to the fermented dough. Each dough was

[DOR: 20.1001.1.16807073.2011.13.7.7.2]

then kneaded by hand for 15 minutes at room temperature. Dough containing 1% dry instant yeast from Klarmaye, Isfahan (based on flour weight) was fermented for 3 hours at 37ºC and 90% relative humidity, whereas the dough containing 1% soda (based on flour weight) was left for 40 minutes at room temperature.

Bread Preparation

Tafton bread was prepared by hand mixing of flour, water, yeast, and NaCl. The dough was then again kneaded by hand for 15 minutes after being fermented for 90 minutes at 34ºC and 90% R.H. 100 g pieces were sheeted to 2.5 mm thickness and 16 cm diameter in a flat bread molder. Molder dough was transferred onto lightly floured stainless steel sheet and punctured to prevent puffing. The process was repeated for each sample. Dough pieces were then transferred onto a preheated oven for 3 minutes at 280ºC. Afterwards, the bread was allowed to cool on racks for 30 minutes and then evaluated.

Phytase Extraction

Half a gram of each milling fraction was homogenized in 5 ml (1:10 w/v) of 50 mM sodium acetate buffer, pH 5.3 employing a power Gen 700 homogenizer at a speed setting of 5 for 1 minute while the sample tube was immersed in ice. The homogenates were soaked over night in a refrigerated cabinet (8ºC) with gentle shakings. The extracts were subsequently centrifuged at 4,000g at 4ºC for 30 minutes. The clear supernatants were collected and re-centrifuged at 15,000g at 4ºC for 30 minutes. Aliquots with known amounts of protein were used for the determination of phytase specific activity. Xylanase was also added as an extraction aid to the acetate extraction buffer before commencing the extraction process. The buffer used in the extraction showed to be optimal for phytase activity and stability. Protein concentration in the extracts was determined using the method of Bradford (1976).

Determination of Phytase Activity

After preparation of all dough samples, they were frozen and dried at 25 mm Hg^{-1} in a freeze drier (Hetoholten, Denmark) up to 7% moisture content and, then, phytase activity was measured. Phytase specific activity was determined colorimetrically (Kilmer *et al*., 1994). Briefly, an aliquot of the clear supernatant (500 µg of protein) had been predetermined to give a linear initial velocity of the reaction. This was mixed with an equal volume of 5 mM sodium phytate as substrate containing 2 mM magnesium chloride in 50 mM sodium acetate buffer at pH 5.3 and incubated at 50ºC for 30 minutes in a shaking water bath. The enzyme activity was stopped by adding an equal volume of 20% aqueous trichloroacetic acid (TCA). The mixture was allowed to stand in cold water for 2 h to precipitate out proteins before centrifugation at 10,000xg for 30 minutes to remove the precipitate. The controls were treated likewise, but the enzyme was inactivated by adding an equal volume of 20% TCA prior to addition of the substrate. An aliquot of the final supernatant was then assayed for released inorganic orthophosphate essentially as described and modified by Nahapetian and Bassiri (1975). FTU is defined as the amount of enzyme that releases one µmol of orthophosphate min^{-1} at 50ºC and pH 5.3. The assay was run in three replicates.

Determination of Phytate

For phytate determination, two grams of the ground sample was extracted with 40 ml of 1.2% HCl containing 10% Na_2SO_4 for 2 hours on a shaker with circular movement. The solution was allowed to stand overnight and shaken again for 1 hour the following

