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Investigating and comparing the enzymatic activity of wheat, Quinoa and Amaranth

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ABSTRACT

Producing high-quality gluten-free products by some pseudo-cereals like Quinoa and Amaranth, which have a high nutritional value has been proved, but their enzymatic activity hasn't been completely known. Considering the effect and importance of enzymes in bakery products, this study, investigated the activity of alpha amylase, protease, lipase, lipoxygenase and Phytase in amaranth, quinoa and wheat. Quinoa and wheat have the highest and the lowest alpha amylase activity, respectively. The lipoxygenase activity in quinoa and amaranth is significantly ($p < 0.05$) greater than its activity in wheat. The activity of lipase and protease enzymes in wheat is significantly ($p < 0.05$) higher than amaranth and quinoa enzymes. Consequently, it seems that using lipase and protease in the production of Quinoa and Amaranth products, could increase the quality of these products. Phytase enzyme activity in amaranth is significantly ($p < 0.05$) higher than this enzyme activity in quinoa and wheat.

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1. Introduction

Celiac Disease (CD) is an autoimmune disease caused by the absorption of gluten protein (present in wheat (*Triticum aestivum*), barley and rye) in those people genetically susceptible to wheat gluten. Its prevalence is estimated to be 1% among the world's population. The only way to treat CD is to use a gluten-free (GF) diet throughout lifetime.

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Increasing demand for GF products is an important nutritional and technological challenge (1-3). There are many alternatives for gluten-containing cereals, including pseudo-cereals such as Quinoa (*Chenopodium quinoa Wild*) and Amaranth (*Amaranthus hypochondriacus*). Quinoa Quinoa is known for its high nutritional value as a super food. The United Nations has named 2013 the Quinoa year to increase the global attention to nutritional value and food security. Amaranth has been a promising food for thousands of

years, and commercial use of Amaranth has been promoted and encouraged (1, 4-8). Alpha amylase (α-amylase) (EC 3.2.1.1) is an enzyme from the glycoside hydrolase group. This enzyme participate in increases the volume of the bread and creating a brown-colored crust in the bread and slowing down the bread staling rate (9,10). Proteases can enhance the quality of GF products. Consequently, using protease enzymes makes it possible to make breads of a higher specific volume, a crumb with a better, softer appearance and lower staling than the control samples (11-13). Lipoxygenase (EC 1.13.11.34) is an enzyme of dioxygenase which performs the oxidation of fatty acids including the Cis-Cis-4.1 Pentadiene (Cis-cis-1,4pentadiene) to hydroperoxides. Lipoxygenase is naturally found in wheat flour and plays a significant role in the baking industry. Lipoxygenase can oxidize the flour pigments, increase the tolerance to mixing in the baking process, improve the rheological properties of the dough and increase the bread volume (14,15). Lipases (EC 3.1.1.3) break down and degrade Ester bonds. Lipases are good alternatives to emulsifiers in food products. (10,16). Considering the beneficial effects of lipases in the production of bakery products, it seems that a higher quality product would appear through using these enzymes in the production of gluten-free products produced from quinoa and amaranth.

Phytase increases the bioavailability of mineral nutrients, by degrading the phytic acid. Phytic acid has anti-nutritional property. Cereal phytases are better substitutes for microbial phytases due to increased consumer acceptance and reduced risk of allergic reactions (18). Various studies have shown these grains are nutritious and their use in the GF diet improves the variety and nutritional quality of GF products. Although their enzymatic activity has remained unknown or rare research has been done in this area. Considering the effect and importance of enzymes in bakery products, the present study, investigated the activity of α-amylase, protease, lipase, lipoxygenase and phytase enzymes in amaranth, quinoa and wheat in order to determine the beneficial or harmful effects of these enzymes in food applications.

