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Malting effect on enzyme activities of quinoa and lupine seed

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Abstract
Quinoa and lupine are considered complete foods because of their nutritional makeup and their nutritional elements have been extensively studied. Malting quinoa and lupine grains have increased nutritional and functional features, allowing for the manufacture of an amylase-rich product for food and beverage manufacturing in households and enterprises. The purpose of this study was to see the effect of malting on enzyme activity of quinoa and lupine seed. Quinoa and lupine seeds were malted by steeping for 6 h and 5 h in distilled water germinating for 24 and 48 h at 28±2 °C, and kilning dry for 4 h at 55 °C under mechanical convection. The results showed that 48 h malted lupine and quinoa extract have significantly ($p \leq 0.05$) high level of α-amylase; 33.4% & 16.8% and β-amylase; 37.4% & 26.9%, respectively. Protease content was also observed significantly high in 48 h malted quinoa and lupine extract 178.5% &108.2% respectively as compared to their un-malted extract. When comparing malted seeds to un-malted seeds extract, amylase and protease activity was shown to be higher in both lupine and quinoa malted extract. Malting causes partial digestion of carbohydrates and proteins by increasing the activity of hydrolytic enzymes in seeds. As a result, both seeds may have the potential to produce higher amylase and protease activities, which can be employed to make gluten-free bakery products with superior quality with greater shelf life.

Keywords: Malting, quinoa, lupine, amylase, protease

Introduction
Malting is the process of germination of cereal grains and legumes under regulated conditions of water, temperature, and humidity and this process activates the enzymes in resting grain, leading in the conversion of starch to fermentable sugars, as well as the partial breakdown of proteins and other macromolecules (Bera, *et al.,* 2018) [1]. Simple conventional food processing methods, such as soaking and germination, have been shown to considerably lower the anti-nutrient content of cereal grains while increasing their nutrient bioavailability (Hejazi and Orsat, 2016). It is the most frequent and successful method which involves soaking, germination, and kilning for improvement in quality of cereal grain (Cheevitsopon and Noomhorm, 2015) [3].

Steeping comprises a series of imbibition in water followed by an air-rest period, during which the water is drained, and carbon dioxide (CO_2) is evacuated using fans. Sufficient aeration of the steep vessel with water under controlled temperature with efficient CO_2 removal is necessary during steeping process as lack of oxygen brings about microbial development, anaerobiosis, and souring while excessive aeration results in unwanted growth and starch loss (Rani and Bhardwaj, 2021) [4]. Germination process is marked by embryo development, manifested by the growth of the rootlets and increment in shoot length, with the concomitant modification of endosperm (Carvalho *et al.*, 2018) [5]. Germination is a pre-digestion process which results in breakdown of the high molecular complex materials like the polysaccharides into oligo and monosaccharide's, the fats into free fatty acids, whereas the proteins into oligopeptides and free amino acid (Chon, 2013). Kilning process aims to reduce the moisture content of malt below 5%, and to ensure the product stability for storage, transport, and to prevent enzyme denaturation. Kilning also promotes the formation of melanoidins via the non-enzymatic Maillard reaction between amino acids and sugars (Smart, 2020) [6]. So, malting contributes to the production of functional foods that have a favorable impact and aid in human health maintenance (Laila and Murtaza, 2014) [7].

Malted grains are high in protein, fibre, and unsaturated fatty acids, as well as being low in carbohydrate Malted legumes improved the amounts of total proteins (prolamins and lysine), fat, essential amino acids, total sugars, B-group vitamins, and starch digestibility by increasing the activities of hydrolytic enzymes like amylase and protease (Dipnaik and Bathere, 2017) [8]. Enzymatic activity has the potential to increase functional qualities aa it helps in production of bioactive substances. Enzymes like amylases and proteases are used to change the dough's rheology, gas retention, and crumb softness. However, depending on the grain variety, growth circumstances, and germination period, the timing of enzyme activation varies (Guzman-Ortiz *et al.*, 2019) [9].

