Evaluation of protease enzymes extracted from germinated barley on the degradation of gluten and biscuit proteins

Abstract
Gluten and gliadin, which are present in wheat, barley, and rye, can cause celiac disease that causes intestinal inflammation and nutrient loss in those who are genetically predisposed. Currently, the only effective treatment is strict lifelong gluten abstinence. This study aimed to evaluate the efficiency of protease enzymes extracted from germinated barley in degrading gluten proteins, with a particular focus on their application in the production of rice biscuits suitable for individuals with gluten sensitivity. Protease enzymes were extracted and partial purified from germinated barley and characterized for their activity and stability at different temperatures and pH levels. The enzymatic hydrolysis of gluten and gliadin proteins was assessed using protein determination, residual gluten quantification and docking study of barley protease and the peptide fragment of gluten and gliadin. The results showed that protease was partially purified by using 60% saturated crude extract salted-out by ammonium sulphate, followed by dialysis. The stability of protease enzyme was by evaluating the enzymatic activity at different pH values and temperature. The results showed that germination significantly enhanced protease activity compared to ungerminated barley, with optimum activity observed at 35–45 °C and pH 7.0. Treatment with the extracted protease caused extensive degradation of gluten (14.75 %). Incorporation of the germinated barley protease into rice biscuit formulations improved dough handling, texture, and sensory properties while reducing gluten levels to a range suitable for gluten-sensitive consumers. These findings highlight the potential of germinated barley protease as a natural, plant-based enzyme source for producing gluten-reduced rice biscuits, offering a promising strategy for developing functional bakery products tailored for individuals affected by celiac disease.

Keywords: Germinated barley, Protease enzyme, Gluten degradation, Rice biscuit, gluten sensitivity, enzymatic hydrolysis.

1. Introduction

Gluten sensitivity, encompassing celiac disease and non-celiac gluten intolerance, affects an increasing portion of the worldwide population, hence boosting the need for gluten-free baked goods that possess satisfactory texture, flavor, and nutritional quality **(Foschia et al., 2016; Catassi et al., 2022; Bianchi et al., 2024)**. Rice flour is one of the most commonly utilized gluten-free substances, attributed to its neutral flavor, hypoallergenic properties, and digestibility **(Sivaramakrishnan et al., 2004; Park and Kim, 2023)**. One of the most significant cereal foods in the world, rice (Oryza sativa L.) is hypoallergenic, naturally gluten-free, and easily digestible. Rice flour, however, is devoid of the viscoelastic gluten network necessary for gas storage and structural development during baking, frequently yielding brittle, dense, and less tasty biscuits **(Renzetti & Arendt, 2009; Phongthai et al., 2017)**.

Enzymatic modification has emerged as a viable technique to enhance the functional and sensory properties of gluten-free doughs **(Pashaei et al., 2025)**. Proteases are essential for hydrolyzing rice proteins into smaller peptides and amino acids, hence improving dough softness, spreadability, and Maillard browning during baking **(Kumari et al., 2020)**. Moreover, protease treatment enhances protein digestion and may elevate the nutritional quality of gluten-free products **(Renzetti & Arendt, 2009)**. Protease enzymes are important in the baking manufacturing industry improving bread volume, texture, dough handling qualities, and overall product quality. These enzymes change the rheology of dough and help retain gas during fermentation and baking by hydrolyzing gluten and other wheat proteins into smaller peptides and amino acids. Improved machinability and consistent crumb structure result from the regulated breakdown of proteins, which lowers dough elasticity **(Liaquat et al., 2025).**

Protease supplementation in bread production can reduce mixing time, increase extensibility, and enhance loaf volume **(Caballero et al., 2007)**. It also facilitates flavor development by producing free amino acids that function as precursors for Maillard reactions during baking **(Hoseney & Rogers, 1990**). Furthermore, protease-treated doughs demonstrate enhanced shelf life and decreased staling owing to superior moisture retention **(Liaquat et al., 2025)**. The flour supplementation with enzyme improvers (technical enzymes) is a normal way for flour standardization and facilitates baking process **(Pedersen et al., 2005).** In food industry, proteases are used in the manufacturing of baked goods like bread, waffles, and crackers. These enzymes may be added to shorten mixing period, to reduce dough elasticity, to promise dough consistency, to adjust gluten strength in bread, and to regulate bread texture and flavors **(Kamel and Stauffer, 2013).** It is noteworthy that rice flour is suitable for biscuits preparation that is low in protein, ash, and damaged starch. The most important features of biscuits are its texture which depends on flour protein content. The application of proteases in dough reduces gluten elasticity due to breakdown of glutenin proteins, which is accountable to the dough elasticity, thus improving the spread ratio of. **(Hassan et al., 2015; Saberi et al., 2024).**

Glutelin is the major fraction, which constitutes about 80% of the total rice endosperm protein **(Martins-Meyer et al., 2013; Pourmohammadi, and Abedi, 2021)**. Protein has been reported to influence rice cooking and eating qualities: the higher the protein content, the firmer the texture of cooked rice **(Pedersen et al., 2005**; **Kamel and Stauffer, 2013)**. The effect of protein on cooking properties has led to aim at improving the texture of rice gel by removing or disrupting its protein. protein-hydrolysing enzyme can be used to remove rice flour protein **(Saberi et al., 2024).** The effect of protease treatments on rice starch. Here, the paste viscosity of rice flour treated with protease was lower than those treated with water (without enzyme). The strength of swollen granules was conferred by protein with intact disulfide bonds. Protease removed mostly starch granule surface protein, hence its starch granules remained more intact and were more tolerant to shear force. protease treatments, played an important role in impairing the gel-forming property of rice flour. Textural quality was observed to improve after their removal, compared to native flour. So, the aim of this work is to extract and purify the protease enzymes from germinated barley

grains, to evaluate its ability for the digestion of dietary gluten and gliadin. Also, its role in improving the quality and rheological properties of doughs. This research will also offer new perspectives to improve enzyme applications for better biscuits composition by conducting an extensive investigation on enzyme treated dough and biscuits. Moreover, outcomes will be helpful for large-scale manufacturers who have concerned about the dough handling and product uniformity

2. MATERIALS AND METHODS

2. 1. Materials

Barley grains (Giza 134) were obtained from Agricultural Research Center, Giza, Egypt, all chemicals used for the experiment including Comassie Brillient Blue G-250, casein (purity of 99.0%), ammonium sulphate ($(NH_4)_2SO_4$) and trichloroacetic acid (TCA) were purchased from Sigma Aldrich, Germany. Deionized water was used in all experiments. The following food ingredients: wheat flour 72% extraction broken rice flour, sugar powder, butter, salt, baking powder, eggs and were obtained from local market, Cairo, Egypt.

2.2. Germination of barley grains.

After being cleaned, the grains were sun-dried and stored. barley kernels were covered with cotton in a Petri dish with a 90 mm diameter that has been filled with 5.5 mL of deionized water. For seven days, the Petri dishes were stored at 25 ºC while being covered with a polyethylene bag. The seeds that had germinated were gathered and kept at -20 ºC (**Shaha., 2002) (see Figure 1)**.

Figure 1. Germination stages of barley grains for 7 days.

2.3. Extraction of crude enzyme from sprouted barley by different methods

Four grams of germinated barley grain after 5 and 7 days were placed in a mortar and pounded into a fine powder with and without liquid nitrogen, thereafter combined with 50 mL of acetate buffer (50 mM) at pH 5.5 for extraction, also extracted protease enzyme from sprouted barley by Ultrasonic at 25ºC for 0., 1, 2 and 4 min, followed by centrifugation of the filtrate for 15 minutes at 10,000 rpm. The transparent supernatant containing enzymes was lyophilized and utilized as crude enzyme (**Gessendorfer et al., 2011)**.

2.4. Barley acidic protease activity assay

Proteases hydrolysed casein into smaller peptides and amino acids, which absorb UV light at 280 nm due to the presence of aromatic amino acids. The increase in absorbance at 280 nm corresponds to proteolytic activity. The protease activity was measured using casein (1% w/v in 50 mM Tris-HCl buffer, pH 7.5) as a substrate. The procedure for the determination of the protease activity was described by **(Ullah *et al.*, 2022)**. The protease activity was calculated using the following Equation:

$$\text{Enzyme activity (U/mL)} = \frac{(\text{A280 of sample} - \text{A280 of blank}) \times V_t}{0.001 \times t \times V_e} \quad :$$

2.5. Determination of protein content.

The Bradford method was used to assess the concentrations of total protein (**Bradford., 1976)**. Rapid Comassie Brillient Blue G-250 dye binding to protein is the basis for the Bradford assay. 300 µL of Bradford reagent was mixed with 10 µL of protein fraction, incubated for five minutes, and then analyzed at 595 nm.

2.6. Partial purification of barley acidic protease.

The enzyme precipitated by salting out the crude barley acidic protease extract by using ammonium sulphate in different ranges of saturation (20 - 100 %) following a slightly modified method to **(Wang et al., 2022**). The solution stirred continuously at room temperature to fully dissolve the salt, then incubated at 4 ºC for 2 hours. The precipitate obtained by centrifugation at 10000 rpm and 4 ºC for 15 minutes, dried, weighted, then redissolved in acetate buffer (50 mM, pH 5.5). fractions undergone several evaluations and started with estimating the protease activity. The selected purified fraction was dialyzed for 24 hours at 4 ºC, the fraction was collected, assayed for their protease activity **(Ullah *et al*., 2022).**

2.7. Optimum pH.

A 0.2M sodium acetate buffer (pH 4- 5.5), a 0.2 M sodium phosphate buffer (pH 6-7.5) and a Tris-HCl buffer (pH 7.5-9) was used as a reaction medium to determine the optimum pH for the enzyme's activity **(Abdulaal, 2018; Ullah *et al*., 2022**).

2.8 Optimum temperature:

200 µL of a Tris-HCl buffer was incubated at a concentration of 50 mM pH 7.5 and 100 µL of the casein reaction solution (1%) at different temperatures ranging from 25-65 °C for 15 mins, then 10 µL of the purified enzyme solution were added to it and left for another 15 mins at the same temperatures, then the reaction was stopped and the enzymatic activity was estimated **(Abdulaal, 2018; Ullah *et al*., 2022**).

2.9. Stability of barley acidic protease.

The purified enzyme solution was incubated at different temperatures ranging from (4 and 25) °C for a period of 15 mins. Then the tubes containing the enzyme solution were transferred to an ice bath, then transferred to a water bath at 35 °C, and the reaction solution of casein and Tris-HCl buffer was added at a concentration of 50 mM, pH 7.5, of the prepared and incubated for a period of time15 mins, then the reaction was stopped and the enzyme activity was estimated **(Abdulaal, 2018; Ullah et al., 2022).**

2.10. Enzymatic hydrolysis (gluten and gliadin degradation by barley acidic protease.

2.10.1. Extraction of gliadin:

Gliadin was extracted by treated of gluten with 70% ethanol and stirred for 45 mins, then centrifuged at 6000 rpm for 10 min, The precipitate was separated, the ethanol was removed, and it was lyophilized **(Hartmann et al., 2006).**

2.10.2. Gluten and gliadin hydrolysis

The partially purified extracted barley protease enzyme concentrations were 1.0 and 2.0 w/v. The treatment was carried out using 100 mL of a 2% gluten or gliadin were prepared by phosphate -citrate buffer pH 6.9 and then of barley acidic protease to proteins solution. The mixture was incubated at 31 ºC for 2h with stirring in ice bath. The hydrolyzed protein solution was heated for 30 second at 100°C to inactivated the protease **(Shaha et al., 2002)**. Then, protein determination in enzymatically treated gluten or gliadin by Bradford method.

