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ORIGINAL RESEARCH PAPER

INSIGHTS ON THE PROTEASE ACTIVITY AND ON TOTAL AND SOLUBLE PROTEINS DURING GERMINATION OF WHEAT AND BARLEY

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Abstract: Malting process leads to changes in the protein content and protease activity of cereal crops. Total protein content and soluble protein content are important indicators of the quality of cereal crops, as they can affect the digestibility and bioavailability of the proteins for human consumption. Protease activity is also important, as it can influence the breakdown and release of amino acids from proteins, which are essential for human nutrition. The study aims to investigate how the total and soluble protein content and protease activity change during the production of malted wheat and barley flours at different germination times (0, 1, 2, 3, and 4 days). The findings indicated that during malting there was an increase in the amount of soluble proteins and protease activity in both wheat and barley. Although the total protein content did not change significantly, the increased amount of soluble proteins suggests that the malting can improve the nutritional value of the cereal crops. Moreover, the increase in protease activity with the extension of the germination time suggests that there is an optimal time for germination that could maximize the nutritional benefits of the malting process. Investigation revealed barley had a higher content of total and soluble proteins and higher protease activity compared to wheat. The outcomes of this study could be valuable for optimizing the malting process for maximum nutritional benefits, and for developing new better nutritional products.

Keywords: *barley, flour, germination, malting, wheat*

INTRODUCTION

Cereals are an important staple food for much of the world's population, especially in developing countries where food shortages and malnutrition are major issues. Improving the nutritional quality of cereal crops through different techniques and technologies is essential to address these challenges [1]. Wheat (*Tritium aestivum* L.), and barley (*Hordeum vulgare* L.) are two of the main cereal crops used for human consumption and also have a long history of being used in the production of malt and beer [2]. The annual global production of malt is substantial (18 to 22 million tons), with the majority of it being prepared from barley for beer production [3]. Wheat, however, has been studied to a lesser extent in this regard [4]. One of the ways to improve the nutritional quality of cereals is through the process of malting, which involves steeping the raw material, germination, and drying. During this process, various hydrolytic enzymes are produced and mobilized, including amylases and proteases, which are involved in the breakdown of starch and proteins into fermentable sugars (through hydrolysis of α-(1→4) and α-(1→6) linkages) [5] and amino acids, respectively. Malting not only improves the nutritional characteristics of cereals, but it also enhances their organoleptic properties, including taste, color, and aroma [6, 7].

The conversion of starch into soluble low-molecular-weight, fermentable sugars are practically the most important change that occurs during malting. Proteins that are synthesized and stored during the maturation of the raw material are broken down into free amino acids for biosynthesis and energy generation. Depending on the solubility, proteins in cereals can be divided into albumins (soluble in water), globulins (soluble in salt), prolamins (soluble in ethanol solutions) and glutelins (soluble in alkaline solutions or acids) [8]. The protein content in barley and wheat is around 10 - 12 % [9, 10]. During steeping and germination, hydrolytic enzymes break down the endosperm cell walls, and this process can impact the protein content in the malted flour, which makes the research field highly interesting [11]. The drying process is important for reducing moisture content and improving microbiological stability, and it also leads to the formation of Maillard products, which contribute to the specific color, taste, and aroma of malt. For these reasons, the use of malt flour can improve dough plasticity, as well as be used in the production of new functional foods, such as cupcakes and cakes [12, 13].