Quinoa is a lesser-known grain that has been cultivated in South America's Andean area. It is a member of the *Chenopodiaceae* family and is frequently referred to as Kinwa. It can withstand a variety of stress situations, including high temperatures, salt, poor sandy soils, and agricultural environments (Bazile *et al.*, 2016) [10]. It has a significant quantity of protein (14 to 20% with strong amino acid digestibility) and crude fibre (Sharma *et al.*, 2015) [11]. It is also high in minerals (calcium, magnesium, iron, copper, potassium, phosphorus, zinc, and iron) and vitamins (thiamin, vitamin C, and folic acid) (Angeli *et al*., 2020) [12]. Their seeds include bioactive substances like as total phenols, total flavonoids, tannins, and polyphenols, which function as potent antioxidants and protect against oxidative stress (Yael, 2012) [13].

Lupin is a legume that belongs to the *Fabaceae* family and is distinguished by its high protein content and well-balanced amino acid composition (Gonzalez, *et al*., 2012). It contains vital fats, fibre, vitamins, and minerals. In comparison to other legumes, "lupin has lower quantities of anti-nutrients such as oligosaccharides, phytic acid, lectins, and trypsin inhibitors" (Barman *et al*., 2018) [15]. It is also high in phytochemicals that have antioxidant properties, including as polyphenols, flavonoids, tannins, and phenolic acids (Siger, *et al*., 2012) [16].

Materials and Methods

Seed Materials: Lupine seeds were purchased from mall of (Bhopal) Madhya Pradesh by golden harvest site whereas red quinoa seeds were procured from Devshree grains and pulses, New Delhi, India for the study. Seeds were authenticated and identified by the expert of Krishi Vigyan Kendra (KVK) of Banasthali Vidyapeeth and stored at 4 °C until used for experiments.

Seed malting: Seeds were soaked in 1 litre of water containing 0.7% sodium hypochlorite solution at room temperature (28 °C) for 30 minutes then washed 3-4 times with distilled water having neutral pH and the remaining water was drained off. Lupine and quinoa seeds had been re-soaked in distilled water for 6 h and 5 h correspondingly and then again the remaining water was drained off. The hydrated seeds were placed in muslin cloth and allowed to germinate in the dark and controlled temperature (25 ±2 º C). The seeds were watered every 3h with 2.5 ml of sterile distilled water to avoid dryness and to maintain the moisture content. Seeds were germinated for 24h and 48h. The duration of these germination periods were strictly based on the laboratory observation. Sprouts corresponding to each stage of germination and ungerminated seeds were dried at 55 °C for 4h in a mechanical convection oven and weighted constant in all samples and then milled to pass through 60 mesh size sieved to obtain homogenous flour samples and stored in air tight containers at 4 ° C (Uchegbu, *et al*, 2015).

Preparation of Extract: 50 g of unmalted and malted quinoa and lupine flour were macerated with 100nml sterile distilled water in a warning blender for 10 min. the macerate was first filtered through double layered muslin cloth and then centrifuged at 4000 rpm for 3 min. The supernatant was filtered through wattmann No-1 filter paper and sterilized at 120 °C for 30 min. The Extract was preserved aseptically in broom bottle at 5°C until further use.

Enzymatic Activity Assay α-Amylase

Alpha-amylase of quinoa and lupine was determined according to the method described by Bernfeld, 1955 with slight modifications (Abeykoon, *et al.*, 2021) [18]. Initially 50 µL of the suspension was mixed with 40 µL of 1% starch solution. Then 860 µL of 100 mM sodium acetate buffer was added to it and incubated at 40oC for 10 minutes. Then 50 µL of alpha-- amylase enzyme (porcine pancreas) was added and further incubated at 40o C for 15 minutes. After that 500 µL of 3, 5-dinitrosalicylic acid (DNS) reagent was added and placed in a boiling water bath for 5 minutes and allowed to cool in a water bath containing ice. Control experiments were carried out in an identical way replacing sample extracts with 100 mM sodium acetate buffer. A sample blank was prepared by replacing the sample extracts, starch and enzyme solution with 100mM sodium acetate buffer. Absorbance was measured at 540 nm against a sample blank using 96 well micro plate reader (Spectr Max plus 384, Molecular Devices, USA). All measurements were done in triplicates.

$$Unit/mg = \frac{micromoles\ maltoes\ relesed}{mg\ enzyme\ in\ recation\ mixture * 3\ min}$$