2.11. Molecular docking of barley acidic protease.

Protein Structure Prediction and Docking Studies

The tertiary structure of the type 1 α2-gliadin 33-mer gliadin peptide fragment was predicted using advanced AI-based modeling tools, including AlphaFold3 (1) and ESMFold (2). Subsequently, AlphaFold3 (1) was employed to predict and dock the Cysteine proteinase EP-B2 enzyme (UniProt ID: P25250) with the same gliadin peptide fragment, using only their amino acid sequences as input. The protein was prepared for docking using the AutoDockTools 1.5.6 suite **(Morris et al., 2009; Kim et al 2021).** All non -protein atoms, including crystallographic waters and ligands, were removed. Polar hydrogen atoms were added, and the protein structure was cleaned by merging non-polar hydrogen atoms and assigning Gasteiger charges. The three-dimensional structure of ligands was obtained from the PubChem database **(O'Boyle et al., 2011).** The ligand structure was prepared using Avogadro 1.2.0 **(Hanwell et al., 2012)** by adding hydrogen atoms, assigning partial charges (Gasteiger method), and performing energy minimization using the UFF force field. The potential binding site on the Cysteine proteinase EP-B 2protein was predicted using the CB-Dock2 web server **(Shib et al., 2021)**, which employs a deep learning-based approach for identifying putative binding pockets and cavities. Molecular docking simulations were performed using AutoDock 4.2 **(Liu et al., 2022)** with the genetic algorithm. The AutoGrid 4 program was used to calculate grid maps for the predicted binding site with a grid box size of 20 Å x 20 Å x 20 Å and a grid spacing of 0.375 Å.

Supplementary materials.

pLDDT: a per-atom confidence estimates on a 0-100 scale where a higher value indicates higher confidence.

pTM score: the predicted template modeling (pTM) score derived from a measure called the template modeling (TM) score. This measures the accuracy of the entire structure**.** pTM score above 0.5 means the overall predicted fold for the complex might be similar to the true structure.

Protein sequence

- >sp|**P25250|CYSP2**_HORVU **Cysteine proteinase EP-B2** OS=Hordeum vulgare OX=4513 GN=EPB2 PE=1 SV=1

MGLLSKKLLVASMVAAVLAVAAVELCSAIPMEDKDLESEEALWDLYERWQSAHRVRRHHA
EKHRRFGTFKSNAHFIHSHNKRGDHPYRLHLNRFGDMDQAEFRATFVGDLRRDTPSKPPS
VPGFMYAALNVSDLPPSVDWRQKGAVTGVKDQGKCGSCWAFSTVVSVEGINAIRTGSLVS
LSEQELIDCDTADNDGCQGGLMDNAFEYIKNNGGLITEAAYPYRAARGTCNVARAAQNSP
VVVHIDGHQDVPANSEEDLARAVANQPVSVAVEASGKAFMFYSEGVFTGECGTELDHGVA
VVGYGVAEDGKAYWTVKNSWGPSWGEQGYIRVEKDSGASGGLCGIAMEASYPVKTYSKPK
PTPRRALGARESL

Peptide sequence
LQLQPFPQPQLPYPQPQLPQPQLPYPQPQPF

Glutelin_AF-Q9ZWJ8:
AVFAPRRGPQQYAEWQINEK
LIPQNFAVVVKARREGFAWVSFKTNHNAVDSQIAGKASILRALPVDVVANAYRLSREDSRHVKFNRGDEM
MASMSTILPLCLGLLLFFQVSMAQFSFGGSPLQSPRGFRGDQDSRHQCRFEHLTALEATHQQRSEAGFTE
YYNIEARNEFRCAGVSVRRLVVESKGLVLPMYANAHKLVYIVQGRGVFGMALPGCPETFQSVRSPFEQEV
ATAGEAQSSIQKMRDEHQQLHQFHQGDVIAVPAGVAHWLYNNGDSPVVAFTVIDTSNNANQLDPKRREFF
LAGKPRSSWQQQSYSYQTEQLSRNQNIFAGFSPDLLSEALSVSKQTVLRLQGLSDPRGAIIRVENGLQAL
QPSLQVEPVKEEQTQAYLPTKQLQPTWLRSGGACGQQNVLDEIMCAFKLRKNIDNPQSSDIFNPHGGRIT
RANSQNFPILNIIQMSATRIVLQNNALLTPHWTVNAHTVMYVTAGQGHIQVVDHRGRSVFDGELHQQQIL

2.12. Aapplication partial purified barley acidic protease on quality and rheological properties of doughs.

2.12.1. Viscosity properties of rice flour.

Viscosity of the batter were measured directly with Brookfield Digital Rheometer, model HA DVIII Ultra (Brookfield Engineering Laboratories INC) according to the methods described by **Brookfield manual (2023**). The sample was placed in a beaker and allowed to rest for 20 min, the selected spindle was RV-7 for the sample measurement speed 10. A thermostatic water bath provided with the instrument was used to regulate the sample temperature. The apparent viscosity of the samples was measured at room temperature (25°C).

2.12.2. Biscuit making

The wheat and rice biscuit without and with barley protease were prepared using the methods described by AOAC **(AOAC 2002)**. the flours (200 g), butter (33 g) and salt (0.2 g) were mixed together manually for 5 min to get a creamy dough. The baking powder (2 g), fortified milk (15 mL), whole eggs (1.25 mL), sugar (1.25 g), vanilla (1.0 g) were mixed thoroughly. Then, 65 mL of water was gradually added using continuous mixing unit good texture, slightly firm dough is obtained. The dough was kneaded on a clean flat surface for 4 mins. It was manually into sheets and cut into shapes using the stamp cutting method. The cut dough pieces were transferred into fluid fat grease pans and baked in an oven at 180 C for 20 min. cooled and packed for further analysis. For the rice biscuit with barley protease, the biscuit was prepared in the same way as described above, with the added 1% barley protease enzyme from rice flour.

2.12.2.1. Texture profile analysis (TPA)

A texture analyzer (Brookfield CT3 Texture Analyzer operating instructions, manual No M08-372-C0113, Stable Micro Systems, USA) was used to specifically measuring the hardness, chewiness, cohesiveness, resilience, adhesive ness, springiness, gumminess, and fracturability parameters. The experiments were conducted according to **(Ashwini, et al., 2009).**

2.12.2.2. Water activity and moisture

Water activity of biscuit was measured at zero time using a Rotronic Hygro Lab water activity meter EA10-SCs (Switzerland) at 25 ± 2 ºC according to Samples were crushed into small pieces and a representative sample was placed into plastic cups and measured one at a time. The methods were carried out as described by **Mathlouthi (2001)**. Determination of moisture according to AOAC **(AOAC 2002)**.

2.13. Statistics

The resulted protease activity of the FPLC fractionations were statistically analysed by the one-way and two-way ANOVA analyses using the SPSS statistical software. The data represented on the graph as the error bars for the mean ± standard deviation for the protease activity of three replicates. The results obtained from the hardness test was statistically analyzed by using the paired - sample T test (to compare between cycle 1 and 2) followed by the Friedman's test (to compare between the hardness after and before the treatment).

3. RESULTS AND DISCUSSION

Proteases represent one of the most important categories of enzymes and are gaining widespread attention in attempts to utilize their industrial and biological applications. In this investigation, partial purifications and biochemical characterizations of a protease from sprouted barley grains. The crude extract contained the acidic protease was extracted successive methods. **Tables 1,2,3 and Figure 2** demonstrates the extraction method markedly influenced the total protein content, total activity, and specific activity of germinated barley protease for 5 and 7 days. Among the tested methods, grinding with mortar and pestle in the presence of liquid nitrogen produced the highest enzyme efficiency, with a total activity of 146.75 ± 0.250 U/mL and specific activity of 633.75 ±32.283 U/mg. The use of liquid nitrogen facilitates efficient tissue

disruption while preventing heat accumulation, thereby maintaining enzyme activity during homogenization **(Kokkat et al., 2011; Shalem et al., 2024)**. Similarly, extraction using mortar and pestle without liquid nitrogen yielded high activity values, suggesting that conventional mechanical disruption is still effective for protease release when temperature is carefully controlled.

Table 1. Total protein, acidic protease enzyme activity and specific activity of germinated barley for 5 days.

Method of extraction	Total protein (mg/mL)	Total activity (U/mL)	Specific activity (U/mg)
Motar and pestle	0.2313±0.01111^{b}	140.5000±2.63391^{a}	608.3967±30.03583^{ab}
Motar and pestle with liquid N_2	0.2320±0.01253^{ab}	146.7500±0.25000^{a}	633.7533±32.28384^{a}
Ultrasonic 0.5 min/ 25ºC	0.2425±0.00231ab	143.5833±6.42424^{a}	592.2900±28.91687^{b}
Ultrasonic 1 min/ 25ºC	0.2393±0.00153^{ab}	65.3333±3.84328^{c}	273.0067±16.62496^{d}
Ultrasonic 2 min/ 25ºC	0.2450±0.00361^{a}	55.9167±5.07650^{d}	228.2333±20.39477^{e}
Ultrasonic 4 min/ 25ºC	0.2420±0.00265^{ab}	52.0833±0.94648^{d}	215.2333±4.16589^{e}
After freeze dryer	0.2407±0.00551^{ab}	131.2500±1.52069^{b}	545.4667±7.62682^{c}

In contrast, ultrasonic-assisted extraction resulted in a progressive decrease in total and specific activity with increasing sonication time. Mild sonication (0.5 min) maintained enzyme activity comparable to the Motar and pestle with and without liquid nitrogen methods, but extended sonication (4 min) significantly reduced activity 52.08±0.946 U/mL, with the lowest value of specific activity (215.23 U/mg) recorded at 4 min). Freeze-drying of the extract resulted in a moderate decrease in enzyme activity (131.25±1.520 U/mL) compared with the freshly prepared sample, although the total protein concentration remained nearly constant. The reduction in specific activity after freeze-drying (545.47±7.626 U/mg) may be due to conformational alterations or partial inactivation during the dehydration and rehydration processes **(Obeidat et al., 2018; Wang *et al*., 2019; Nowak and Jakubczyk, 2020).** Nevertheless, the retention of considerable activity suggests that freeze-drying is a suitable approach for long-term storage of barley protease without major loss of enzymatic function. Overall, these results indicate that mechanical grinding with liquid nitrogen is the most effective and stable extraction method for barley protease enzymes. Ultrasonic extraction, although rapid and efficient, must be carefully optimized to avoid enzyme damage.

Table 2. Total protein, acidic protease enzyme activity and specific activity of germinated barley for 7 days.

Method of extraction	Total protein (mg/mL)	Total activity (U/mL)	Specific activity (U/mg)
Motar and pestle	0.2153±0.00025^{a}	193.5000±0.66144^{a}	898.8244±2.13318^{a}
Motar and pestle with liquid N_2	0.2153±0.00017^{a}	197.3083±0.17017^{a}	916.4495±0.49041^{a}
Ultrasonic 0.5 min/ 25ºC	0.2150±0.00006^{a}	156.4167±3.75278^{c}	727.3922±17.64883^{b}
Ultrasonic 1 min/ 25ºC	0.2156±0.00049^{a}	128.7500±1.14564^{d}	597.1054±5.69117^{c}
Ultrasonic 2 min/ 25ºC	0.2085±0.00280^{b}	125.8333±1.66458^{d}	603.5146±16.26471^{c}
Ultrasonic 4 min/ 25ºC	0.2150±0.00015^{a}	113.4167±2.80995^{e}	527.5267±13.4240^{d}
After freeze dryer	0.2053±0.00020^{c}	188.5833±3.00347^{b}	918.5499±14.09387^{a}

The findings shown in **Table 3** show that the total protease activity recovered from barley was highly impacted by both the extraction technique and the germination time. Enzyme activity increased after 7 days of germination in all extraction procedures compared to 5 days, suggesting that enzyme production progressed throughout seed germination. During germination, protease enzymes are essential for mobilizing storage proteins, and as the embryo grows and hydrolytic enzymes are activated, their concentration usually increases **(Zafar et al., 2005; Bretträger et al., 2025).** The extraction technique that produced the highest overall activity at both germination phases was crushing with a mortar and pestle while liquid nitrogen was present. By reducing thermal denaturation during homogenization, this technique effectively disrupts cells while maintaining enzyme integrity, which accounts for its higher performance. Although somewhat less than that obtained with liquid nitrogen, the traditional mortar and pestle method still yielded comparatively high activity, suggesting that stabilizing plant proteases during extraction benefits from a low temperature.