2. Materials and Method

2.1. Materials

Five different types of Wheat was obtained from an agricultural Research Center, and Five different types of Quinoa (grown in Peru), and Five different types of Amaranth (grown in Peru) were purchased in a local whole food store. Bovine hemoglobin, Tween 20 and L-tyrosine were used from Sigma Aldrich Company. Other solvents and chemicals were manufactured by Merck Company.

2.2. Method

2.2.1. Moisture content

Moisture content was measured based on AACC methods (01-44) and by moisture detector (X-50 model) (AND, Iran, Tehran) (19).

2.2.2. Ash, Protein and Lipid

Measuring the amount of ash, protein and Lipid respectively was done based on approved methods of AACC Standard (08-01), (46-10), (30-10).

2.2.3. Crude fiber

The amount of crude fiber was measured based on ICC Standard, No. 113 (20).

2.2.4. α-amylase

In order to measure the enzyme activity, the Ceralpha method was used based on AACC Standard (02-22) by using an enzyme kit manufactured by Megazyme Company, Ireland (Megazyme International Ireland Inc., Bray, Ireland).

2.2.5. Protease

Enzyme activity was measured based on modified methods of Caussette et al. (1997) (5).

2.2.5.1. Enzyme extraction

In order to extract protease enzyme, 1 g of flour was mixed with 10 ml of acetate buffer (0.2 Molar with pH = 5) and incubated for 30 min. Then, the suspension was centrifuged at about 13/400 g for 10 min. The upper liquid separated from the suspension was filtered by microfilters with a diameter of 0.45 µm. In control samples, the extract was exposed to heat for 5 min at 98°C and then centrifuged. The obtained clear solution was used to evaluate the enzyme activity. The boiled extract had no protease activity.

2.2.5.2. Preparation of substrate solution

In order to prepare substrate solution, 1 g of bovine hemoglobin was dissolved in 100 ml acetate buffer (0.1 molar with pH = 5) to obtain a 1% w/v solution of hemoglobin.

2.2.5.3. Enzyme measurement

First, 1 ml of the extract was mixed with 2 ml substrate. The resulting mixture was incubated for 30 min at 37°C. The reaction was then stopped by adding 2 ml of Trichloroacetic acid (TCA) solution of 14% w/v. In order to accurately express the enzyme, the mixture was stored at 40°C for 1 h and centrifuged at 7000 g for 20 min and filtered by microfilter 0.45 µm and the absorbance of the clear solution was read at 700 nm. The amount of enzyme activity was determined using the standard curve of soluble Tyrosine in acidic distilled water and the result was reported in µg of Tyrosine produced in the test mixture per minute (units) per ml of extract (units/ml). The boiled extract was examined for 60 min, without showing any enzymatic activity. The calculation formula is as follows:

$$Units/ml = \frac{E*V}{V1*V2*T}$$

E: µ mole tyrosine equivalents release, ***V***: Total volume (in ml) of assay, ***V1***: Volume of Enzyme (in ml) of enzyme used, ***V2***: Volume (in ml) used in Colorimetric Determination, ***T***: Time of assay (in min) as per the unit definition

2.2.6. Lipoxygenase

This enzyme was measured based on the modified method of Sun et al (2012) (21).

2.2.6.1. Enzyme extraction

For enzyme extraction, 5 gr of the flour was mixed with 25 ml of cold phosphate buffer (0.1 Molar with pH = 6.5 and temperature of 4-5°C) and stored for 2 h at a temperature of 4°C and at 10 min intervals, the resulting suspension was stirred. Then, the resulting

suspension was poured into 15 ml falcons and centrifuged at 7000 g for 30 min. In the next stage, the upper liquid was passed through 0.2 syringe microfilter and the clear solution was used as a source of lipoxygenase enzyme. During measurement times, the enzyme extract was stored in ice.

2.2.6.2. Preparation of substrate solution

At this stage, 1 ml of pure ethanol was added to 0.06 g linoleic acid and the mixture container was shaken. Then, 100 ml of phosphate buffer (0.1 Molar with pH=6.8) containing 0.05 g Tween 20 was added to the linoleic acid and ethanol mixture and was mixed well.