[DOR: 20.1001.1.16807073.2011.13.7.7.2]

day. The extract was centrifuged at about 650g for 15 minutes. Five ml of cleared extract was added to five ml of distilled water and, after adding 6 ml of 4% $FeCl_3.6H_2O$ in .07N HClsolutions, it was mixed and heated in a boiling water bath for 15 minutes. The precipitated ferric salt of phytate was isolated by centrifugation at 4,000g for 15 minutes. The precipitate was washed with 5 ml of 4% Na_2SO_4 in .07N HCl solution. After centrifugation at 4,000g for 15 minutes, the precipitate was dissolved in 3 ml of concentrated H_2SO_4. One ml of the solution with 1 ml of 65% HNO_3 and 1 ml of distilled water were transferred into a Kjeldahl flask of 30 ml capacity and, after addition of three glass beads, the solution was left over night at room temperature and wet digestion was carried out the next day. Five ml of distilled water was added to the digest with care while the digest was still warm, then, it was heated in a boiling water bath for 15 minutes to destroy pyrophosphate. The solution was diluted to 100 ml and inorganic P was determined after addition of a color complex (mixture of ammonium vanadate and ammonium hepta molibdate in perchloric acid) by reading absorbance at 420 nm. Two ml of KH_2PO_4 solution containing 3.2×10^{-3} mg of P was used for making standard samples (Nahapetian and Bassiri, 1975).

Hydrothermal Treatment

In hydrothermal treatment (Fredlund *et al*., 1997) the bran (300 g) was wet steeped in 2 volumes of acetate buffer (pH= 4.8) at 55ºC until a weight gain of 40 g 100 g^{-1} sample was achieved (*ca*. 60 minutes), then, buffer was exchanged and soaking continued for further 23 hours. Finally, the bran was dried in a convection oven at 40-50ºC for 10 hours.

Statistical Analysis

Statistical analysis of the data for chemical and rheological characteristics was carried out using two and three-way analysis of variance and using Duncan's test separated means. Analysis was done using SAS and MSDAT softwares.

RESULTS AND DISCUSSION

Comparison of Phytase Activity of Bran and Different Flour Extractions

Phytase activity of the wheat flours and the corresponding brans is shown in Figures 1 and 2. It can be seen that there were higher

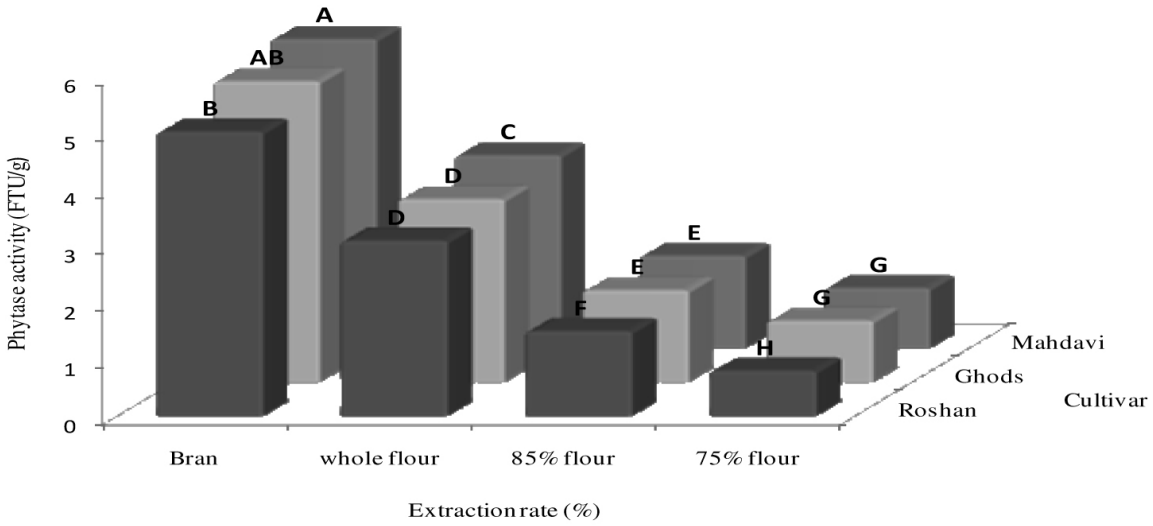

Figure 1. Comparison of phytase activities in bran and different flour obtained from three wheat cultivars.