β-Amylase

β-amylase activity of quinoa and lupine was determined according to the method described by Bernfeld, 1955 with slight modifications (Srivastava and Kayastha, 2014) [19]. Reaction mixture was prepared by taking 0.5 mL of suitably diluted enzyme and 1% starch prepared in 50 mM sodium acetate buffer, pH 5.0. This was incubated at 30°C for 3 min, reaction was stopped by addition of 1 mL of 3,5-dinitrosalicylic acid. Test tubes were then placed in boiling water bath for 5 min and were allowed to cool down to room temperature, followed by addition of 10 mL of distilled water. Absorbance was measured at 540 nm against a sample blank using 96 well micro plate reader (Spectr Max Plus 384, Molecular Devices, USA). All measurements were done in triplicates. One unit of β-amylase is defined as the amount required for release of 1 μM of β-maltose per min at 30°C and pH 5.0 under the specified condition.

$$Unit/mg = \frac{micromoles\ maltoes\ relesed}{3\ min * mg\ enzyme\ in\ recation\ mixture}$$

Protease Activity

Protease Activity was assayed according to the procedure described by Kunitz, 1947 with slight modification (Mughal, *et al.*, 2020) [20]. 1 mL aliquot of an appropriate dilution of the extract sample was combined with an equivalent amount of trypsin (1000 units/mg) and incubated for 15 minutes at 37 °C. 2 mL of 1% casein was then added, and the resulting mixture was allowed to stand for 30 minutes at 37 °C. To halt the reaction, 2.5 mL of 5% trichloroacetic acid (TCA)

solution was added. The absorbance at 280 nm was determined after centrifugation of the reaction mixture (12,000 rpm, 15 minutes). Under normal test conditions, one unit of protease activity (PU) was defined as a one-unit reduction in absorbance at 280 nm of TCA soluble casein hydrolysis product released by trypsin action per minute.

$$Inhibitory\ activity\ (\%) = \frac{Amount\ of\ tyrosine\ with\ inhibitor}{Amount\ of\ tyrosine\ resealed\ without\ inhibitor} \text{ x } 100$$

Statistical Analysis

Result were expressed as mean value and standard deviation of three determinations and also statistically analyzed to ascertain its significance. The analytical data obtained for enzymatic activity of malted quinoa and lupine flour were subjected to student t-test. The significant difference at ($p \leq 0.05$ level) was estimated.

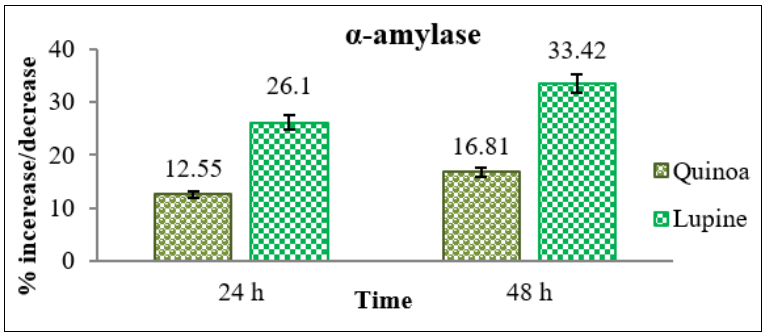
Results and Discussion

Table 1: Amylase Activity of Unmalted and Malted Quinoa and Lupine Aqueous Extract

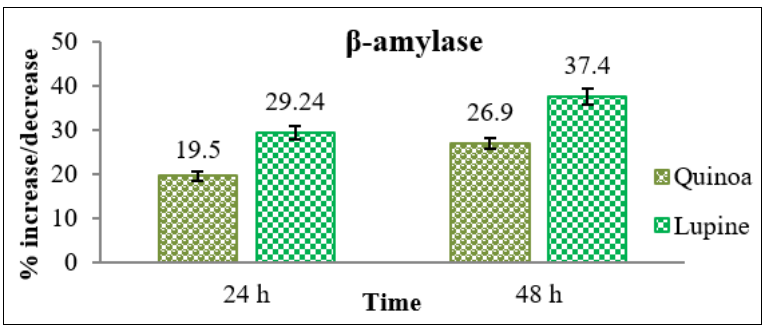
(a)			
α-amylase (N moles of Maltose/mg/min/g)			
Sample	Unmalted	Malted	
		24h	48h
Quinoa	30.16±0.56^{c}	41.65±0.35^{b*}	54.81±0.46^{a*}
		(31.59%↑)	
Lupine	46.02±0.66^{c}	56.73±0.45^{b*}	72.64±0.57^{a*}
		(28.04%↑)	