The freeze-dried extract exhibited a notably high overall activity, particularly following 7 days of germination. Despite the potential for structural alterations caused by ice crystal formation and dehydration stress during freeze-drying, the retention of significant activity indicates that this technique efficiently preserves enzyme stability throughout storage. This discovery endorses freeze-drying as an appropriate post-extraction method for the long-term preservation of barley protease. These data indicate that germination length and extraction conditions are critical factors affecting the recovery of active barley protease. Prolonging germination from 5 to 7 days augments enzyme production, whereas extraction via liquid nitrogen-assisted grinding yields the most effective recovery of active enzyme. Ultrasonic extraction has opportunities for expedited processing; however, it requires meticulous optimization to prevent activity loss from excessive exposure.

Table 3. Total protein, acidic protease enzyme activity and specific activity of germinated barley for 5 and 7 days.

	motar and pestle	motar and pestle with liqued N2	ultrasonic 0.5 min 25C	ultrasonic 1 min 25C	ultrasonic 2 min 25C	ultrasonic	after freez dryer	mean
				Protein (mg/mL)				
5 days	0.225^{d}	0.225^{d}	0.241^{bc}	0.239^{c}	0.247^{a}	0.241^{bc}	0.243^{b}	**0.237**
7 days	0.215^{e}	0.215^{e}	0.215^{e}	0.216^{e}	0.212^{f}	0.215^{e}	0.205^{g}	**0.213**
LSD	**G: 0.001**	**M: 0.002**	**MG:0.003**					
				Total Activity (U/mL)				
5 days	140.500^{d}	146.750^{c}	143.583^{e}	65.333^{g}	55.917^{h}	52.083^{h}	131.250^{f}	**105.060**
7 days	193.500^{a}	197.308^{a}	156.417^{b}	128.750^{e}	125.833^{e}	113.416^{f}	188.583^{a}	**157.687**
LSD	**G: 8.72**	**M: 16.31**	**MG: 23.07**					
				Specific Activity (U/mg)				
5 days	624.854^{e}	652.965^{d}	596.678^{de}	273.007^{i}	226.436^{j}	216.568^{j}	539.384^{f}	**447.128**
7 days	898.824^{b}	916.449^{a}	727.392^{c}	597.105f^{g}	594.146^{g}	527.526^{h}	918.549^{b}	**739.999**
LSD	**G: 1.908**	**M: 3.570**	**MG: 5.049**					

G: germination, **M:** Method of extraction and **MG**: interaction between G and M, Letters beside values: Duncan's multiple range test

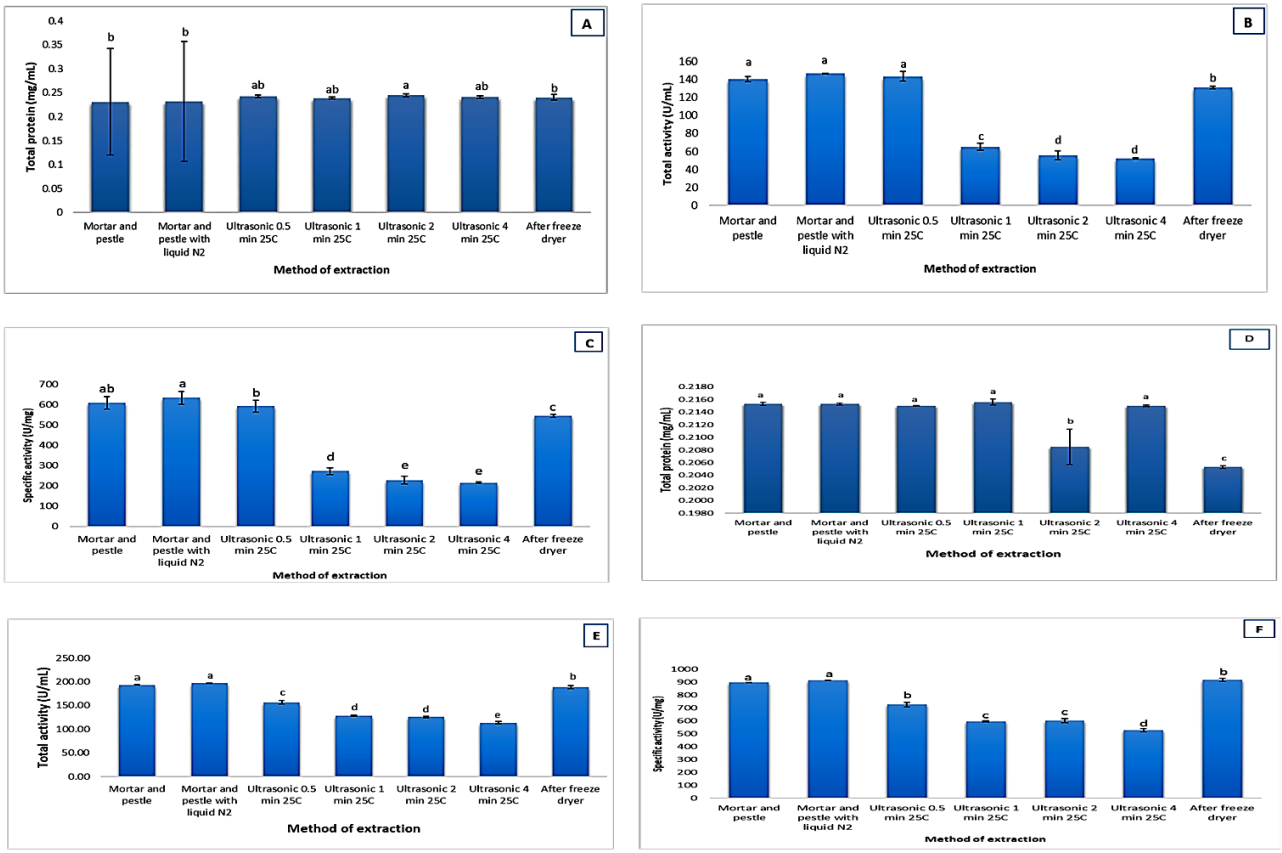

Figure 2. Total protein **(A)**, acidic protease enzyme activity **(B)** and specific activity **(C)** of germinated barley for 5 days, Total protein **(D)**, acidic protease enzyme activity **(E)** and specific activity **(F)** of germinated barley for 7 days

Results of partial purification of protease extracted from barley grains germinated for 7 days by ammonium sulfate is illustrated in **Tables 4, 5 and Figure 3**. Protease activity was evident at all saturation levels; however, the extent of enzyme recovery varied significantly based on the concentration of ammonium sulphate. The maximum protease activity occurred in the pellet fraction at 60% saturation, reaching around 1100 U/mg/mL, indicating this as the optimal precipitation threshold for barley protease. This concentration presumably provided sufficient ionic strength to promote selective protein aggregation without causing irreversible enzyme denaturation. At diminished saturation levels (20–40%), significant enzymatic activity was seen in both supernatant and pellet fractions, suggesting insufficient precipitation of the target enzyme. The incomplete preservation of activity in the supernatant at 40% saturation. As the concentration of ammonium sulphate increased to 60%, the majority of the enzyme was successfully precipitated into the pellet, signifying the balance between reduced solubility and protein aggregation.

Increased salt concentrations (80–100%) led to a decrease in protease activity in the pellets, perhaps due to elevated ionic strength causing enzyme aggregation or structural changes that reduced catalytic efficiency (**Rao et al., 1998**). Moreover, the residual enzyme activity seen in the supernatant at high saturation levels suggests that excessive ammonium sulphate may impede the full precipitation of some enzyme isoforms by altering the hydration shells encasing the protein molecules **(Scopes, 1982)**. In the result, a 60% saturation of ammonium sulphate was identified as the best concentration for the precipitation and concentration of barley protease. This method enhanced enzyme recovery while reducing activity loss, hence supporting its use as an initial purification stage prior to further processing.

Table 4: Summary for the barley protease fractions purified by ammonium sulfate

Ammonium Sulphate (%)	Total protein (mg/mL)	Total activity (U/mL)	Specific activity (U/mg)
20% (in supernatent)	0.0423 ± 0.00153^{i}	264.3333 ± 1.52753^{i}	6267.25 ± 176.13084^{cd}
20% (in pellets)	0.0327 ± 0.00115^{g}	163.3333 ± 1.52753^{g}	4989.46 ± 84.84436^{de}
40% (in supernatant)	$0.0510\pm.00100^{e}$	394.3333 ± 1.52753^{e}	$7705.48\pm105.40006^{abc}$
40% (in pellets)	$0.0273\pm.00577^{d}$	435.3333 ± 7.02377^{d}	8611.57 ± 572.29842^{ab}
60% (in supernatant)	$0.0407\pm.00208^{f}$	290.0000 ± 1.52753^{f}	7115.61 ± 337.93846^{bc}
60% (in pellets)	$0.1067\pm.00058^{a}$	960.3333 ± 13.45362^{a}	9010.67 ± 131.86291^{a}
80% (in supernatant)	$0.0090\pm.00100^{j}$	74.0000 ± 5.56776^{j}	$8529.75\pm1627.40755^{ab}$
80% (in pellets)	$0.1140\pm.00100^{b}$	712.0000 ± 5.29150^{b}	6232.52 ± 37.42327^{cd}
100% (in supernatent)	$0.0077\pm.00115^{h}$	68.3333 ± 6.65833^{h}	$8573.21\pm1834.68070^{ab}$
100% (in pellets)	$0.1163\pm.00153^{c}$	522.3333 ± 15.17674^{c}	4478.30 ± 77.84223^{e}

The purification profile of the protease enzyme obtained from sprouted barley demonstrated a consistent increase in specific activity at every purification stage, indicating the effective removal of non-enzymatic proteins and the concentration of the target enzyme **(Table 5)**. The crude extract had a total protein concentration of 0.215 mg/mL and a total activity of 189 U/mL, resulting in a specific activity of 875.89 U/mg, reflecting the fundamental enzyme quality of the raw extract. Following 60% ammonium sulphate precipitation, substantial changes occurred in both total protein and total activity. The total protein concentration decreased to 0.1067 mg/mL, but overall activity increased to 960.33 U/mL, resulting in an almost 10-fold enhancement in specific activity (9010.67 U/mg). This result demonstrates that salting-out successfully precipitated the protease fraction while eliminating impurities (**Verma et al., 2019)**. Ammonium sulphate precipitation is an established technique in protease purification, efficient for stabilising enzyme conformation and separating protein fractions based on solubility differences **(Gupta et al., 2002)**. Following dialysis enhanced enzyme purity, resulting in a total protein concentration of 0.0513 mg/mL and an increase in total activity to 1018.33 U, achieving a maximum specific activity of 19920.69 U/mg—over 22 times more than that of the crude extract. This notable improvement suggests that salts and low-molecular-weight contaminants that can impair enzyme performance have been successfully removed **(Rao et al., 1998).** By removing excess ammonium sulphate, dialysis helps restore enzyme structure and catalytic function **(Verma et al., 2019).**