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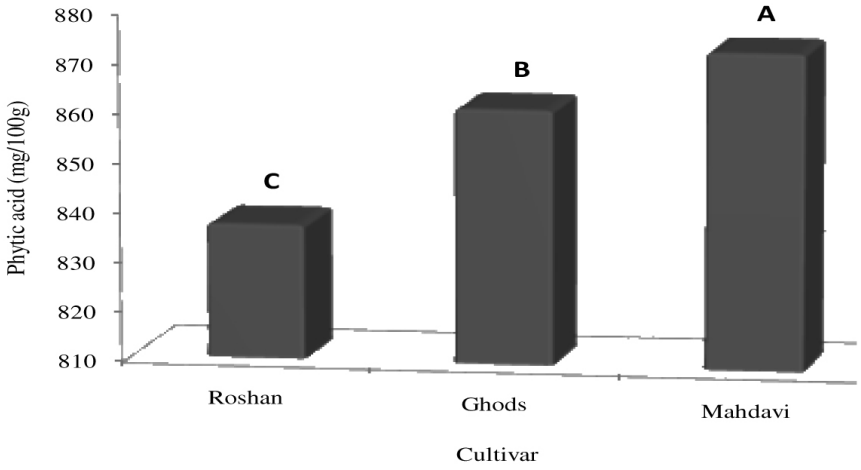

Figure 2. Comparison of phytate content in bran of three wheat cultivars.

phytase activity levels observed in the bran fractions of the cultivars ranging from 5.03 to 5.48 FTU g^{-1}. The results also indicate that by decreasing the extraction rate, the phytase activity also decreases. The average phytase activity in 75% flour was 0.99 FTU g^{-1} and reached 5.21 FTU g^{-1} in bran separated from the flours. This would be a good indication that phytase should be located in the bran section of the seed. However, it is not clear whether the activity observed in the flour fractions was due to the presence of trace amounts of bran in the fractions or that the endosperm itself has enzyme activity. This is consistent with the observation by Okot-Kotber (2003) who reported that bran had higher phytase activity than wheat flour. Also, the result show significant differences among the different cultivars, where the average phytase activity of 2.61 FTU g^{-1} in Roshan increased to 2.94 FTU g^{-1} in Mahdavi cultivar. The phytase content reported in other studies show a wide variability depending on flour yield, extraction method, and flour types. Analysis of variance of the data shows that the differences among the cultivars as well as those among milling fractions were significant ($P< 0.01$) Moreover, interactions between milling fractions and cultivars were also significant ($P< 0.05$).

Effects of Yeast Fermentation and Soda on Phytase Activity

Wheat phytase has an optimum pH of 5.5 (Leenhardt *et al.*, 2005). Phytase activity was significantly higher in dough leavened with yeast than in others (Table 1). On the other hand, the reduction in phytate was significantly higher in dough with added yeast than those without it. During fermentation, the pH of the dough decreases and approaches the optimum pH of endogenous wheat phytase. This shift of pH along with suitability of temperature (37ºC) during fermentation seems to have an exciting effect on phytase activity (Moses *et al*. 2003). Results of Leenhardt *et al.,* (2005) also support this finding that a fermentation process providing a pH value of 5.5 was the optimal condition for endogenous phytase activity. The extent of phytase activity (during fermentation) has been shown to be greater in dough made with whole meal flour than those at lower extraction rates. Phytase activity in dough made from whole wheat, 85%, and 75% flours were higher by 51.5%, 30.3%, and 20.6%, respectively, compared to the control sample (Table 1). The reduction in phytase was significantly higher in dough leavened with added soda than those made with yeast (Table 1).

[DOR: 20.1001.1.16807073.2011.13.7.7.2]

Table 1. Comparison of the effect of leavening procedure on phytase activity in doughs obtained from three wheat cultivars.