2.2.6.3. Enzyme measurement

In order to measure the amount of enzyme, 60 µl of the enzyme extract and 2 ml of the substrate were mixed and 5 ml of 0.5 M Sodium hydroxide solution was added and the mixture was kept for 4 min until completing the reaction. The Sodium hydroxide, denatured the enzyme and led linoleic acid to the formation of its salt. As a result, a clear system was obtained. Then, the absorbance of the solution was read by spectrophotometer at 234 nm. A unit of lipoxygenase activity is defined as an increase in the absorption of 0.001 at 234 nm in 1 min. The calculation formula is as follows:

$$x = \frac{\Delta OD\ 234\ \times V2}{\Delta t M(1-W)V1} \times 1000$$

***ΔOD234*:** Absorption rate at 234 nm, **Δt**: Reaction time in minute (4 min), ***M*:** Flour mass (in grams), ***W*:** Moisture of the flour, ***V1*:** Volume of the crude enzymatic extract, which added to substrate (in milliliters), ***V2*:** Volume of the crude enzymatic extract (in milliliters).

2.2.7. Lipase

The method of Rose and Pike (2006) was used to measure the enzyme activity (24).

2.2.8. Phytase

Measuring phytase enzyme was done according to Zimmermann et al. (2002) and direct incubation (22).

2.3. Data Analysis

The obtained results of the current study were statistically analyzed in a completely randomized design with SPSS v.22 (SPSS INC). All experiments were performed in three replicates. ANOVA was used for data analysis and Duncan's multiple range test was used in order to compare the means. The graphs were plotted using Excel 2013 software. The results indicated that the difference between the samples in all factors is significant at 5% level.

3. Results

To compare means of Amaranth, Quinoa and Wheat enzyme activities and material contents, the mean of physicochemical properties of each flour type and each enzyme activity has been analyzed by One Way ANOVA Comparing means and Duncan test (Table 1 and Table 2).

Table 1. Physicochemical properties of Amaranth, Quinoa and Wheat (%)

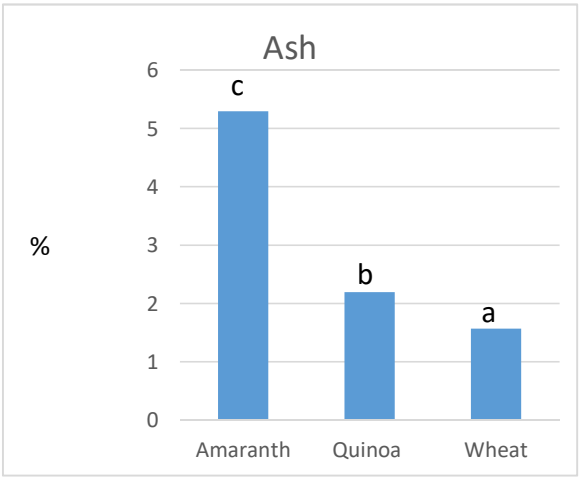
Independent Variable Level	Fiber	Lipid	Protein	Ash	Moisture
Amaranth	1.990 a	8.645 c	16.035c	5.295c	7.795 b
Quinoa	3.130 c	7.000 b	12.135 b	2.190 b	9.400 c
Wheat	2.220 a	2.450 a	8.185 a	1.565 a	6.885 a

*The difference in numbers with the same letters is not statistically significant on the basis of Duncan's test ($p < 0.05$)

Table 2. Enzyme activity of Amaranth, Quinoa and Wheat

Independent Variable Level	Alpha amylase	Protease	Lipase	Lipoxygenase	Phytase
Amaranth	0.176 b	9.425 b	1.780 b	13899.8 b	7433.0 c
Quinoa	1.485 c	7.020 a	1.615 a	14534.0 c	1659.50 b
Wheat	0.159 a	18.050 c	2.160 c	10766.9 a	1536.0 a

