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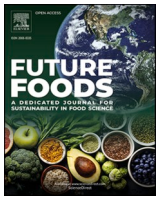

Impact of a protease and xylanase cocktail on the physicochemical properties of wafer batter and sheets

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ABSTRACT

This study investigated enzymatic treatment as a mean of regulating viscosity in wheat flour batter and its impact on the quality of wafer sheets. Parameters such as enzyme concentration and hydrolysis time were evaluated. Changes in the secondary structures of proteins in flour and commercial gluten were further investigated using attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR), confirming a decrease in α-helix and β-sheet structures. Enzymatic hydrolysis of gluten led to the unfolding of protein structure, resulting in an increase in free sulfhydryl group content. Rheological analysis of batters revealed a decrease in apparent viscosity in enzymatically treated batters compared to control ones. Enzymatic proteolysis caused a decrease in both storage modulus (G′) and loss modulus (G″) compared to control, indicating a weakened elastic structure and a more liquid-like behaviour. Enzyme-treated wafer sheets showed lower hardness and fracturability compared to control ones. These findings suggest that proteolytic enzymes could alter the chemical structure of gluten and the rheological properties of wafer batter leading to a reduction in batter viscosity, structural protein matrix, and the production of softer wafer sheets.

1. Introduction

For years, wafers have been integral to the confectionery world and play a vital role in numerous confectionery products (Verma, 2019). During its short baking process, wafer batter loses its moisture, resulting in a porous and crunchy structure, characterized by a light and thin texture (Manley, 2011a). Controlling the water level in batter enables better distribution of ingredients, while appropriate batter viscosity is required for a pumpable material and for achieving a desired texture in baked sheets (Dogan, 2006). Incorrect viscosity of the wafer batter, as well as the presence of lumps and air bubbles, results in the production of wafers with uneven thickness, poor appearance, and increased fragility (Chetrariu and Dabija, 2022).

Flours with strong gluten network pose various challenges in the confectionary industry, especially for wafer making, which requires flour to be milled to specific standards and to derive from soft wheat (Dogan, 2006; Irigoytia et al., 2025). While low-protein wheat flour can be used to reduce gluten in wafer formulations, its availability and consistency may vary, making it less reliable for large-scale production. Enzymatic treatment, particularly with proteases, could offer a more controlled and targeted approach to modifying gluten, allowing manufacturers to use standard flours (Pourmohammadi and Abedi, 2021; Naderi et al., 2022).

Enzymatic modification of batter affects its water holding capacity, viscoelasticity, and rheological properties, leading to changes in flavour, volume, crumb structure, and shelf life of final confectionary products (Manley, 2011b). Specifically, proteases are used in the wafer industry to break down gluten, preventing the formation of a strong gluten network, unlike in other baked goods where gluten development is encouraged for elasticity and volume (Tiefenbacher, 2017). This is particularly beneficial when aiming for uniform layers between wafer sheets, thereby providing consumers with a consistent mouthfeel bite.

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Studies suggest that protease treatment breaks down larger protein structures into smaller peptides, with altered secondary structure and reduced molecular sizes; such structural changes increase protein solubility and decrease batter viscosity (Naderi et al., 2022; Ismail et al., 2019). Moreover, fungal-derived proteases have been shown to enhance the water absorption capacity of proteins, leading to higher dough consistency (Ekramian et al., 2021).

Hemicellulase enzymes, such as xylanases, effectively reduce batter viscosity by hydrolysing pentosans, particularly arabinoxylans, which are major contributors to the high viscosity of wafer batter. Studies on wheat flour batters have demonstrated that xylanase treatment led to a significant viscosity reduction (Ahmad et al., 2013; Courtin et al., 2001). This effect is mainly due to the enzymatic breakdown of water-extractable arabinoxylans, resulting in lower molecular weight and reduced water-binding capacity (Redgwell et al., 2001). The hydrolysis also disrupts non-covalent interactions between arabinoxylans, starch, and gluten, and alters the pattern of arabinose substitution along the arabinoxylans backbone, further diminishing their thickening ability (Redgwell et al., 2001). In wafer production, there is a knowledge gap with regards to the specific mechanisms through which enzymes interact with batter substrates and how these changes affect the physical properties of batters (Naderi et al., 2022).

In this study, a protease-xylanase cocktail was used to investigate its influence on the physical and rheological attributes of wafer batter. Insights on the biochemical properties of protease and xylanase activities in the enzyme cocktail used for wafer production were given, whereas the rheological properties of wafer batter and the quality of subsequent baked sheets were also assessed.