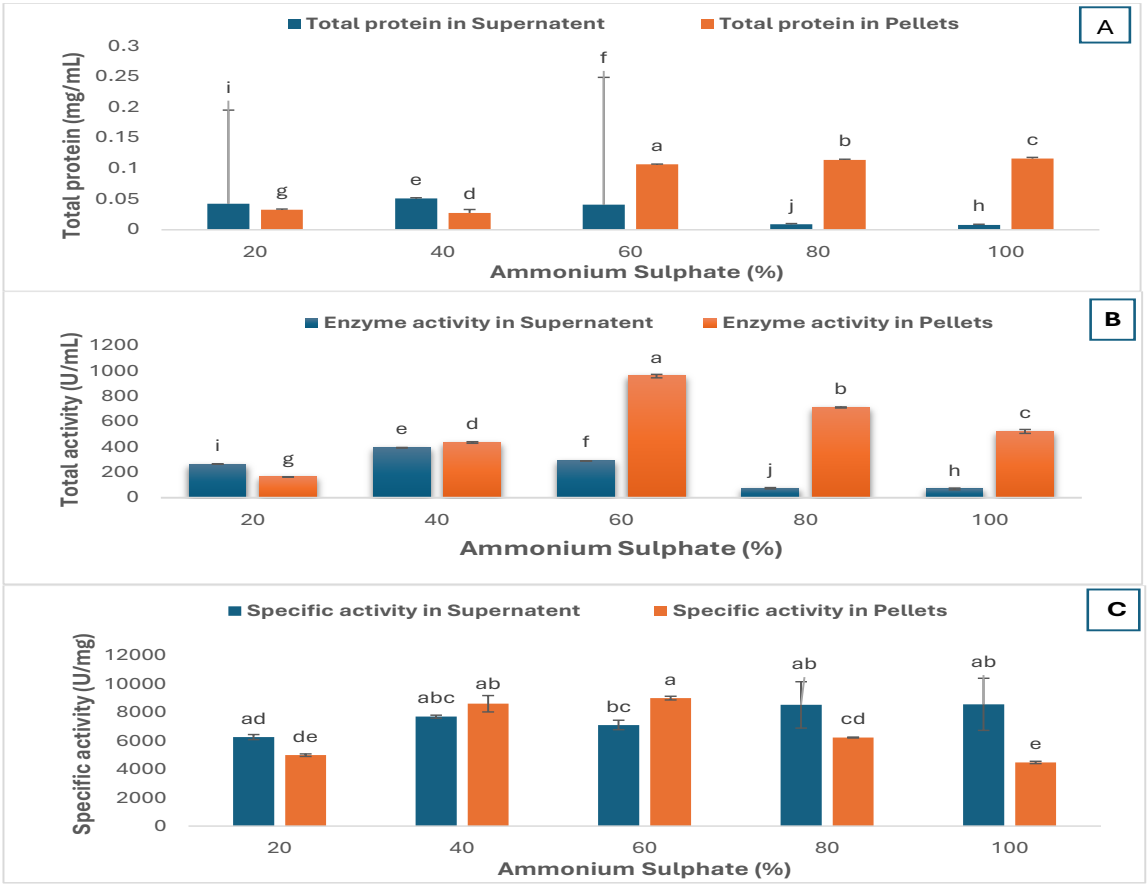

Figure 3. Total protein (A); Specific activity in purification by ammonium sulphate precipitation.

Table 5. Summary of purification steps of protease enzyme from germinated barley (Giza 134 grains) for 7 days.

Purification steps	Enzyme volume (mL)	Protein contents (mg/mL)	Total protein (mg/mL)	Protease activity (U/mL)	Total activity ((U/mL)	Specific activity (U/mg)
Crude enzyme	100	0.2150±.00000[a]	21.5	189.0000±3.00000[c]	18900	875.89±13.46018[c]
Precipitate by ammonium sulphate (NH_2SO_4) 60%	20	0.1067±.00208[b]	2.134	960.3333±1.52753[b]	19206	9010.67±131.86291[b]
Dialysis	10	0.0513±.00058[c]	0.513	1018.3333±15.27525[a]	10188.333	19920.69±541.65901[a]

Figure 4 illustrates the activity of the partial purified protease enzyme extracted from sprouted barley was evaluated under different temperatures and pH conditions to determine its optimal catalytic parameters. The protease activity varied significantly with temperature **(see Figure 4-A)**. The enzyme exhibited maximum activity at 35°C (1206.67 ± 1.53 U/mL), followed by a moderate activity at 45°C (1094.33 ± 5.03 U/mL) and 25°C (1024.67 ± 8.33 U/mL). However, a sharp decline was observed at higher temperatures, with activity dropping to 601.67 ± 8.50 U/mL at 55°C and further decreasing at 65°C. This temperature-dependent trend indicates that the barley-derived protease is a mesophilic enzyme, with an optimum around 35°C. At lower temperatures, reduced enzyme activity may be attributed to decreased molecular motion and insufficient energy for effective enzyme–substrate interactions. Conversely, beyond 45°C, the decline in activity likely results from thermal denaturation, where heat disrupts the enzyme's tertiary and quaternary structures, leading to the deformation of its active site **(Sumantha et al., 2006)**. Similar temperature optima have been reported for plant-derived proteases, such as those from wheat, papaya, and barley, which typically exhibit maximal activity between 30°C and 40°C **(Kaur et al., 2024)**.

The loss of activity at elevated temperatures suggests that this protease is not thermostable and should be applied in moderate-temperature food processes such as dough fermentation, biscuit preparation, and protein modification, where excessive heating is avoided. The protease retained high activity across a broad pH range from 3 to 9, with the maximum at pH 7 **(Figure 4-B)**. Although minor variations were observed, enzyme activity remained above 90% of the maximum throughout the tested range, indicating strong pH tolerance and structural stability. This broad pH adaptability is characteristic of plant proteases, which often maintain functionality in both acidic and slightly alkaline conditions **(Kaur et al., 2024; Chaiwut et al., 2010)**. The stability over a wide pH range suggests that the enzyme's active site residues are well-protected against protonation or deprotonation changes that could otherwise alter catalytic efficiency. Such behavior enhances the enzyme's potential applicability in diverse food industry processes, including protein hydrolysis, baking, beverage clarification, and meat tenderization **(Rao et**

al., 1998). The results demonstrate that the sprouted barley protease possesses desirable biochemical characteristics for industrial application namely, a moderate optimum temperature (35°C), wide pH stability, and high catalytic efficiency. These properties make it suitable for incorporation into gluten-free food formulations, such as rice-flour-based biscuits, where controlled proteolysis can improve dough handling, texture, and digestibility. Results in **Figure 4-C** shows barley protease stability at 25 °C and 40 °C for eight days. It clearly shows a faster decline in enzymatic activity at the higher temperature, confirming the discussion that thermal exposure accelerates enzyme denaturation and activity loss.

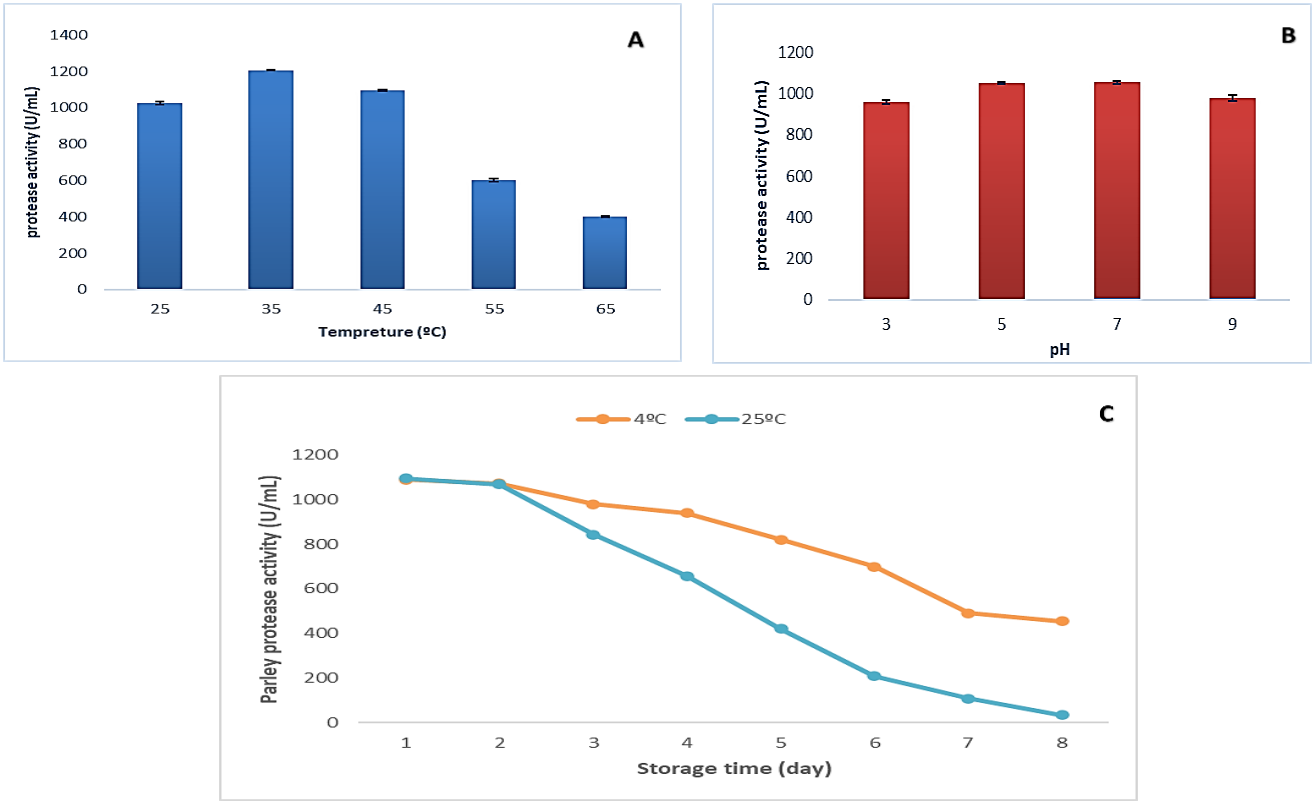

Figure 4. Effect of temperature **(A)**, pH **(B)**, and storage conditions on the activity and stability of protease enzyme extracted from germinated barley **(C)** Stability of barley protease during storage at 4°C and 25°C.

The stability of barley protease was assessed at 25 °C and 40 °C during an eight-day period, revealing that temperature significantly influenced enzyme stability. At 25 °C, the enzyme preserved greater residual activity during the storage duration, sustaining 843 U/ mL after three days, 421 U/mL after five days and 34 U by the eighth day. Conversely, at 4 °C, the activity diminished more swiftly, with a steep reduction from 1090 U on day one to 455 U by day eight. This pattern indicates that elevated temperatures hasten enzyme denaturation and the subsequent decline in catalytic activity, aligning with the typical behaviour of enzyme stability **(Aehle, 2007).** The reduced rate of activity loss at 25 °C indicates that barley protease demonstrates moderate thermostability, however remains susceptible to extended exposure to high temperatures. The diminished activity at 4 °C may be ascribed to the thermal unfolding of the enzyme's tertiary structure, resulting in irreversible denaturation **(Vieille & Zeikus, 2001)**.

Moreover, storage stability is a critical factor for industrial and culinary applications. The noted reduction at both temperatures indicates that barley protease ought to be preserved at lower temperatures, potentially under refrigeration, to sustain its activity over prolonged durations. **Xiaoxu et al. (2025)** reported analogous findings, indicating that cereal-derived proteases exhibited enhanced activity when held below 30 °C in contrast to ambient or increased temperatures. The results affirm that temperature is essential for preserving the structural and catalytic integrity of barley protease. For practical applications like baking or the hydrolysis of cereal proteins, it is advisable to keep the enzyme below 30 °C to sustain its functional efficacy over time. Subsequent research should use stabilising substances like polyols or salts to improve enzyme longevity (**Haki & Rakshit, 2003)**.

Table 6 provides a summary of the gluten concentration results following one and two hours of treatment using two protease concentrations at a concentration of 1 and 2%. the protease extracted from barley grains that had the lowest percentage of protein hydrolyzed (9.31 %) at Protease 1 % for 1 hour. The percentage of protein hydrolyzed by 1% barley protease was comparatively high after 2 hours (11.58%). The highest percentage of protein hydrolyzed (14.75 %) at 2% protease concentrate after two hours from gluten hydrolysis. The findings show that both enzyme concentration and incubation period gradually increased the amount of gluten hydrolysed. This implies that the proteolytic activity of barley-derived enzymes is both amount-dependent and time-dependent, comparable with typical enzymatic kinetics in protein degradation **(Rizzello et al., 2016)**.