*The difference in numbers with the same letters is not statistically significant on the basis of Duncan's test ($p < 0.05$)



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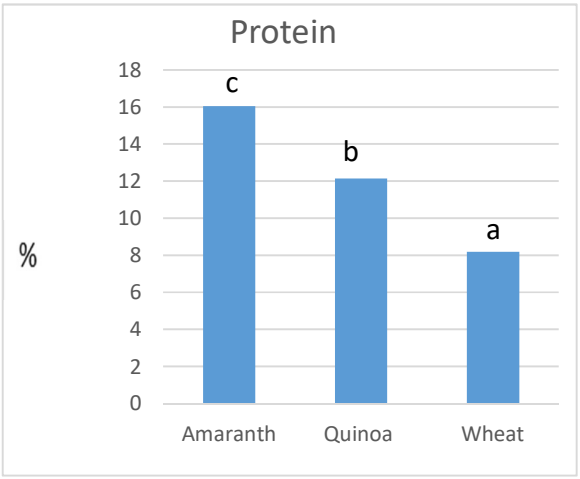

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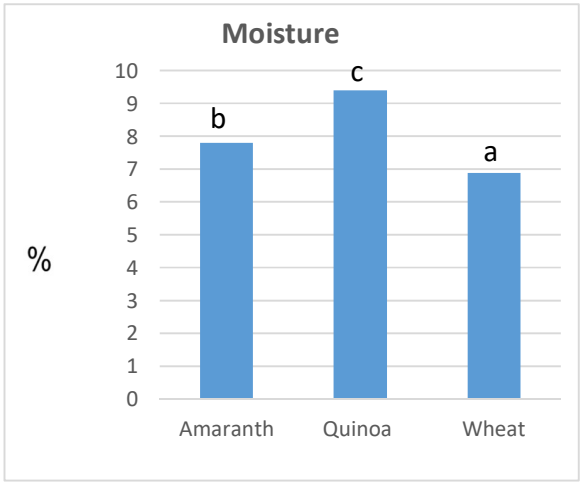

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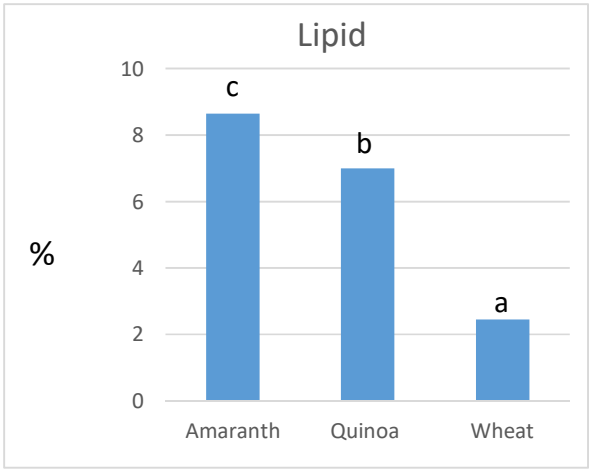

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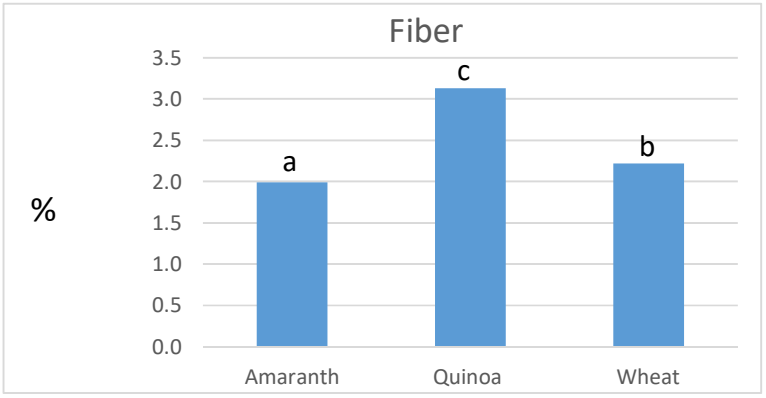