Malt flour is mostly used in the beer production and not so much as a functional component in the creation of functional products. On the other hand, there is a lack of data on the changes in the nutritional composition and biologically active properties that are occurring during the malting process of cereals. The influence of germination time on the physicochemical properties and nutritional profile of chickpea flour has been investigated, and it was found that germination time significantly affects the nutritional profile and functional properties of the flour. In addition, a significant increase in protein and crude fiber content was also observed [1]. Helland et *al.* [14] investigated the effect of corn germination on the suspension viscosity. The study found that a three-day germination period resulted in a significant reduction in the viscosity of the suspension, making it easier to handle and process. However, it is important to note that the activation of hydrolytic enzymes during uncontrolled germination can have a negative impact on the technological characteristics of the finished product, and using sprouted wheat has been found to reduce gluten strength [2, 15]. Yang et *al.* [13] in their study show that germination time plays an important role in improving the nutritional and antioxidant

properties of cereals. The authors have developed a technology in which wheat flour is replaced by malt flour that was germinated for 3 - 4 days in the preparation of biscuits. It has been shown that biscuits with malt flour have a higher content of dietary fiber and phenols alongside with better appearance and structure.
The aim of this paper is to provide insights into the effects of malting process of wheat and barley at different germination times (0, 24, 48, 72, and 96 hours) by determination of the change in the total and soluble proteins content and protease activity during this process.

MATERIAL AND METHODS

Material

The two wheat cultivars (Lorena - sample I and Snaša - sample II) and the four barley cultivars (Zlatko – sample III; Tristan - sample IV; Osvit – sample V; Mandatar – sample VI) used for analyzes were obtained from the Agricultural Institute Osijek (Croatia). Protease Assay kit (Cat No. 539125) was used to determine the protease activity. All other reagents were purchased from Sigma-Aldrich.

Methods

Production of malt flour

Wheat and barley grain for production of malt flour were malted in a micro-malting Automated Joe White Malting Systems Unit (Perth, Australia) according to Jukić et *al.* [16].

Extraction procedure for soluble and non-soluble proteins

Soluble and non-soluble protein fractions were extracted using 0.05 M sodium phosphate buffer (*p*H 6.8) with the sole difference that for non-soluble ones 2 % sodium dodecyl sulfate (SDS) was used. 10 mL of SDS sodium phosphate buffer was added to 1 g of the sample. Using a shaker, the samples were mixed for 1 hour at 225 rpm at a temperature of 4 °C. Then the samples were centrifuged (10 min at 8000 rpm) and the supernatant was separated from the precipitate. First, soluble fractions were extracted and desalted, followed by the extractions of the non-soluble fractions on the same samples. Three repetitions were made for each trial.

Determination of proteins

The determination of soluble fractions of proteins and total proteins of malt flours was done using a spectrophotometer (UV-Vis Spectrophotometer - UV-1800, Shimadzu, Japan) at $\lambda = 760$ nm according to Lowry's method [17].

Determination of protease activity

The protease activity of the malt flours was determined using the Protease Assay Kit (Cat. No. 539125). Preliminary extraction of the samples was done in the following way: 5 mL (50 mM Na-phosphate buffer *p*H 7.5) was added to 1 g of flour. The samples were then allowed to homogenize (2 hours at 225 rpm) using a shaker at 4 °C. After the time for homogenization, the samples were centrifuged for 15 min (8000 g). Furthermore, the

supernatant was used to determine the protease activity. Trypsin was used as standard. The results for protease activity were expressed unit Trypsin activity nmol·min.

Statistical analysis

Analysis of variance (ANOVA) and Fisher's Least Significant Difference test (LSD) at $p < 0.05$ were performed with the software XLSTAT 2019 and Microsoft Office Excel 2016.

RESULTS AND DISCUSSION

During grain ripening, proteins are synthesized and stored in the endosperm of the grain. During the malting process, enzymes break down these proteins into free amino acids, which can then be used by the developing embryo for energy and biosynthesis [8]. The total amount of proteins, which were determined in the different types of non-malted wheat and barley flours, are presented in Table 1.

Table 1. *Total quantity of proteins in different non-malted wheat and barley`s flours*

Sample	Type	Total proteins [%]	Soluble proteins [%]
I	Wheat flour	13.56 ± 0.24	2.56 ± 0.01
II	Wheat flour	12.58 ± 0.01	2.36 ± 0.06
III	Barley flour	13.75 ± 0.06	3.08 ± 0.05
IV	Barley flour	13.78 ± 0.12	3.11 ± 0.01
V	Barley flour	13.28 ± 0.06	3.91 ± 0.02
VI	Barley flour	13.70 ± 0.17	3.85 ± 0.01

The values are the average of three repetitions (±standard deviation).