Table 6. Gluten degradation by partial purified barley protease at two enzyme concentration for 1 and 2 hours.

Protease concentration	Total gluten (mg/mL)	Remaining gluten (mg/mL)	Remaining gluten (%)	Hydrolyzed gluten (mg/mL)	Hydrolyzed gluten (%)
Gluten hydrolysis for 1 hour					
Protease 1 %	0.0400±.00001[a]	90.6902±.03072[a]	0.0041±.00001[d]	0.0041±.00001[d]	9.3098±.03072[d]
Protease 2 %	0.0390±.00001[b]	88.4198±.03072[b]	0.0051±.00001[c]	0.0051±.00001[c]	11.5802±.03072[c]
Gluten hydrolysis for 2 hours					
Protease 1 %	0.0385±.00001[c]	87.3379±.03072[c]	0.0056±.00001[b]	0.0056±.00001[b]	12.6621±.03072[b]
Protease 2 %	0.0376±.00006[d]	85.2449±.14078[d]	0.0065±.00006[a]	0.0065±.00006[b]	14.7551±.14078[a]

The data in **Table 7** shows Gliadin amount after 1 and 2 hours of treatment two concentration from protease extracted and partial purified from barley germinated for seven days. The barley protease extracted from barley grains that had the lowest percentage of protein hydrolyzed (4.97 %) at protease 1 % for 1 hour. While, the percentage of gliadin hydrolyzed by 1% barley protease was comparatively high after 2 hours (9.88 %). The highest percentage of gliadin hydrolyzed (12.16 %) at 2% protease concentrate after two hours from gliadin n hydrolysis.

Table 7. Gliadin degradation by partial purified barley protease at two enzyme concentration for 1 and 2 hours.

Protease concentration	Total gliadin (mg/mL)	Remaining gliadin (mg/mL)	Remaining gliadin (%)	Hydrolyzed gliadin (mg/mL)	Hydrolyzed gliadin (%)
Gliadin hydrolysis for 1 hour					
Protease 1 %	0.0314±.00037[a]	0.0298±.00013[a]	95.0318±1.52960[a]	0.0016±.00050[b]	4.9682±1.52960[b]
Protease 2 %	0.0314±.00037[a]	0.0283±.00011[b]	90.1203±.74577[b]	0.0031±.00027[a]	9.8797±.74577[a]
Gliadin hydrolysis for 2 hours					
Protease 1 %	0.0314±.00037[a]	0.0281±.0001[c]	89.4894±1.06895[b]	0.0033±.00037[a]	10.5106±1.06895[a]
Protease 2 %	0.0314±.00037[a]	0.0276±.00012[d]	87.8346±1.40518[b]	0.0038±.00048[a]	12.1654±1.40518[a]

Figures 5-8 illustrate docking study of Cysteine proteinase EP-B2 (UniProt ID: P25250) and the peptide fragment using AlphaFold3 AI protein structure prediction from the amino acid sequence. Different AI-based modeling tools were employed to predict the structure of the type 1 α2-gliadin 33-mer peptide fragment. AlphaFold3 (1) **(see Figure 5),** which incorporates multiple sequence alignment (MSA), and ESMFold (2) **(Figure 6)**, which omits MSA for faster computation, were both utilized. Both methods produced moderate prediction confidence scores based on the Predicted Local Distance Difference Test (pLDDT), with AlphaFold3 (1) yielding slightly higher values. The structural alignment between the two predicted models showed a close correspondence, with a Root Mean Square Deviation (RMSD) of 5.717 Å (based on 33 aligned atoms) **(Figure 7)**. These results are considered acceptable given the short length of the peptide fragment.

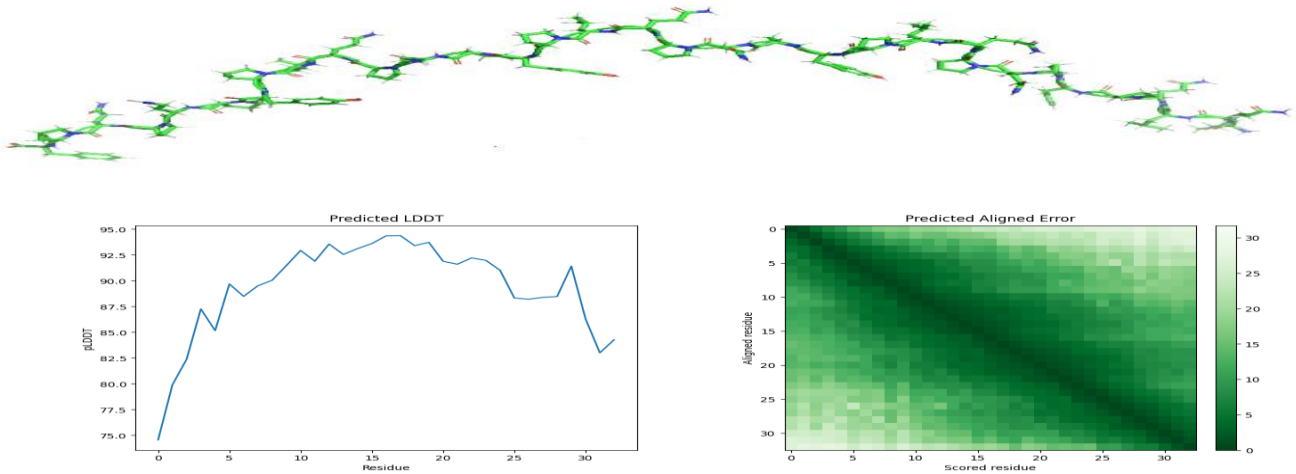

Figure 5. Predicted three-dimensional structure of the type 1 α2-gliadin 33-mer peptide fragment generated using AlphaFold3 (1), showing the Predicted Local Distance Difference Test (pLDDT) confidence scores and the corresponding residue–residue contact map.

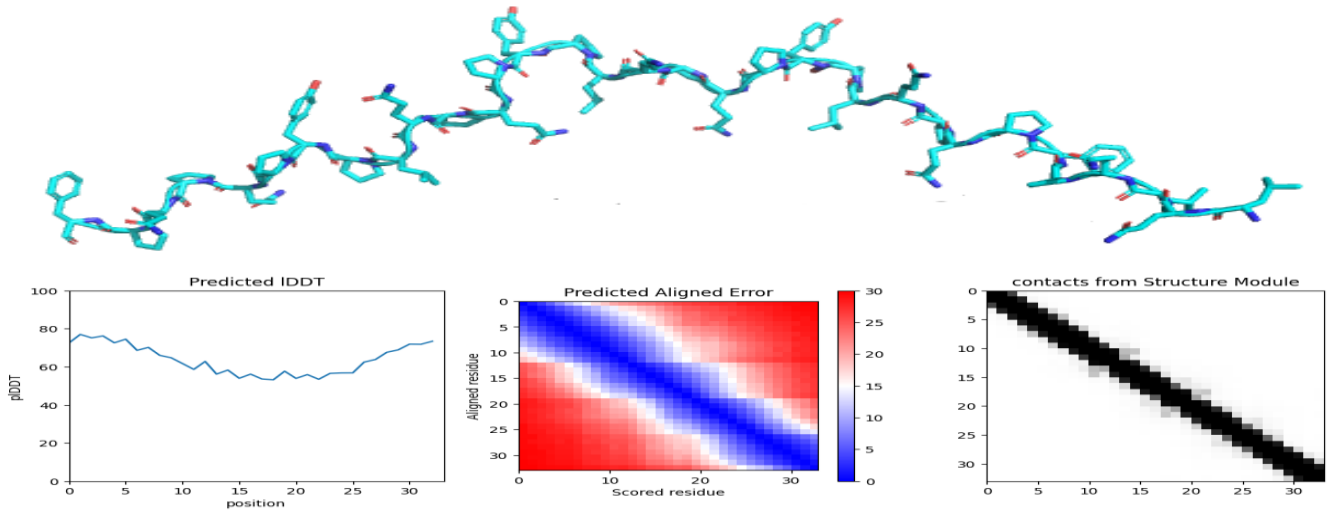

Figure 6. Predicted tertiary structure of the type 1 α2-gliadin 33-mer peptide fragment generated using ESMFold (2), illustrating the Predicted Local Distance Difference Test (pLDDT) confidence scores along with the corresponding residue–residue contact map.

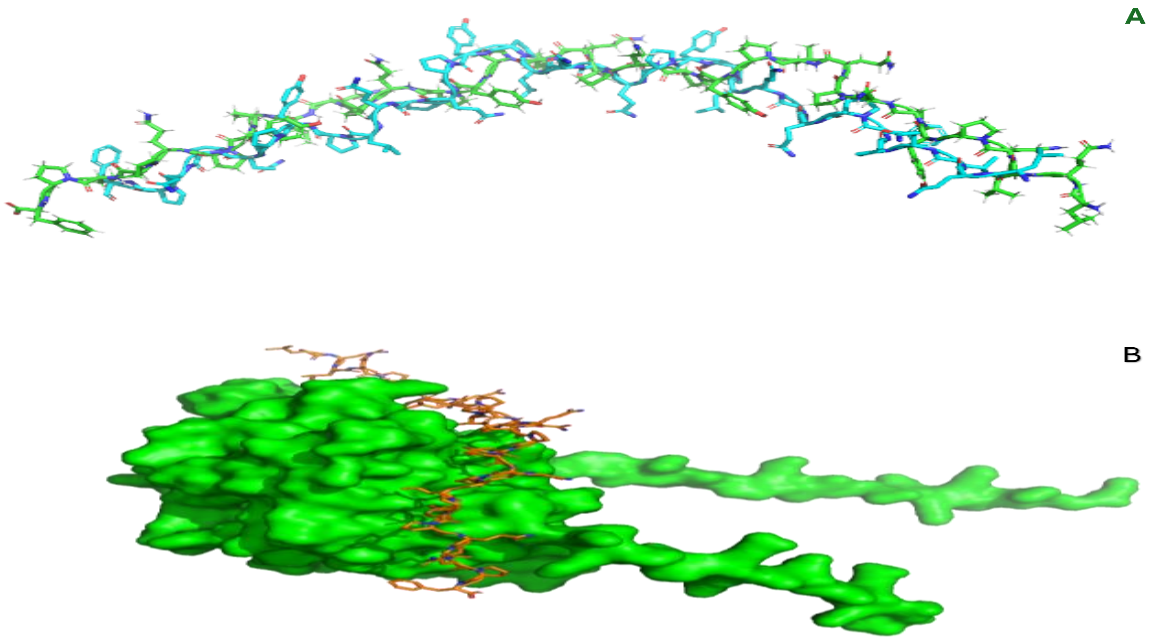

Figure 7. (A) Structural alignment between AlphaFold3 (green) and ESMFold (cyan) models of the type 1 α2-gliadin 33-mer peptide fragment, showing a Root Mean Square Deviation (RMSD) of 5.717 Å based on 33 aligned atoms; **(B)** Surface representation of the Cysteine proteinase EP-B2 (green) docked with the type 1 α2-gliadin 33-mer peptide fragment (orange), as predicted using AlphaFold3 (1).

Subsequently, AlphaFold3 (1) was employed to predict and dock the Cysteine proteinase EP-B2 enzyme with the gliadin peptide fragment. The EP-B2 enzyme exhibited a consistently high pLDDT score (typically above 90), indicative of a well-defined and confidently predicted structure. In contrast, the peptide fragment displayed a lower pLDDT score, as expected due to its short length and inherent conformational flexibility. The Predicted Template Modeling (pTM) score of the docked complex was 0.78, suggesting a high level of confidence and a close approximation to the native binding conformation **(Figure 7&8)**. Overall, these results demonstrate satisfactory model quality and provide a reliable structural basis for subsequent interaction analyses.