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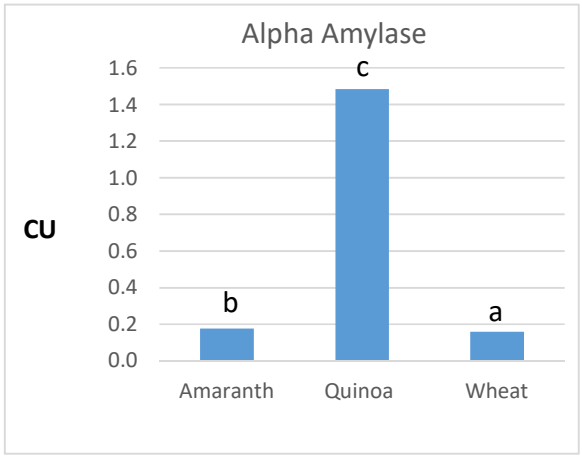
Figure 1. Comparing of percentage of Ash (Chart. 1), Protein (Chart. 2), Moisture (Chart. 3), Lipid (Chart. 4) and Fiber (Chart. 5)



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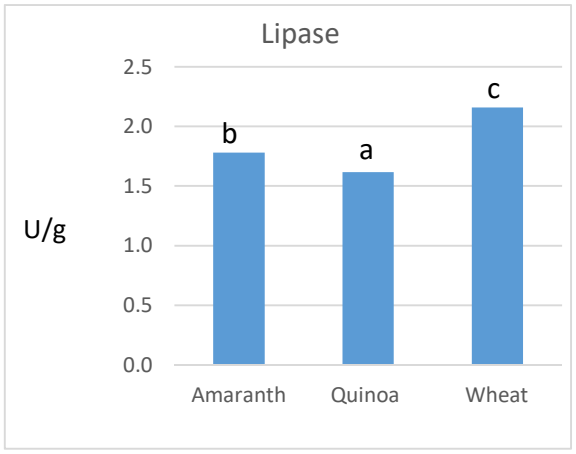

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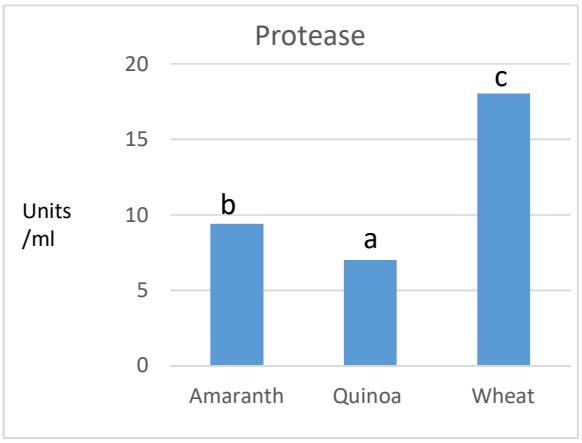

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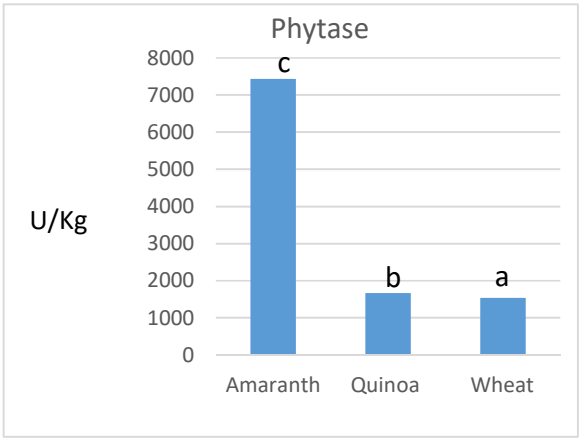