Cultivars	Phytase activity (FTU g^{-1}) in dough		
	Fermented with yeast	Leavened with Soda	Control sample*
Roshan			
Whole flour	$3.30^{ab} \pm 0.15$	$2.10^{f} \pm 0.11$	$2.67^{d} \pm 0.11$
85% Flour	$1.48^{gh} \pm 0.10$	$0.55^{o} \pm 0.08$	$1.22^{ij} \pm 0.13$
75% Flour	$1.14^{jk} \pm 0.06$	$0.55^{o} \pm 0.09$	$0.73^{lmno} \pm 0.15$
Ghods			
Whole flour	$3.30^{bc} \pm 0.12$	$2.34^{e} \pm 0.14$	$2.86^{d} \pm 0.15$
85% Flour	$1.91^{f} \pm 0.07$	$0.85^{lmn} \pm 0.10$	$1.43^{ghi} \pm 0.15$
75% Flour	$1.32^{hij} \pm 0.08$	$0.64^{no} \pm 0.07$	$0.86^{lmn} \pm 0.08$
Mahdavi			
Whole flour	$3.58^{a} \pm 0.20$	$2.39^{e} \pm 0.16$	$3.10^{c} \pm 0.20$
85% Flour	$1.96^{f} \pm 0.07$	$0.91^{klm} \pm 0.09$	$1.57^{g} \pm 0.09$
75% Flour	$1.39^{ghi} \pm 0.12$	$0.69^{mno} \pm 0.05$	$0.96^{kl} \pm 0.06$

The same letter in the same row indicates no significant difference ($P< 0.05$)
* Mixture of flour and water without any kind of leavening agent.

Adding soda increases pH of the dough to a higher point far enough from the optimum pH of endogenous phytase so that phytase activity decreases by 33%. This is consistent with the observation by Almana (2000). Faridi *et al.* (1983) also explained that baking soda had a retarding effect on the hydrolysis of phytate. The variance analysis table shows that the differences among different types of dough are significant (P< 0/01). The analysis also shows that the interactions among milling fractions, cultivars, and different dough are also significant (P< 0/01).

Effect of NaCl on Phytase Activity

By increasing the NaCl content, phytase activity also increased. For example, phytase activity in fermented dough prepared from 75% flour of Mahdavi cultivar increased from 0.806 FTU g^{-1} to 1.188 FTU g^{-1} with increasing the amount of salt from 0 to 1.5%, indicating an almost 50% growth in enzyme activity (Table 2). In general, protein solubility is variable and is influenced by the number of polar and apolar amino groups and their arrangement along the molecule. It also depends on pH as well as NaCl concentration in the latter solubility rises with increase in ionic strength. It can, therefore, be concluded that adding NaCl increases phytase solubility and its effect on phytate. Inyang (1996) reported that protein solubility was affected by ionic strength of the dispersing medium and that its activity increased by progressive increase of ionic strength of NaCl. Zhou (2005) also reported the effect of NaCl on solubility of protein and showed that it could increase at low salt concentrations. The variance analysis shows that the differences among doughs with different amounts of NaCl are significant (P< 0.01). The analysis also reveals that interactions among milling fractions, cultivars, and different amounts of NaCl are significant (ρ<0.05).

Effect of Hydrothermal on Phytase Activity

Hydrothermal treatment of whole grains is

Table 2. Comparison of phytase activities in fermented dough containing different amounts of salt.