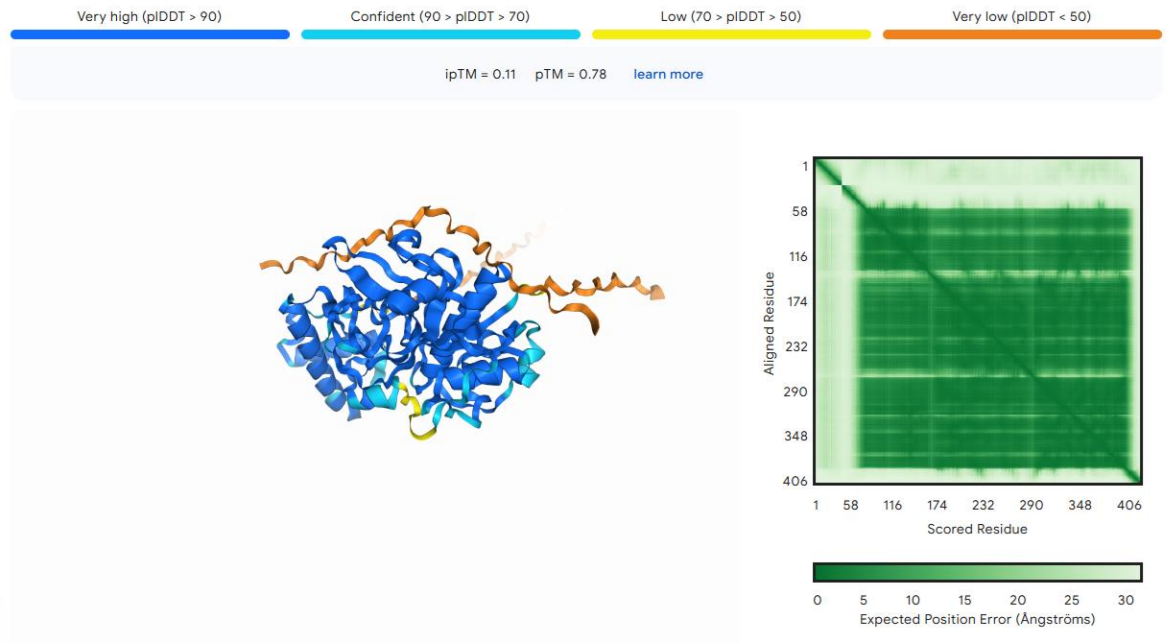

Figure 8. Structural model generated by AlphaFold3 (1) depicting the Cysteine proteinase EP-B2 (blue) docked with the type 1 α2-gliadin 33-mer peptide fragment (orange). The figure highlights the Predicted Local Distance Difference Test (pLDDT) and Predicted Template Modeling (pTM) scores, reflecting the overall confidence and accuracy of the predicted complex.

Figures 9 and 10 show docking Study of Cysteine Proteinase EP-B2 and Glutelin (*Oryza sativa*, Rice). AlphaFold3 was utilized to predict and dock the Cysteine proteinase EP-B2 enzyme with Glutelin from *Oryza sativa* (UniProt ID: Q9ZWJ8). The predicted complex achieved a Predicted Template Modeling (pTM) score of 0.55, indicating a reliable and biologically relevant interaction model. The docking results suggest a stable binding interface between EP-B2 and Glutelin, closely approximating the native conformation (**Figure 9 & 10**). These findings confirm satisfactory model quality and provide a dependable structural basis for further interaction and functional analyses.

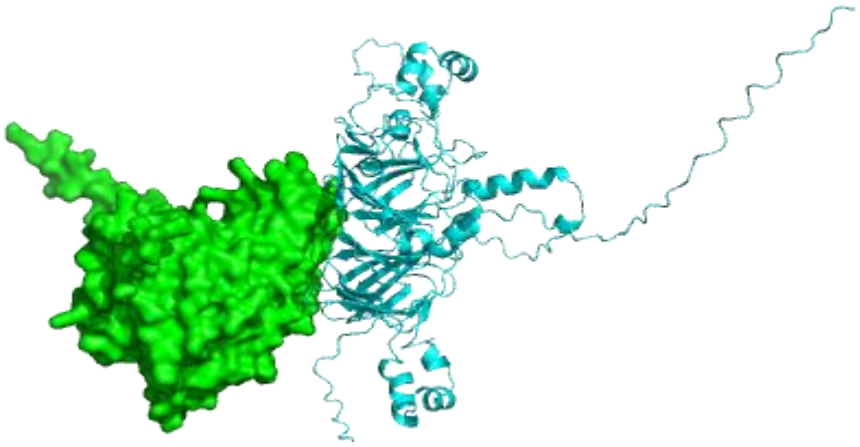

Figure 9. Docking and predicted structure of Cysteine proteinase EP-B2 and Glutelin (*Oryza sativa*, rice). The EP-B2 enzyme is shown as a surface representation in green, while Glutelin is displayed as a cartoon model in cyan.

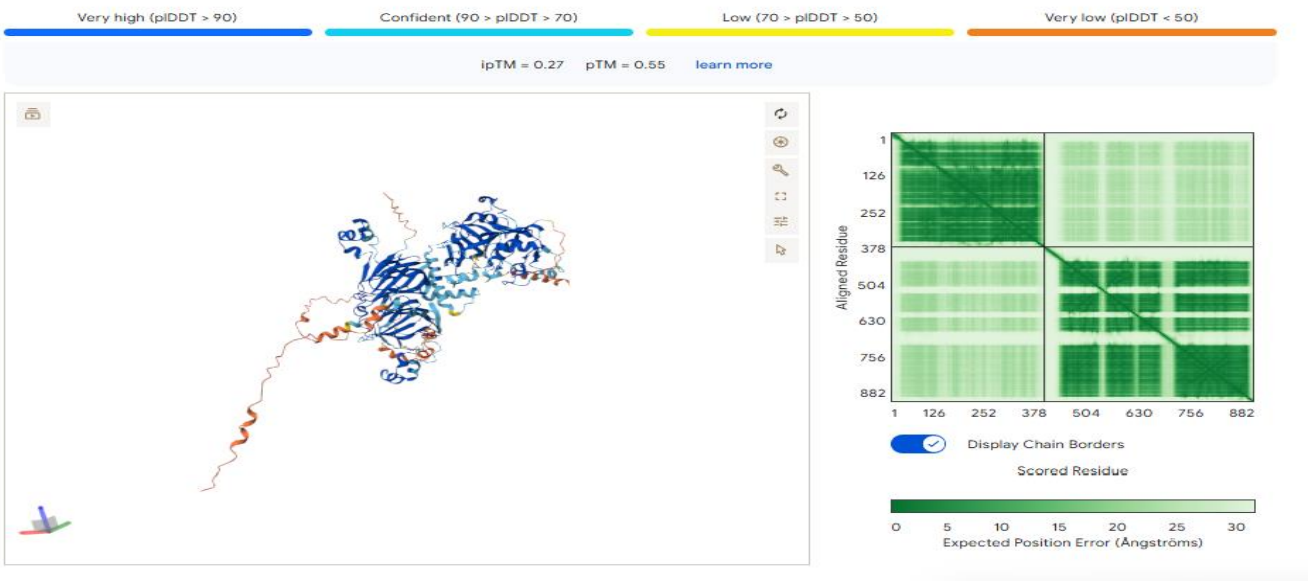

Figure 10. AlphaFold3 docking and predicted complex structure of Cysteine proteinase EP-B2 and Glutelin (*Oryza sativa*, rice), showing a Predicted Template Modeling (pTM) score of 0.55, indicating moderate to high confidence in the docking prediction.

The addition of protease enzyme isolated from sprouted barley for rice flour at different concentrations had a substantial impact on the viscosity of rice flour paste. Because of the high starch content and intact protein network structure, the control sample (rice flour without enzyme) had the greatest viscosity value (11933.33 ± 503.32 cp), suggesting a thick paste. However, the addition of protease caused a noticeable drop in viscosity, which intensified as the concentration of the enzyme decreased. The viscosity dropped to 6933.33 ± 1006.64 cp at 0.005% protease, and it dropped to 5600.00 ± 400.00 cp and 5366.66 ± 321.45 cp at 0.01% and 0.02% enzyme doses, respectively. This pattern implies that the paste's consistency and viscosity were decreased as a result of proteolytic activity partially hydrolyzing the protein matrix and potentially disrupting starch-protein connections. Furthermore, protein hydrolysis may also have contributed to increase accessibility of disulphide bonds,explaining the increase in the relative concentration of the a and b glutelin subunits with rice protein 0.01 under reducing conditions. Protease treatment at 0.01% addition significantly affected the pasting profile of rice flour ($p < 0.05$), by reducing peak and final viscosity and breakdown **(Table 8)**, thus confirming previous results **(Derycke et al., 2005; Xie et al., 2008; Hasjim, J., Li, E., & Dhital, S)**. In conclusion, the reduction in viscosity upon the addition of barley protease concentration suggests that the rheological characteristics of rice flour are successfully altered by enzyme treatment, which may enhance its processability for biscuit or baked product applications intended for people with gluten sensitivity.

Table 8. Viscosity properties of untreated and protease-treated rice batters**.**

Treatments	Viscosity (cp)
rice flour	11933.33±503.32[a]
rice flour +0.005% protease	6933.33±1006.64[b]
rice flour +0.01% protease	5600.00±400.00[c]
rice flour +0.02% protease	5366.66±321.45[c]

Table 9 shows that wheat biscuits had superior water activity and moisture content values of 0.49 and 7.81%, respectively, in contrast to rice biscuits, which recorded values of 0.39 and 6.81%. The incorporation of barley protease marginally enhanced both parameters (0.45 and 7.04%), likely due to the generation of low-molecular-weight peptides and amino acids that augment water binding ability. Nonetheless, all levels remained within the permissible range for dry baked products, guaranteeing adequate shelf stability. Controlling aw in food is one of the most significant preservation methods to assure food safety against microbiological and chemical degradation, which can lengthen shelf life and enhance convenience with new food products.

As a result, several food preservation procedures concentrate on reducing the aw to slow microbial development and chemical reactions **(Deepika et al., 2022)**. The profile of pasting functionalities of flour treated with different concentrations of enzyme is illustrated in **Table 9**. Samples were observed under controlled condition of cooling, heating, and holding. The result recorded was significantly varied ($p < .05$). Although the enzyme treatments significantly alter the moisture content of the rice biscuits, they notably improved biscuits quality.

Table 9. Physiochemical analysis of enzyme treated biscuits.

Properties	Wheat biscuits	Rice biscuits	Rice biscuits with barley protease
Width (mm)	40.3333±.57735[b]	43.6667±.57735[a]	44.6667±.57735[a]
thickness(mm)	48.3333±.57735[a]	34.6667±.57735[c]	38.6667±.57735[b]
spread ratio	0.8343±.00231[c]	1.2597±.00462[b]	1.1553±.00231[a]
water activity	0.4927±0.00902[a]	0.3907±0.02419[c]	0.4543±0.01124[b]
moisture	7.8133±6.8133[a]	6.8133±0.09504[c]	7.0400±0.02646[b]

The physical and moisture-related properties of wheat biscuits, rice biscuits, and rice biscuits enhanced with barley protease exhibited significant variations, indicating the impact of flour composition and enzymatic treatment on product quality. The width of the biscuits markedly increased in rice-based formulations relative to wheat biscuits. Rice biscuits exhibited a width of 43.67 ± 0.58 mm, whereas those treated with barley protease demonstrated the largest width at 44.67 ± 0.58 mm, suggesting that protease treatment improved dough spreadability after baking **(see Table 9)**. This may be due to the partial breakdown of rice proteins by protease, resulting in a softer dough with enhanced flow characteristics. **Adebowale et al. (2012)** similarly reported that enzymatic or protein modification enhanced cookie distribution by compromising dough structure. The thickness of the biscuit demonstrated a contrary tendency. Wheat biscuits exhibited the greatest thickness (48.33 ± 0.58 mm), whereas rice biscuits were comparatively thinner (34.67 ± 0.58 mm). The addition of protease marginally elevated thickness to 38.67 0.58 mm, likely attributable to enhanced gas retention from protein degradation products that improve dough flexibility **(Ding & Yang, 2013)**. The spread ratio, an essential quality metric, was greatest in rice biscuits (1.26 ± 0.00), succeeded by rice biscuits with protease (1.16 ± 0.00) and wheat biscuits (0.83 ± 0.00). The elevated spread ratios in rice-based formulations suggest enhanced dough flow during baking, perhaps resulting from the lack of gluten and the influence of protease on diminishing protein-starch connections, which facilitates larger expansion **(Pareyt & Delcour, 2008)**.