The ANOVA (not presented) highlighted significant differences ($p < 0.05$) between the content of total proteins in the examined samples of wheat and barley flour with average values of 13.07 % and 13.63 %, respectively. Habschied et *al.* [18] performed a protein content analysis of different varieties of barley, cultivated in the period from 2016 to 2019. In their research they examined the same barley varieties as our samples marked as V and VI (cultivar: Osvit and Mandatar, respectively) with the protein content ranging from 11.07 % to 13.31 %. The amount of total proteins in cereal crops is influenced by climatic conditions during the growing period, with greater precipitation leading to a higher yield but lower nitrogen content in the grain [19]. Šimić et *al.* [20] in their research determined the amount of protein in different fractions of hull-less barley flour, variety Mandatar (marked as sample VI). The protein content in the different fractions varied from 12.77 to 16.93 %. Germination can change the solubility of the numerous components present in the grains, and can even modify their biological activity. One of the ways to assess the effect of germination on the reserve proteins contained in the grain is by determining the soluble nitrogen (soluble proteins) [21]. The soluble proteins in the samples are respectively: 2.56 % (sample I); 2.36 % (sample II); 3.08 % (sample III); 3.11 % (sample IV); 3.91 % (sample V) and 3.85 % (sample VI). The changes in the content of the total proteins in the different samples, grown for 0, 1, 2, 3 and 4 days are presented in Figure 1.

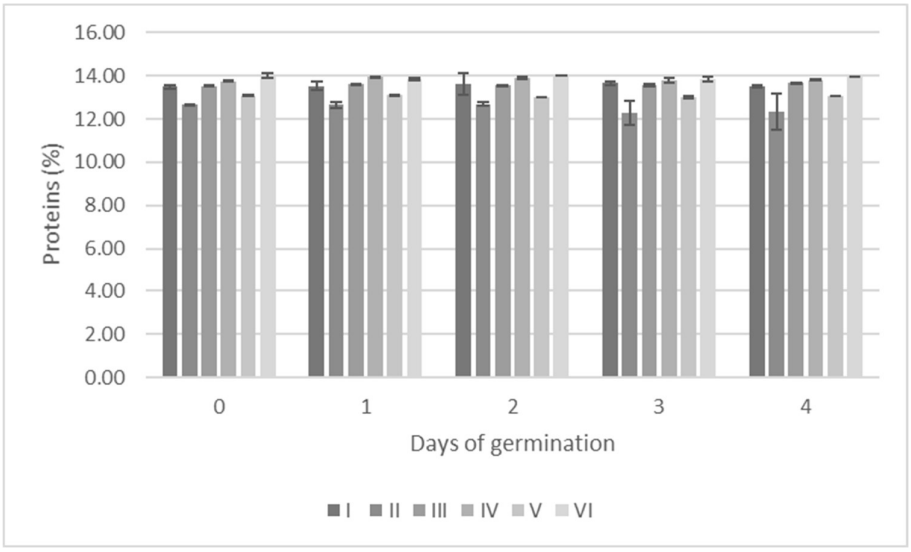

Figure 1. *Total proteins during different days of germination of wheat and barley cultivars*

From the LSD test (data not shown), it can be noted that the amount of total proteins in barley is higher (average 13.60 %) compared to the total proteins determined in wheat (average 13.05 %) and it is statistically significant ($p < 0.05$). Regarding the days of germination, the change in the total proteins content is insignificant, with the average amount of proteins for samples I, II, III, IV, V and VI as 13.56 %, 12.52 %, 13.58 %, 13.83 %, 13.05 % and 13.92 %, respectively.