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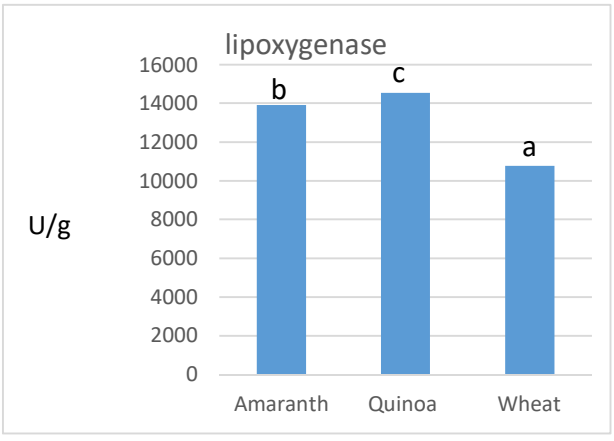

(10)

Figure 2. Enzyme activity of Alpha amylase (Chart. 6), Lipase (Chart. 7), Protease (Chart. 8), Phytase (Chart. 9) and Lipoxygenase (Chart. 10)

4. Discussion

4.1. Moisture

As for the ash and protein, the moisture amount of Amaranth, Quinoa and Wheat were significantly different. Quinoa had the highest (9.400) and Wheat had the lowest amount (6.885) of moisture (Fig. 1-chart 3).

4.2. Ash, Protein and Lipid

The ash amount of Amaranth, Quinoa and Wheat was significantly different. Amaranth has the highest amount of ash (5.295) and Wheat has the lowest amount (1.565) (Fig. 1-chart 1).

The protein amount of Amaranth, Quinoa and Wheat was significantly different. The amount of protein of Amaranth (16.035) was higher than the amount of Quinoa protein (12.135). In the current study, Wheat had the lowest amount of protein (8.185) (Fig. 1- chart 2).

The lipid amount of Amaranth, Quinoa and Wheat was also significantly different. The highest and lowest amount is related to Amaranth and Wheat, respectively (Fig. 1-chart 4). Since the Lipid content of Amaranth and Quinoa was higher than Wheat, there is the possibility of rancidity of Quinoa flour and Wheat if they are kept in inappropriate conditions and in long periods of time (16).

4.3. Fiber

The fiber content of Amaranth and Wheat was not significantly different while the fiber percentage of Quinoa was significantly higher than Amaranth and Wheat (Fig. 1- chart 5).

4.4. α-amylase activity

The α-amylase activity of Quinoa was significantly higher than Amaranth and Wheat, while no significant difference was observed between Amaranth and Wheat in this regard (Fig. 2- chart 6). In order to measure the α-amylase activity, the current study implemented Ceralpha method and the enzyme kit produced by Megazyme Company. So far, the enzyme activity of amaranth has not been evaluated in this way. The results are inconsistent with the study of Elgeti et al. (2014), which reported higher α-amylase activity in Wheat than Quinoa (17). Caussette et al. (1997) reported a high level of α-amylase activity in Quinoa (5). In another study, Hidalgo et al. (2013) investigated the activity of alpha and beta amylase and phenolase in different subspecies of wheat. The researchers used the Ceralpha method and the Megazyme Enzyme Measurement Kit to measure the minimum and maximum α-amylase activity in different subspecies of wheat. They indicated that the most enzymatic activity is in the germ, bran, and endosperm, respectively (23). The α-amylase activity obtained in this research is similar to the enzymatic activity of many species in the study of Hidalgo et al (23).