	Phytase activity (FTU g^{-1})			
	Added Salt			
Cultivars	0.0٪	0.5٪	1.0٪	1.5٪
Roshan				
Whole flour	$3.10^{de} \pm 0.07$	$3.14^{de} \pm 0.11$	$3.30^{cd} \pm 0.47$	3.59 ± 0.09^{b}
85% Flour	$0.93^{q} \pm 0.32$	$1.24^{mnop} \pm 0.25$	$1.61^{hij} \pm 0.16$	$2.112^{g} \pm 0.16^{g}$
75% Flour	$1.03^{pq} \pm 0.09$	$1.07^{opq} \pm 0.05$	$1.19^{nop} \pm 0.10$	$1.27^{lmno} \pm 0.18^{lmno}$
Ghods				
Whole flour	3.06 ± 0.03^{e}	$3.22^{cde} \pm 0.04$	$3.32^{cd} \pm 0.49$	$3.60^{b} \pm 0.45$
85% Flour	1.56 ± 0.34^{hijk}	$1.7^{hi} \pm 0.26$	$2.03^{g} \pm 0.10$	$2.35^{f} \pm 0.23^{f}$
75% Flour	1.16 ± 0.04^{nop}	$1.2^{nop} \pm 0.08$	$1.34^{klmn} \pm 0.16$	$1.57^{hij} \pm 0.21^{hij}$
Mahdavi				
Whole flour	$3.25^{cde} \pm 0.10$	$3.40^{bc} \pm 0.12$	$3.59^{b} \pm 0.66$	$4.07^{a} \pm 0.10$
85% Flour	$1.48^{ijkl} \pm 0.34$	$1.69^{hi} \pm 0.25$	$2.12^{g} \pm 0.17$	$2.55^{f} \pm 0.13$
75% Flour	$1.15^{nopq} \pm 0.16$	$1.24^{mnop} \pm 0.08$	$1.46^{jklm} \pm 0.13$	$1.72^{h} \pm 0.21$

The same letters indicates no significant difference (p<0.05).

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traditionally practiced to facilitate seed dehulling. Phytase is generally inactive in dry cereals but it becomes active when the seeds are moistened. Figures 3 and 4 show that there were higher levels of phytase activity in normal brans (ranging from 5.04 to 5.47 FTU g^{-1}) than in brans subjected to hydrothermal treatment (ranging from 0.02 to 0.009 FTU g^{-1}), along with higher levels of phytate content in normal bran (837.7 to 874 mg 100 g^{-1}) compared with its treated counterparts (124.7 to 130.2 mg 100 g^{-1}). Mosharraf *et al.* (2009) also reported 50-65% reduction in phytic acid content of Sangak bread after hydrothermal treatment of the bran. The much lower activities observed in brans subjected to hydrothermal treatment might be due to enzyme leaching and/or inactivation during drying. On the other hand, a considerable reduction in phytate (85%) might be also due to hydrolysis of phytate by phytase just before enzyme inactivation. This is consistent with the observations of Fredlund *et al.* (1997) and Urbano *et al.* (2003) who reported that, under the experimental conditions, this reduction might be due to the fact that the endogenous phytase is activated, although it could be affected by mild hydrothermal treatment itself.

Phytate Content in Fermented Dough and Bread

Phytate contents of the fermented doughs (containing 1.5% NaCl and 1.5% yeast) made from reconstituted 85% Roshan, Ghods, and Mahdavi flours were, respectively, 217.4, 236.7 and 262.3 (mg/100g). In this regard, brans of corresponding flours were initially cut back added to flours to obtain an 85% extraction rate, which is usually used for baking Tafton bread. Results showed a greater loss of phytate by about 30% after baking (Figure 5). This is consistent with the results of Garcia-Estepa *et al*. (1999) who reported 37% reduction in phytic acid content after baking of dough prepared with whole wheat flour. Almana (2000) observed the same trends for phytate reduction. Also Ter-sarkissian *et al*. (1975) reported 18% loss of phytate after a short fermentation time (1.5 hours) of those made of whole wheat flour followed by 39% losses after baking for 3-4 minutes in the Iranian flat bread baked in villages. Phytic acid is a heat resistant compound and a significant reduction in phytate is not expected after high temperatures treatment due to its association with the cations. However, thermal degradation of phytic acid is accelerated by low pH (Servi *et al*., 2008). It seems that most of phytate reduction occurre during the