2. Materials and methods

2.1. Materials

For wafer batter preparation, wheat flour was used (14.20 % moisture, 0.72 % ash, 8.80 % protein, Carr's Mill, Kirkcaldy, UK). A commercial enzyme cocktail (protease with xylanase side activity) was used in the study. Wheat gluten (WG) (CAS Number: 8002–80–0, ≥75 % protein, 5–7.5 % moisture content), O-phthalaldehyde (OPA), Dithiothreitol (DTT), methanol, sodium tetraborate decahydrate, Trichloroacetic acid (TCA), Sodium hydroxide (NaOH), Hydrochloric acid (HCl), urea, Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB), Sodium dodecyl sulphate (SDS), sodium sulphite, Bovine serum albumin (BSA), Copper(II) sulphate ($CuSO_4 \cdot 5H_2O$), Bicinchoninic acid (BCA), glycerol, Ethylenediaminetetraacetic acid (EDTA), iodoacetamide, SimplyBlue SafeStain buffer, Rhodamine B, Fluorescein isothiocyanate (FITC) were obtained from Sigma-Aldrich. 3,5-Dinitrosalicylic acid (DNS), and potassium sodium tartrate were purchased from Sigma-Aldrich. Beechwood xylan and arabinoxylan were purchased from Megazyme Ltd (Ireland).

2.2. Determination of protease and xylanase activity in the enzyme cocktail

Protease activity was carried out as outlined in a prior study (Merz et al., 2016), using wheat gluten (WG) as substrate. Briefly, a stock suspension of WG was prepared by dissolving WG powder (based on its protein content) in 0.1 M NaOH to achieve a concentration of 10 mg/mL, ensuring complete solubilization. This stock was then diluted with 150 mM phosphate buffer (pH 6.0) at ratio of 1:4 (v/v, stock solution to buffer), to obtain a working solution with a final protein concentration of 2 mg/mL. After dilution, the final pH of the WG suspension was measured and adjusted to pH 6.5. For enzyme hydrolysis, 300 μL of WG suspension were incubated with 200 μL of phosphate buffer at 35 °C for 5 min, followed by the addition of the 100 μL enzyme solution. The mixture was incubated at 35 °C for 30 min in a water bath, and the enzymatic reaction was terminated by the addition of 100 μL TCA (1.5 M). The mixture was centrifuged at 10,000 × *g*, at 4 °C for 5 min and the supernatant was collected. Then, 125 μL of supernatant were mixed with 875 μL of OPA reagent, consisting of 11 mM OPA and 20 mM DTT dissolved in 11.25 mL methanol and made to 100 mL using disodium tetraborate buffer (pH 9.8). Samples were incubated at 37 °C for 1 min and their optical density was measured at 340 nm using a spectrophotometer. A standard curve using serine was constructed to calculate the proteolytic activity of the sample. One unit (U) of protease activity was defined as the release of 1 μmol serine from WG per min at 35 °C and pH 6.5 reflecting the actual conditions of the reaction.

For xylanase activity assessment, the reaction mixture consisted of either 1 % (w/v) beechwood xylan or 1 % (w/v) arabinoxylan (400 μL) diluted in 150 mM phosphate buffer (pH 6.0) with the final pH adjusted to 6.50. This was combined with 100 μL of enzyme solution and incubated at 35 °C for 30 min. To inactivate the enzyme, the samples were heated at 100 °C for 10 min and centrifuged (10,000 × *g*, 20 °C, 1 min). Following this, 100 μL of sample supernatant was mixed with an equal volume of DNS reagent and incubated at 100 °C for 5 min. Subsequently, 1 mL of water was added to the mixture, and absorbance was measured at 540 nm (Miller, 1959). A calibration curve was generated using xylose (0.1–1 mg/mL). One unit (U) of xylanase activity was defined as the amount of μmol of xylose released per min per mL of reaction mixture at 35 °C and pH 6.5. Three replicates were carried out for each enzymatic assay.

2.3. Kinetic studies

To determine kinetic parameters such as Michaelis-Menten constants (K_m and V_{max}), catalytic constant (K_{cat}), and catalytic efficiency (K_{cat}/K_m), various substrate concentrations were used. Specifically, the enzyme cocktail was incubated with different concentrations of gluten (for protease activity) and beechwood xylan and arabinoxylan (for xylanase activity) ranging from 2 to 50 mg/mL. The enzyme activity was then assessed as described in Section 2.2.