(b)			
β-amylase (N moles of Maltose/mg/min/g)			
Sample	Unmalted	Malted	
		24h	48h
Quinoa	39.17±0.61^{c}	49.84±0.20^{b*}	67.96±0.8^{a*}
		(36.35%↑)	
Lupine	49.02±0.75^{c}	59.66±0.56^{b*}	76.31±0.78^{a*}
		(34.68%↑)	

1 maltose unit is equivalent to the enzyme activity, which liberate 1 µmol of maltose in 3 min; Values are expressed as Mean ± SD of triplicate determinations of unmalted and malted quinoa and lupine flours on dry weight basis. * Show significant difference at ($p \leq 0.05$) level; NS show non-significant difference at ($p \leq 0.05$) level. Values sharing same superscript letter between columns are not significantly different at $p \leq 0.05$.



(a)



(b)

Fig 1: Percent Increase of Amylase Activity of Malted Quinoa and Lupine Extract

The α-amylase content indicated that malted flour extract is higher than unmalted extract due to amylase is usually found in seedlings of many species during malting. It is produced during malting to mobilize storage macromolecules in the endosperm because simpler molecules are needed to provide energy to the seed during malting (Kalita *et al*., 2017) [21]. The

Mean ± SD of α-amylase content (N moles of Maltose/mg/min/g) had 30.16±056 and 46.02±0.66 respectively for of unmalted quinoa and lupine flour as showed in table 1and figure 1 The Mean± SD for malted quinoa flours were 41.65±0.35 and 54.81±0.46 respectively at 24 and 48 hour which were significantly increased by 12.55% and 16.81% ($P \leq 0.05$) when compared to unmalted quinoa flour. Whereas malted lupine flour had 56.73±0.45 and 72.64±0.57 respectively which were significantly increased by 26.10% and 33.42% at 24 and 48 hour when compared to unmalted lupine flour at $P \leq 0.05$. The α-amylase content was found to significantly increased percent in malted quinoa 31.59% as compared to malted lupine 28.04% at $P \leq 0.05$ level. Nam and Park, 2018 [22] who reported that the approximately closer value of present data that α-amylase activity of quinoa was the lowest in the 6 h (29.28 ± 1.07) germination and increased in proportion to the germination period until 24 h (34.39 ± 0.54) at $p < 0.05$. Similarly, Ghavidel & Davoodi (2011) [23] analysed that "α amylase activity as a function of germination time, the control samples being non germinated exhibited very low amylase activity which ranged from 7.0 to 18.1 maltose units/g dry matters. The α-amylase activity improved by 10 to 150% over the initial value with the lowest in cowpea and highest in mung bean samples and increased significantly at $P < 0.05$ level".

β-amylase content was higher in malted flour when compared with unmalted flour extract due to β-amylase, is synthesized during grain development, and during germination acts to liberate the disaccharide maltose from the non-reducing end of starch molecules (Aubert *et al*., 2018) [24]. The Mean ±SD of β-amylase content (N moles of Maltose/mg/min/g) had 39.17±0.61 and 49.02±0.75 respectively for unmalted quinoa and lupine flour as showed in table 1.b and figure 1.b. After malting the Mean±SD for quinoa flours were 49.84±0.20 and 67.96±0.8 respectively at 24 and 48 hour which were significantly increased by 19.52% and 26.96% ($P \leq 0.05$) when compared to unmated quinoa flour. Whereas malted lupine flour had 5.66±0.56 and 76.31±0.78 respectively which were significantly increased by 29.24% and 37.40% at 24 and 48 hour when compared to unmalted lupine flour at $P \leq 0.05$level. The α-amylase content was found to significantly increased percent in malted lupine flour (34.68%) as compared to malted quinoa flour (36.35%). The data represent that there was significant difference observed at $P \leq 0.05$ level. The present data was comparable and in agreement with Gujjaiah and Kumari, 2013 [25] who stated that the β- amylase (Nmoles of Maltose/mg/min/g) activity was significantly increased in germinated rice (88.10), wheat 133.70, ragi 56.85 and bajra 109.80 at 48 hours as compared to 6 and 12 and 24 h germination. Likewise, Ortiz *et al*., 2018 who affirmed that during this process the amylase activity was 2.87 times higher at 7 days of germination than at 1 day of corn. The increase in amylase activity with an increase in steeping time is due to enzymes' activation during steeping and the penetration of the gibberellic acid by diffusion to the aleurone layers to prompt enzyme synthesis (Adetokunboh *et al*., 2022) [26]