Protein solubility is important in food production and it defines the type of food that can be produced (liquid or solid), the type of processing that is required (thermal processing, mixing, etc.) and the time required to carry out these processes. On the other hand during storage, protein solubility affects various quality characteristics such as appearance, sedimentation, viscosity and flavor [22]. Contrary to the total proteins, the amount of soluble proteins tends to increase with extended germination time due to enzymes activity that breaks down the storage proteins in the grain into smaller soluble peptides and amino acids, which can then be used as nutrients for the developing plant (Figure 2).

In general, the lowest amount of soluble proteins in all samples was examined at the beginning of germination at day 0 (2.49 % for I; 2.74 % for II; 3.14 % for III; 3.17 % for IV; 3.95 % for V and 3.95 % for VI). Soluble proteins in the cultivars Osvit and Mandatar (samples V and VI in the current research) from 2016 contained 4.68 and 4.68 % respectively, from harvest of 2017 contained 3.54 and 3.56 % respectively; in 2018, their amount was 2.64 % and 3.44 %, respectively and in 2019 - 2.44 % and 2.76 %, respectively [20]. The increase in soluble proteins during germination as is a result of enzymatic degradation of storage proteins with the extension of the germination time has been proven by many auth. The influence of germination time and temperature on the biochemical and structural properties of HomChaiya rice was determined by Leitgeb et *al.* [23]. The authors indicate that the time of germination has a greater influence on the soluble proteins content than the temperature at which this process takes place. Soluble proteins are the main source of nitrogen for the germinated grain with the quantity dependent on the protease activity besides the type of grain. By extending the germination time from 12 to 72 hours, Paucar-Menacho et *al.* [24]

observed that soluble proteins in soybean cultivar BRS-2508 increase. del Rosario Moguel Concha et *al.* [21] also confirmed that the total protein content in *P. Vulgaris* after 72 hours of germination increased by 80 % from the initial content. Endopeptidases located in the cotyledons cleave peptide bonds within the protein molecule, producing smaller peptides, while exopeptidases hydrolyze the peptides from the ends to produce free amino acids. These free amino acids are then used for the synthesis of new proteins and other essential molecules. This process is critical for the growth and development of the germinating seedling, as it provides a source of nitrogen for the developing plant [25 – 27].

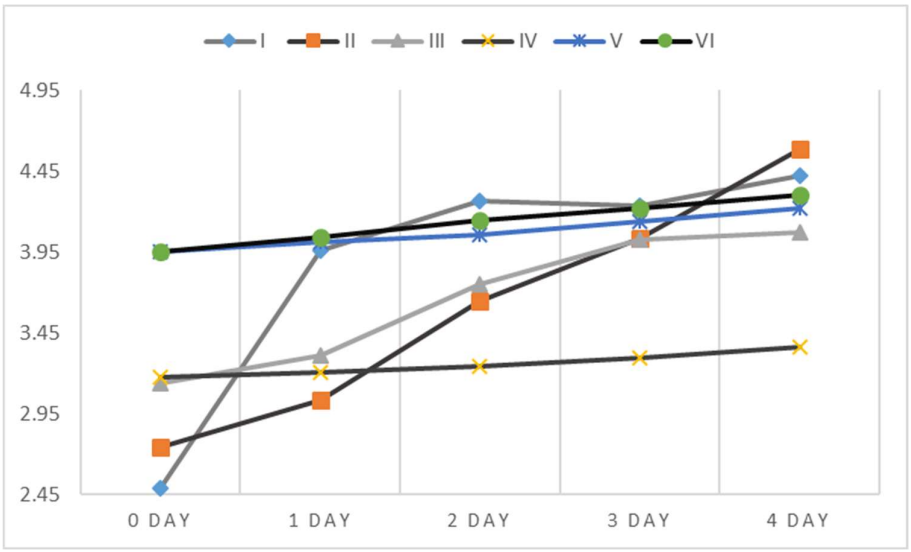

Figure 2. *Soluble proteins during different days of germination of wheat and barley*