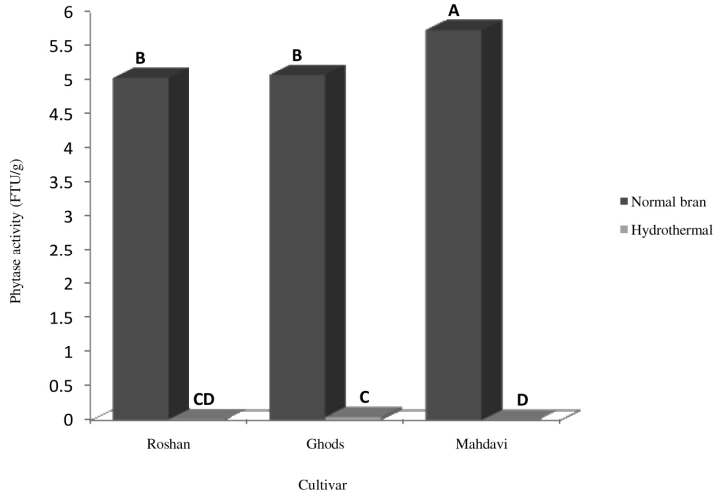

Figure 3. Comparison of phytase activity of normal and hydrothermaled brans obtained from three Iranian wheat cultivars.

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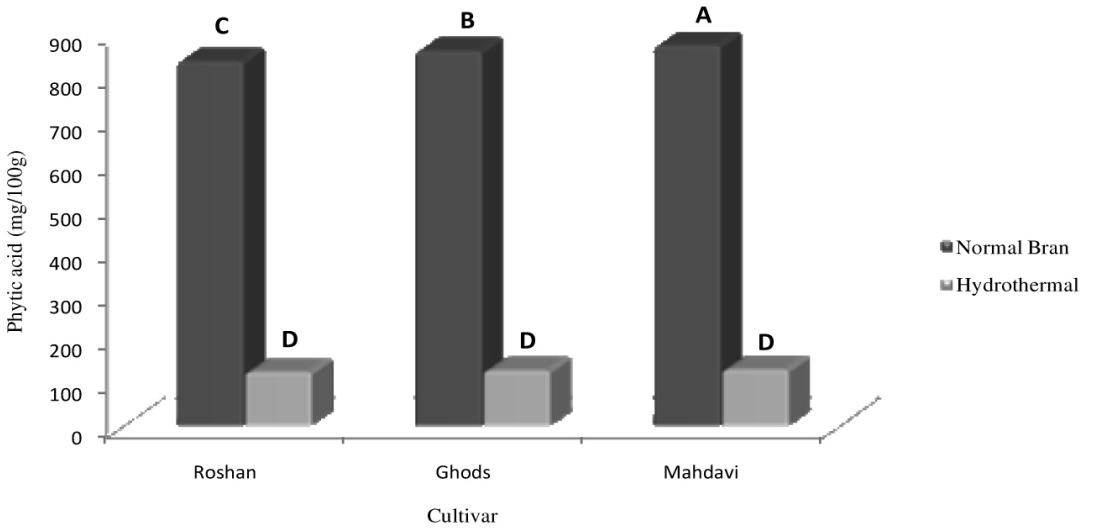

Figure 4. Comparison of phytate content of normal and hydrothermaled brans obtained from three Iranian wheat cultivars.