Table 2: Protease Activity of Unmalted and Malted Quinoa and Lupine Aqueous Extract

Protease (Protease unit/g)			
Sample	Unmalted	Malted	
		24h	48h
Quinoa	2.8±0.8^{c}	3.7±0.2^{b*}	7.80±0.7^{a*}
		(124.7%↑)	
Lupine	6.3±0.2^{c}	8.2±0.6^{b*}	12.5±0.8^{a*}
		(52.4%↑)	

1Protease unit (PU) is equivalent to the enzyme activity, which liberates 1µmol tyrosine in 60 min; Values are expressed as Mean ± SD of triplicate determinations of unmalted and malted quinoa and lupine flours on dry weight basis. * Show significant difference at ($p \leq 0.05$) level; NS show non-significant difference at ($p \leq 0.05$) level. Values sharing same superscript letter between columns are not significantly different at $p \leq 0.05$.

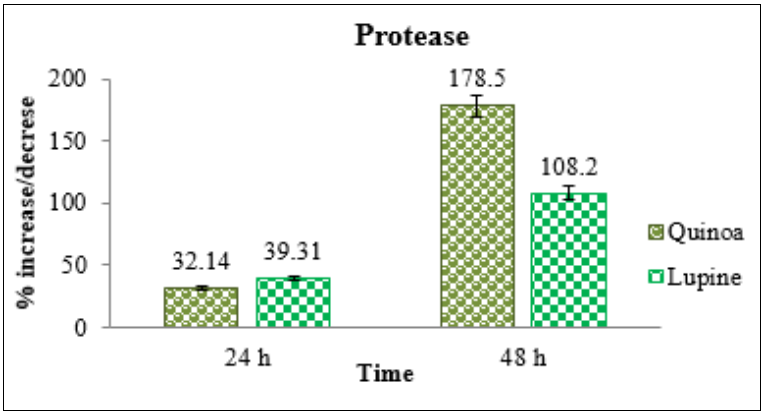


Fig 2: Percent Increase of Protease Activity of Malted Quinoa and Lupine Extract

Table 2 and figure 2 showed protease activity of quinoa and lupin. The Mean±SD of protease content (Protease Unit/g) had 2.8±0.8 and 6.3±02 respectively for unmalted quinoa and lupine extract. After malting the Mean±SD for malted quinoa flours extract were 3.7±0.2 and 7.80±0.7 respectively at 24 and 48 hour which were significantly increased by 32.14% and 178.5% ($P \leq 0.05$) when compared to unmalted quinoa flour extract. Whereas malted lupine flour had 8.2±0.6 and 12.5±0.8 respectively at 24 and 48 hour which were significantly increased by 39.31% and 108.2% when compared to unmalted lupine flour at $P \leq 0.05$. The protease content was found to significantly increased percent in malted lupine flour (124.7%) as compared to malted quinoa flour (52.4%) at $P \leq 0.05$ level. The study was comparable and in agreement with Rahman *et al*., 2007 [27] who stated that Mung bean varieties showed the significant increase in protease activity 7.14- 8.16 unit/g at 48 hours. According to Ghavidel and Davoodi (2011) [23] reported that protease activity of non-germinated legumes had ranged 0.71 to 1.53 protease unit/g, after malting chickpea had the highest protease activity 6.21PU/g d w at 72 followed by lentil, cowpea and mung bean.

Conclusion

When comparing malted seeds to un-malted seeds extract, amylase and protease activity was shown to be significantly high in both lupine and quinoa malted extract. Malting causes partial digestion of carbohydrates and proteins by increasing the activity of hydrolytic enzymes in seeds. As a result, both seeds may have the potential to produce higher amylase and protease activities, which can be employed to make gluten-free bakery products with superior quality and shelf life without the need of starch. Overall, the potential of understanding the features of seed enzymes activities together with germination as a biotechnological alternative to provide great prevention measures in protein-caused illnesses will open a great field of research with new goals.

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