4.5. Lipase enzyme activity

The level of lipase enzyme activity was significantly different in Amaranth, Quinoa and Wheat. In addition, Wheat had the highest and Quinoa the lowest levels of enzyme activity, respectively (Fig. 2-chart 7). By measuring the activity of lipase enzyme, Rose and Pike (2006) determined the optimal conditions for its measurement. They reported that the activity of lipase in the whole wheat grain ranges between 1.05 and 3.54 U/g. The result of the enzyme activity is expressed as

unit/gram in which 1 unit equals to micro-equivalent linoleic acid which is released per h (24). In the current study, the enzymatic activity of wheat was 2.6 U/g, which is consistent with the results of Rose and Pike (2006). The level of lipase enzyme activity in wheat was significantly higher than in Quinoa (1.615 U/G) and A (1.780 U/g).

4.6. Protease enzyme activity

The level of protease enzyme activity was significantly different in Amaranth, Quinoa and Wheat. Further, Wheat had the highest and Quinoa the lowest levels of enzyme activity, respectively (Fig. 2-chart 8). In the current study, the absorption rate was first read at 500 nm. The spectrophotometer failed to indicate the absorption rate for wheat at this wavelength causing an error. In the next stage of the experiment, 700 nm wavelength was used to read the absorbance and protease activity of the samples. At this wavelength, the protease activity of Amaranth, Quinoa and Wheat samples were obtained. It is worth noting that the calculated protease activity level for Quinoa and Amaranth at 700 nm wavelength was higher than the calculated protease activity at 500 nm. Thus, the 700 nm wavelength is more appropriate for protease assay, and the result of this study is in line with the results of the study conducted by Caussette et al., (1997) (5). It is worth noting acidic distilled water and ultrasonic waves were used in order to plot the standard tyrosine curve for the dissolution of this amino acid.

4.7. Phytase enzyme activity

Phytase enzyme activity was significantly high in Amaranth than Quinoa and Wheat, but this activity was not significant in Quinoa and Wheat (Fig. 2-chart 9).

The results of phytase enzyme analysis showed that phytase enzyme activity is similar in Wheat and Quinoa, but Amaranth enzyme activity was significantly higher in Wheat and Quinoa. The enzymatic activity of Quinoa was 1536 U/Kg which is similar to the result obtained by Zimmermann et al (2002) (22). It is worth noting that phytase enzyme activity of Quinoa and Amaranth was first evaluated in the present study.

4.8. Lipoxygenase enzyme activity

The level of lipoxygenase enzyme activity was significantly different in Amaranth, Quinoa and Wheat. Further, Quinoa had the highest and Wheat the lowest levels of enzyme activity, respectively (Fig. 2-chart 10). The method used in the current study to evaluate the activity of lipoxygenase was an appropriate method and could determine the activity of lipoxygenase in Quinoa, Amaranth and Wheat well. Furthermore, the level of lipoxygenase activity was high in Quinoa. The method used by Caussette et al. (1997) failed to measure and detect lipoxygenase enzyme activity in Quinoa (5). In another study, Sun et al. (2012) examined the properties of lipoxygenase in wheat malt at different temperatures and pH conditions. They found that the optimal activity of the lipoxygenase enzyme was determined at 35°C and pH=6.8 and the inactivation temperature of this enzyme was 70°C (21).

5. Conclusion

In conclusion the content of protein, lipid and ash in Amaranth and Quinoa are significantly higher than Wheat. The fiber content of Wheat and Quinoa are equal and the fiber content of Amaranth is less. The

Amaranth is significantly higher than Wheat. Thus, by adding the flour of these pseudo-cereals to wheat flour, the flour can be enriched in terms of fiber and protein, and the qualitative properties of wheat flour products can be improved. The activity of lipase and protease enzymes in Wheat is significantly higher than Amaranth and Quinoa. As a result, it seems that using these enzymes in the production of gluten-free products can increase the quality of these products. Phytase enzyme activity in Amaranth is significantly higher than Quinoa and Wheat. Due to the short duration of fermentation in gluten-free products, Amaranth can be safely used to produce gluten-free products.

Conflict of interest

The authors declare that there is no conflict of interest.

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