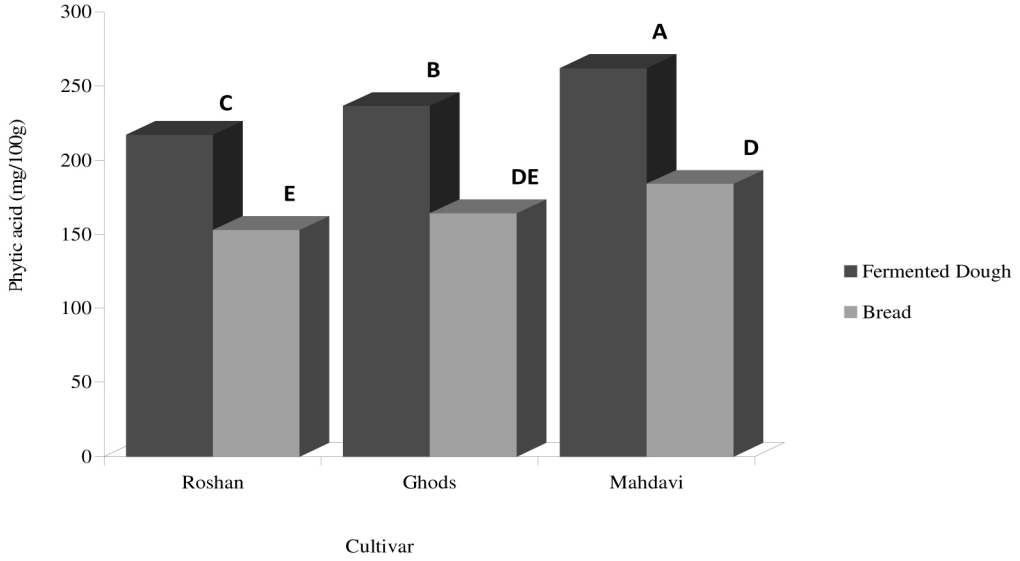

Figure 5. Comparison of phytic acid content in dough and bread obtained from three Iranian wheat cultivars.

early stage of baking when temperature reaches the optimum point for the best phytase activity.

CONCLUSIONS

Results indicate varietal differences in the phytase activity and phytate content between the three cultivars and milling fractions i.e. those with higher phytate content had more phytase activity. Considering their chemical structure, in both components phosphorous has a major role. However, more investigations are needed on more wheat varieties to gain a clearer picture of the phenomenon. Fermentation with reduction of pH and hydrothermal treatment with increasing temperature provided suitable conditions for endogenous phytase activity. NaCl addition had positive effects on phytase solubility and phytate reduction as well as heat treatment that could lessen phytate content, whereas adding soda

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showed a negative influence on the enzyme activity and, therefore, more phytate was left in the samples.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support from Isfahan University of Technology.

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تاثیر تیمارهای تخمیر، هیدراتاسیون گرم و افزودن جوش شیرین و نمک بر فعالیت فیتازی و مقدار فیتات سه رقم گندم ایرانی

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چکیده

این تحقیق به منظور بررسی تاثیر فرایند تخمیر، هیدراتاسیون گرم و افزودن جوش شیرین و نمک بر فعالیت فیتازی و مقدار فیتات در سه رقم گندم ایرانی مهدوی، قدس و روشن انجام شد.نمونه ها پس از آرد شدن با سه درصد استخراج مختلف (آرد کامل، ۸۵ و ۷۵ درصد) به دست آمدند و سه نوع روش عمل آوری (تخمیری، جوش شیرین و شاهد) و چهار مقدار نمک (۰، ۰/۵، ۱ و ۱/۵ درصد) برای تهیه خمیر در سه تکرار استفاده شد. به منظور بررسی اثر فرایند حرارتی بر میزان اسید فیتیک فرایند پخت بر روی خمیرها انجام گرفت.نتایج حاصل از تحقیق نشان داد در بین ارقام گندم رقم مهدوی بیشترین

[DOR: 20.1001.1.16807073.2011.13.7.7.2]

فعالیت فیتازی و در عین حال بیشترین مقدار اسید فیتیک را دارد و ارقام قدس و روشن به همین شکل در ادامه قرار داشتند. ضمن آنکه بیشترین مقدار فیتات در سبوس همه ارقام گندم یافت شد و به همین دلیل آردهای با درصد استخراج بیشتر، فعالیت فیتازی بالاتری نشان دادند. فرایند تخمیر (۱٪ مخمر در $۳۷^{o}C$ به مدت ۳ ساعت)، فرایند هیدراتاسیون گرم (pHبرابر ۴/۸ در $۵۵^{o}C$ به مدت ۱۲ ساعت) و تیمار افزودن نمک (تا ۱/۵%) منجر به افزایش فعالیت فیتازی می شود در حالیکه افزودن جوش شیرین (۱٪) سبب کاهش فعالیت فیتازی می شود. حرارت پخت تاثیر معنی داری بر مقدار اسید فیتیک داشته و سبب کاهش قابل توجهی در مقدار آن شد.

[DOR: 20.1001.1.16807073.2011.13.7.7.2]