Enzymes present in cereals initiate many chemical changes that affect the compositional and functional properties of flour. Enzymes in cereals and flour are usually present in an inactive form during storage, but when exposed to water, they become activated and start catalyzing reactions that affect the functional properties of flour. Cellulases, amalyses, and pentonases are enzymes that hydrolyze carbohydrates while proteases hydrolyze proteins [23]. The protease plays an essential role in plant physiology and development. During seed germination, high protease activity cleave gluten and from a technological point of view, reduces the mixing time, improves the dough knead ability, and increases the extensibility and retention of gases in the dough [28]. Figure 3 shows the protease activity of the flours obtained from grains that were germinated for a different period of time from 0 to 4 days. It's interesting to note that barley has a higher proteolytic activity compared to wheat during germination. Additionally, the proteolytic activity increases with the extension of germination time and is not much affected by kilning temperature. The greatest increase in proteolytic activity occurs after 48 hours from the start of the germination process, according to Zafar et *al.* [29]. Furthermore, Silveira et *al.* [30] suggest that *p*H values can influence protease activity. This highlights the importance of controlling various factors, such as grain type, germination time, temperature, and *p*H, to optimize the proteolytic activity during the germination process.

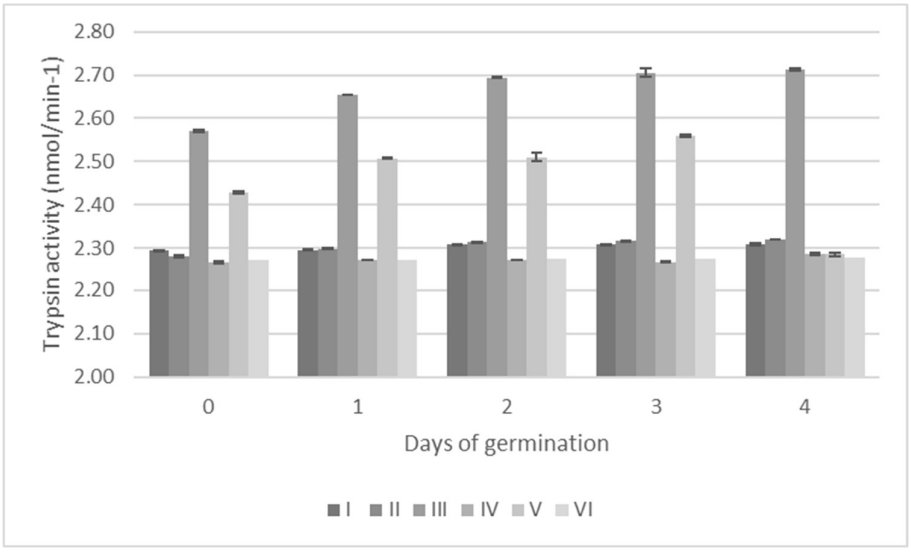

Figure 3. *Protease activity in different days of germination of wheat and barley*

CONCLUSION

Malting process can significantly improve the nutritional and functional properties of cereals, making them more valuable as food and beverage ingredients. Additionally, the malting process can also lead to the production of enzymes such as α-amylase and β-glucanase, which can break down carbohydrates in the grain into simpler sugars, making them more available for yeast fermentation. It is interesting to note that while the content of total proteins did not significantly change during the germination process, there were significant differences in the soluble protein content between the samples. This suggests that while the total amount of proteins may remain relatively stable, the nature of the proteins present may change during germination, possibly due to the breakdown of storage proteins into smaller, more soluble peptides and amino acids. It would be informative to investigate the specific protein profiles and changes in protease activity during the germination process to better understand these observed changes in protein content. In general, protease activity also increased with increasing of the germination time. When the different studied cereals (wheat and barley) are compared, germinated barley grains have a statistically higher ($p < 0.05$) content of total and soluble proteins and higher protease activity. The extent and duration of the malting process can be adjusted to optimize these properties based on the specific needs of the end product.

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