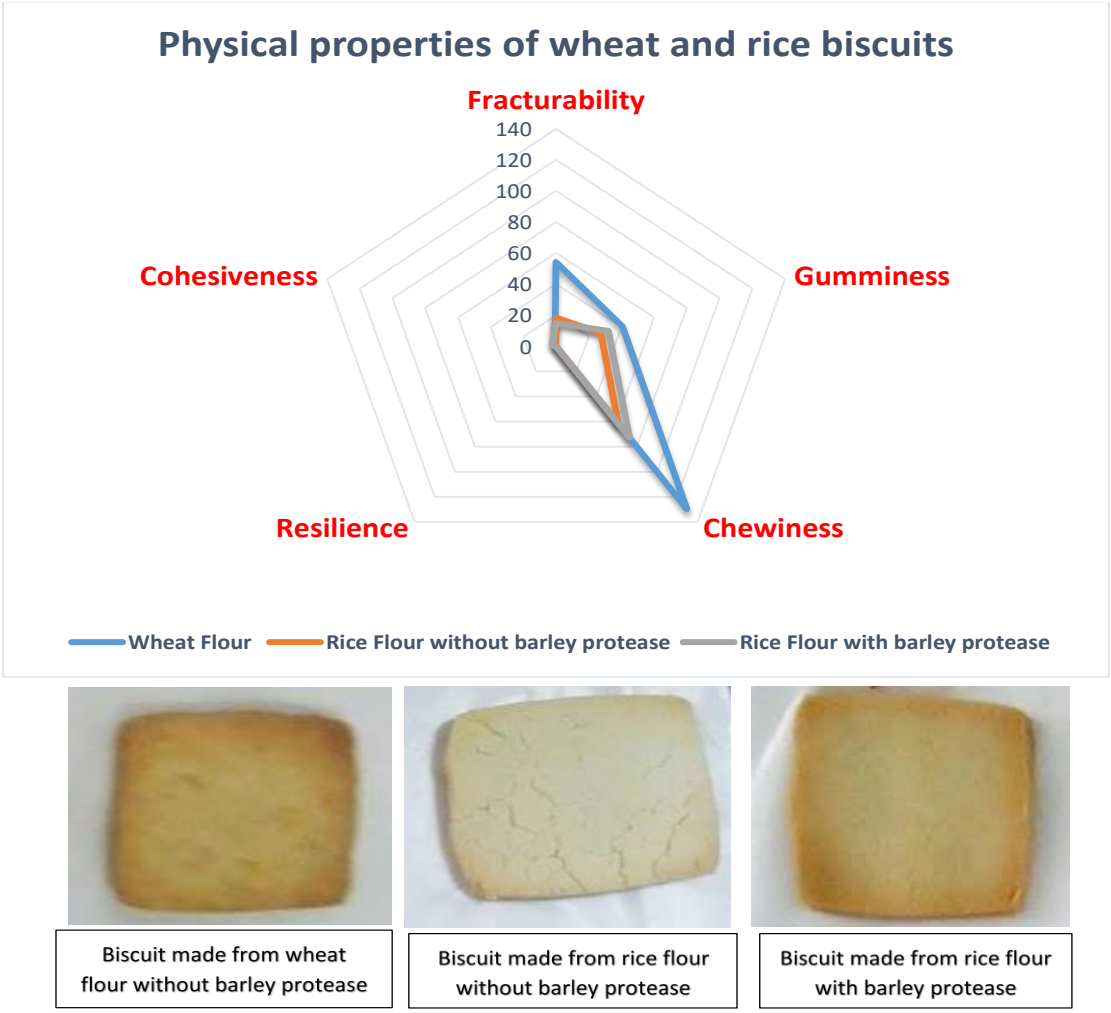

Figure 11. Effect of barley protease enzyme on the physical properties and appearance of rice flour biscuits compared to wheat flour biscuits.

Figure 11 demonstrated the enzymatic effects rice biscuits with 0.01% protease compared with rice biscuits and wheat biscuits. biscuits with 0.01% protease concentration on the dimensional properties of biscuits e.g., thickness, spread ratio, and diameter. The diameter of biscuits with 0.01% higher significant compared with rice without enzyme and wheat rice. It was observed that the diameter increased This increase in diameter is owed to protein break down that allowed the dough to spread more during baking. The slight decrease at higher concentrations could be due to excessive breakdown, leading to a dough became too soft to hold its shape. However, the trend was It is noteworthy inconsistent trend could be due to different effects of fat breakdown at various enzyme concentrations, impacting dough elasticity. Regarding the thickness, biscuits became slight decrease than wheat biscuits The reason behind a decrease in thickness is the weakening of the gluten network that likely led to reduced ability of the dough to retain its height during baking. However, the thickness remains relatively consistent with protease. The spread ratio increases significantly with protease enzyme 0.01% concentration, compared with wheat biscuits but less than rice biscuits. This increase corresponds to the larger diameter and thinner biscuits. However, addition of enzymes has improved the functionality of flour for cookies.

The physical qualities of biscuits made from wheat flour, rice flour without barley protease, and rice flour with barley protease **(Figure 11)**. e xhibit notable differences in texture and appearance.

Wheat flour biscuits had superior fracturability, chewiness, and cohesiveness, reflecting a robust and elastic structure attributed to gluten, wh ich facilitates network development and gas retention during baking **(Day et al., 2006).** Conversely, rice flour biscuits devoid of protease exhibited diminished textural values and apparent surface fissures, indicative of the inadequate binding characteristics of gluten-free dough **(Gujral & Rosell, 2004)**.

The incorporation of barley protease enzyme into rice flour enhanced various textural characteristics, including fracturability, chewiness, and cohesiveness, in contrast to rice flour biscuits devoid of the enzyme. This enhancement indicates that the protease partially hydrolysed rice proteins, hence improving dough extensibility and facilitating superior interaction between starch and protein molecules. The biscuit structure became more refined and less brittle, yielding a texture akin to that of wheat-based products.

The biscuit composed of rice flour and barley protease exhibited a more uniform surface and a golden-brown hue, signifying superior dough spread and consistent baking. The findings validate that the enzymatic alteration of rice flour proteins can enhance the quality of gluten-free baked goods by improving their mechanical and sensory attributes **(Rai et al., 2018).** Integrating barley-derived protease into rice flour formulations demonstrates significant potential for creating gluten-free biscuits appropriate for individuals with coeliac disease or gluten sensitivity, while preserving desirable textural and visual attributes akin to those of traditional wheat biscuits.

CONCLUSIONS

In conclusion, this work efficiently indicates the capacity of protease extracted from germinated barley for 7 days to degrade gluten and gliadin proteins, which is especially pertinent for persons with gluten sensitivity and coeliac disease. The effective integration of the isolated protease into rice biscuit formulations enhanced dough handling and sensory qualities while substantially lowering gluten levels to acceptable thresholds for gluten-sensitive individuals. The results highlight the efficacy of germinated barley protease as a natural, plant-derived alternative in creating gluten-reduced baked goods, presenting a significant opportunity to improve dietary choices for those with coeliac disease. Subsequent research may investigate the utilisation of these enzymes in diverse food products, thereby expanding the range of gluten-free alternatives in the market.

CONFLICT OF INTEREST

The authors of this article declare that they have no conflict of interest.

REFERENCES

[1] Foschia M.; Horstmann S.; Arendt E.K. and Zannini E. (2016). Nutritional therapy - Facing the gap between coeliac disease and gluten-free food. Int J Food Microbiol. 2016 Dec 19;239:113-124. doi: 10.1016/j.ijfoodmicro.2016.06.014. Epub 2016 Jun 15. PMID: 27321352.

[2] Catassi C.; Verdu E.F.; Bai J.C. and Lionetti E.(2022). Coeliac disease. Lancet. 25;399(10344):2413-2426. doi: 10.1016/S0140-6736(22)00794-2.

[3] Bianchi, P. I.; Aronico, N.; Santacroce, G.; Broglio, G.; Lenti, M. V., and Di Sabatino, A. (2024). Nutritional Consequences of Celiac Disease and Gluten-Free Diet. Gastroenterology Insights, 15(4), 878-894. https://doi.org/10.3390/gastroent15040061.

[4] Sivaramakrishnan, H. P.; Senge, B. and Chattopadhyay, P. K. (2004). Rheological properties of rice dough for making rice bread. Journal of Food Engineering, 62(1), 37–45.

[5] Park J. and Kim H.S. (2023). Rice-Based Gluten-Free Foods and Technologies: A Review. Foods. 2023 Nov 13;12(22): 4110. doi: 10.3390/foods12224110. PMID: 38002168; PMCID: PMC10670158.

[6] Renzetti, S. and Arendt, E. K. (2009). Effect of protease treatment on proteins in gluten-free breads. Journal of Cereal Science, 50(3), 337–344.

[7] Phongthai, S.; D'Amico, S.; Schoenlechner, R.; Homthawornchoo, W. and Rawdkuen, S. (2017). Effects of protein enrichment on structural, textural, and sensory properties of rice flour-based gluten-free biscuits. Food Science and Technology International, 23(4), 326–337.

[8] Pashaei M.; Fereshte B.; Sima T. ; SarahSanaei N.; ElhamKhalili S.; Mollakhalili-Meybodi N. and Leila M. (2025). The role of enzymes in gluten-free bakery products: A review of technological and nutritional perspectives. Applied Food Research 5 (2025) 100923. https://doi.org/10.1016/j.afres.2025.100923.

[9] Liaquat, A.; Ashraf, H.; Ahsan, M.; Ul-Haq, I.; Mugabi, R.; Alsulami, T. and Nayik, G. A. (2025). Enzymatic influence on dough rheology and cookie quality: protease and lipase as functional modifiers. International Journal of Food Properties, 28(1). https://doi.org/10.1080/10942912.2025.2489490.

[10] Caballero, P. A.; Gómez, M., and Rosell, C. M. (2007). Improvement of dough rheology, bread quality and bread shelf-life by enzymes combination. LWT – Journal of Food Engineering, 81,1,42-53. https://doi.org/10.1016/j.jfoodeng.2006.10.007.

[11] Hoseney R. C.; Rogers D. E. (1990). The formation and properties of wheat flour doughs. Crit Rev Food Sci Nutr. 1990;29(2):73-93. doi: 10.1080/10408399009527517. PMID: 2198884.

[12] Pedersen, L.; Kaack, K.; Bergsøe, M. N.; Adler-Nissen, J. (2005). Effects of Chemical and Enzymatic Modification on Dough Rheology and Biscuit Characteristics. J. Food Sci. 2005, 70(2), E152–E158. DOI: 10.1111/j.1365-2621.2005. tb07089.x .

[13] Kamel, B. S and Stauffer, C. E. (2013). Advances in Baking Technology. Springer: New York, 2013. DOI: 10.1007/978-1-4899- 7256-9 .

[14] Hassan, C. Z.and Elgubbi, H. M.; Agbaje, R. Physicochemical and Textural Properties of White Bread Made from Different Commercial Brands of High Gluten Flours. Int. J. Rev. Appl Soc Sci 2015, 1, 17–30

[15] Saberi, F.; Kouhsari, F. and Gasparre, N. (2024). Green Alternative for Sodium Metabisulfite Substitution: Comparison of Bacterial and Fungal Proteases Effect in Hard Biscuit Making. Food Sci. Technol. Int. 2024, 30(5), 407–417. DOI: 10.1177/10820132231152279 .

[16] Martins-Meyer, T. S.; Figueiredo, E.; Lobo, B. and Dellamora-Ortiz, G. (2013). Enzymes in Bakery: Current and Future Trends. In Food Industry, Muzzalupo, I., Eds.; IntechOpen: London, UK, 2013; pp. 278–321.

[17] Pourmohammadi, K. and Abedi, E. (2021). Enzymatic Modifications of Gluten Protein: Oxidative Enzymes. Food Chem. 2021, 356, 129679. DOI: 10.1016/j.foodchem.2021.129679 .

[18] Shaha, RK. (2002). *Conformation and surface properties of gluten treated with protease from germinating wheat. Pakistan Journal of Biological Sciences, 2002: 5 (10): 1077-1080.*

[19] Gessendorfer B.; Hartmann G.; Wieser H. and Koehler P. (2011). Determination of celiac disease-specific peptidase activity of germinated cereals. In European Food Research and Technology, 2011: 232: 205-209.

[20] Bradford M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem, 1976: 72:248.

[21] Wang, H.; Xu, J.; Kong, B.; Liu, Q.; Xia, X. and Sun, F. (2022). Purification and characterization of the protease from Staphylococcus xylosus A2 isolated from harbin dry Sausages. Foods, 11: 1094.

[22] Ullah, N.; Ur Rehman, M.; Sarwar, A.; Nadeem, M.; Nelofer, R.; Shakir, H. A.; Irfan, M.; Idrees, M.; Naz , S.; Nabi, G.; Shah, S.; Aziz, T.; Alharbi, M.; Alshammari, A. and Alqahtani, F. (2022). Purification, characterization, and application of alkaline protease enzyme from a locally isolated Bacillus cereus strain. Fermentation; 8: 628.

[23] Abdulaal, W.H. (2018). Purification and characterization of cysteine protease from miswak Salvadora persica. BMC Biochem 19, 10 (2018). https://doi.org/10.1186/s12858-018-0100-1

[24] Hartmann G.; Koehler P. and Wieser H. (2006). Rapid degradation of gliadin peptides toxic for coeliac disease patients by proteases from germinating cereals. J. Cereal Sci., 2006: 44: 368-371.

[25] Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., & Olson, A. J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. Journal of Computational Chemistry, 30(16), 2785–2791. https://doi.org/10.1002/jcc.21256 2.

[26] Hanwell, M. D., Curtis, D. E., Lonie, D. C., Vandermeersch, T., Zurek, E., & Hutchison, G. R. (2012). Avogadro: An advanced semantic chemical editor, visualization, and analysis platform. Journal of Cheminformatics, 4, 17. https://doi.org/10.1186/1758-2946-4-17.

[27] Shib, A., Bhardwaj, G., Kumari, U., Singh, J., & Kaur, S. (2021). DeepSite: An ensemble based deep learning approach for accurate and reliable prediction of protein binding sites. bioRxiv, 2021.03.08.434460. https://doi.org/10.1101/2021.03.08.434460

[28] Liu, Y., Yang, X., Gan, J., Chen, S., Xiao, Z. X., & Cao, Y. (2022). CB-Dock2: improved protein-ligand blind docking by integrating cavity detection, docking and homologous template fitting. Nucleic acids research, 50(W1), W159–W164. https://doi.org/10.1093/nar/gkac394.

[29] Pal, Rahul and Pandey, Prachi. (2023). Physical Pharmaceutics Experiments: Determination of Viscosity of Semisolid by using Brookfield Viscometer. 10.13140/RG.2.2.28874.54729.

[30] AOAC, 2002. Official methods of analysis of the association of chemists. Washington, DC: analysis of the association of chemists.

[31] Ashwini, A.; Jyotsna R. and Indrani, D. (2009). Effect of hydrocolloids and emulsifiers on the rheological, microstructural and quality characteristics of eggless cake. Food Hydrocolloids, 23(3), pp.700–707. https://doi.org/10.1016/j.foodhyd.2008.06.002

[32] Mathlouthi, M. (2001). Water content, water activity, water structure and the stability of foodstuffs. Food Control, 12 (7), 409–417. doi:10.1016/s0956-7135 (01)00032-9.

[33] Kokkat, T. J., McGarvey, D., Lovecchio, L. C., & LiVolsi, V. A. (2011). Effect of thaw temperatures in reducing enzyme activity in human thyroid tissues. Biopreservation and Biobanking. https://doi.org/10.1089/BIO.2011.0026.

[34] Shalem, A., Yehezkeli, O., & Fishman, A. (2024). Enzymatic degradation of polylactic acid (PLA). Applied Microbiology and Biotechnology. https://doi.org/10.1007/s00253-024-13212-4.

[35] Obeidat, W.M., Sahni, E., Kessler, W. et al. (2018). Development of a Mini-Freeze Dryer for Material-Sparing Laboratory Processing with Representative Product Temperature History. AAPS PharmSciTech 19, 599–609 (2018). https://doi.org/10.1208/s12249-017-0871-5.

[36] Nowak, D., & Jakubczyk, E. (2020). The Freeze-Drying of Foods—The Characteristic of the Process Course and the Effect of Its Parameters on the Physical Properties of Food Materials. Foods, 9(10), 1488. https://doi.org/10.3390/foods9101488.

[37] Sara Zafar, M. Yasin Ashraf and M. Ashraf (2005). Protease Activity and Associated Changes During Germination and Early Seedling Stages of Cotton Grown under Saline Conditions. International Journal of Botany, 1: 103-107.

[38] Bretträger, M., Franz, V., Sacher, B. et al. (2025). Gene expression studies of malting induced germination of barley seeds in response to Alternaria alternata infection. Eur Food Res Technol 251, 1061–1073 (2025). https://doi.org/10.1007/s00217-025-04688-z

[39] Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998). Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev. 1998 Sep;62(3):597-635. doi: 10.1128/MMBR.62.3.597-635.1998. PMID: 9729602; PMCID: PMC98927.

[40] Scopes, R.K. (1982). Protein Purification. Principles and Practice. Springer-Verlag New York Heidelberg Berlin, 356 p.

[41] **Verma, D., Kumar, R., and Satyanarayana, T. (2019).** "Diversity in xylan-degrading prokaryotes and xylanolytic enzymes and their bioprospects," in Microbial Diversity in Ecosystem Sustainability and Biotechnological Applications, eds T. Satyanarayana, S. K. Das, and B. N. Johri (London: Springer Nature), 325–373. doi: 10.1007/978-981-13-8487-5_14.

[42] **Gupta R, Beg QK, Lorenz P. (2002).** Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol. 2002 Jun;59(1):15-32. doi: 10.1007/s00253-002-0975-y. Epub 2002 Apr 20. PMID: 12073127.

[43] **Sumantha, A., Larroche, C., & Pandey, A. (2006).** Microbiology and industrial biotechnology of food-grade proteases: a perspective. Food Technology and Biotechnology, 44(2), 211–220.

[44] **Kaur S. T. Huppertz and T. Vasiljevic (2024).** Plant proteases and their application in dairy systems. International Dairy Journal, 154, 105925. https://doi.org/10.1016/j.idairyj.2024.105925.

[45] **Chaiwut, P., Pintathong, P., & Rawdkuen, S. (2010).** Extraction and three-phase partitioning behavior of proteases from papaya peels. Process Biochemistry, 45(7), 1172–1175.

[46] **Aehle, W. (2007)** Enzymes in Industry: Production and Applications. Wiley-VCH/ Verlag GmbH and Co., Weinheim. https://doi.org/10.1002/9783527617098.

[47] **Vieille, C. and Zeikus, G.J. (2001)** Hyperthermophilic enzymes: Sources, uses, and molecular mechanisms for thermostability. Microbiology and Molecular Biology Reviews, 65, 1-43. http://dx.doi.org/10.1128/MMBR.65.1.1-43.2001.

[47] **Vieille, C. and Zeikus, G.J. (2001)** Hyperthermophilic enzymes: Sources, uses, and molecular mechanisms for thermostability. Microbiology and Molecular Biology Reviews, 65, 1-43. http://dx.doi.org/10.1128/MMBR.65.1.1-43.2001

[48] **Xiaoxu Z., Kexin F., Xianjun D. (2025).** Separation and identification of microbial enzymes on cereal grains/oilseeds and their activity assessment and effects on the quality of processing products - A comprehensive review, LWT, Food science and technology, 227,118031, https://doi.org/10.1016/j.lwt.2025.118031.

[49] **Haki GD, Rakshit SK (2003).** Developments in industrially important thermostable enzymes: a review. Bioresour Technol. 2003 Aug;89(1):17-34. doi: 10.1016/s0960-8524(03)00033-6.

[50] **Rizzello, C. G., Nionelli, L., Coda, R., De Angelis, M., & Gobbetti, M. (2010).** Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. *Food Chemistry*.

[51] **Derycke, V., Veraverbeke, W. S., Vandeputte, G. E., Man, W. D., Hoseney, R. C., & Delcour, J. (2005**). Impact of protein on pasting and cooking properties of nonparboiled and parboiled rice. Journal of Cereal Chemistry, 82(4), 468–474.

[52] **Xie, L., Chen, N., Duan, B., Zhu, Z., & Liao, X. (2008).** Impact of proteins on pasting and cooking properties of waxy and non-waxy rice. *Journal of Cereal Science*, **47**(2), 372–379. **https://doi.org/10.1016/j.jcs.2007.05.018**

[53] **Hasjim, J., Li, E., & Dhital, S. (2013).** Milling of rice grains: Effects of starch/flour structures on gelatinization and pasting properties. *Carbohydrate Polymers*, 92(1), 682–690. https://doi.org/10.1016/j.carbpol.2012.09.023

[54] ***Deepika, R., Dhanapal, K., Madhavan, N., & Kumar, P. (2022).*** Functional and Biochemical Characteristics of Extruded Snacks Flourished with Fish Powder and Shrimp Head Exudate During Storage Conditions. World Journal of Nutrition and Food Science, 2, 1006.

[55] **Adebowale, A. A., Adegoke, M. T., Sanni, S. A., Adegunwa, M. O., & Fetuga, G. O. (2012**). Functional properties and biscuit making potentials of sorghum–wheat flour composite. *American Journal of Food Technology*, *7*(6), 372–379. https://doi.org/10.3923/ajft.2012.372.379

[56] **Ding S. and Yang J. (2013).** The influence of emulsifiers on the rheological properties of wheat flour dough and quality of fried instant noodles, LWT - Food Science and Technology,53, 1, 61-69,https://doi.org/10.1016/j.lwt.2013.01.031.

[57] **Pareyt, B., & Delcour, J. A. (2008).** The role of wheat flour constituents, sugar, and fat in low moisture cereal-based products: A review on sugar-snap cookies. *Critical Reviews in Food Science and Nutrition*, *48*(9), 824–839. https://doi.org/10.1080/10408390701719223

[58] ***Day, L., Augustin, M. A., Batey, I. L., & Wrigley, C. W. (2006). Wheat-gluten uses and industry needs. Trends in Food Science & Technology, 17(2), 82–90.***

[59] **Rai S, Kaur A, Singh B. (2014).** Quality characteristics of gluten free cookies prepared from different flour combinations. J Food Sci Technol. 2014 Apr;51(4):785-9. doi: 10.1007/s13197-011-0547-1

[60] **Gujral, H. S., & Rosell, C. M. (2004). Improvement of the breadmaking quality of rice flour by glucose oxidase. Food Research International, 37(1), 75–81. https://doi.org/10.1016/j.foodres.2003.08.001**