2.4. Effect of enzyme dosage on hydrolysis of wheat protein

To investigate the effect of different enzyme cocktail concentrations on protease substrates under isoproteic conditions, suspensions of gluten and wheat flour were prepared. Based on the protein content of each material, appropriate amounts of substrate were weighed and suspended in 0.1 M NaOH to achieve a stock protein concentration of 10 mg/mL then diluting with 150 mM phosphate buffer (pH 6.0) at a 1:4 (v/v) ratio (stock solution to buffer), resulting in a final concentration of 2 mg/mL. The pH of the resulting solution was measured and, if necessary, adjusted to pH 6.5. Enzyme powders were prepared at concentrations of 2, 4, 8, and 12 mg/mL in 150 mM phosphate buffer (pH 6.5). Then, 100 μL of each enzyme solution was added to the substrate solution to achieve final enzyme activities of 0.228, 0.403, 0.555, and 0.790 U/mL, respectively. The mixtures were incubated for 60 min at 35 °C. Then, the mixture was heated for 10 min at 95 °C to inactivate the enzyme followed by centrifuging at 10,000 × *g*, 4 °C for 5 min. Supernatants were collected and the degree of hydrolysis (DH) was measured using the OPA assay (Nielsen et al., 2001). Briefly, 125 μL of samples were mixed with 875 μL of OPA and incubated at 37 °C for 1 min. Colour development was recorded at 340 nm using a spectrophotometer. The DH of samples was calculated using Equation 1 (Merz et al., 2015):

$$\mathrm{DH}\ (\%) = \frac{h}{h_{total}} \times 100 \quad (1)$$

where :

h (mol/L) is the concentration of free amino groups; and
h_{tot} (mol/L) is the maximum concentration of free amino groups at complete hydrolysis

The maximum concentration of free amino groups at complete hydrolysis (h_{tot}) was measured as described in Equation (2) (Merz et al., 2015):

$$\text{htotal (mol / L)} = \frac{C_{protein}}{M^{*} - M_{H2O}} \quad (2)$$

where:

$C_{Protein}$ (mg/mL) is the concentration of wheat flour protein and gluten protein;
M* is the average molecular mass of the amino acids in WG protein (133.50 g/mol).

The molecular mass of water ($M_{H2O} = 18$ g/mol) is subtracted due to the addition of a water molecule during hydrolysis of peptide bonds (Merz et al., 2015).

2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Wheat flour and gluten protein hydrolysates were subjected to SDS-PAGE analysis as described in a previous study (Chatzifragkou et al., 2016). Briefly, supernatants were mixed with NuPAGE LDS sample buffer and NuPAGE reducing agent. Samples were loaded onto a 1.0 mm 4–12 % Bis–Tris pre-casted gel (NuPAGE Novex, UK) and electrophoresis was conducted using Xcell SurelockTM unit (Invitrogen, UK) at constant voltage (200 V) for 35 min. Then, the gel was washed three times with distilled water followed by staining using SimplyBlue SafeStain buffer (Life Technologies, UK) for 60 min at room temperature. Finally, the gel was washed once again with distilled water to visualize protein bands.

2.6. Measurement of secondary structure and free SH content of protein hydrolysates

To measure the secondary structure and free SH content of the wheat flour and gluten protein, a maximum enzyme dosage of 0.790 U/mL (protease activity) was selected, to ensure extensive hydrolysis and maximize any protein structural modifications. Wheat flour and gluten protein were firstly dissolved in 0.1 M NaOH to achieve a stock protein concentration of 50 mg/mL. This stock was then diluted with phosphate buffer (pH 6.0) at a 1:4 (v/v) ratio (stock to buffer), resulting in a final protein concentration of 10 mg/mL. The pH of the resulting solution was measured and adjusted to pH 6.5. To this, the enzyme cocktail was added at an activity of 0.790 U/mL and hydrolysis took place of 120 min at 35 °C. Samples were taken at 15, 30, 60, and 120 min of hydrolysis and heated at 95 °C for 10 min to inactivate the enzyme. The, samples were centrifuged at 10,000 × *g* at a 4 °C for 5 min and supernatants were collected and stored at −20 °C.

Gluten and wheat flour hydrolysates were freeze-dried and analysed by attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR) (100 FT-IR, PerkinElmer). Scanning was carried out in the range of 4000–650 cm^{-1} with a resolution of 4 cm^{-1}; 32 scans were applied for each sample. The amide I band (1600–1700 cm^{-1}) was analysed and deconvolution was applied using OMNIC software.

The free sulfhydryl (-SH) content was measured using the DTNB assay as described in former studies with some modifications (Žilić et al., 2012; Ren and Li, 2022). Specifically, 400 μL of WG and flour hydrolysates were diluted with 600 μL of Tris-glycine buffer containing 86 mM Tris, 90 mM glycine, 4 mM EDTA, 8 M urea (pH 8.0). To this mixture, 50 μL of 4 mg/mL DTNB solution prepared in 10 mM Tris-glycine buffer (pH 8.0), was added. After 1 hour incubation at 25 °C, the absorbance was recorded at 412 nm against blank. The free SH content (μmol/g) were calculated using the following equation:

$$\text{Free SH } (\mu\text{mol / g}) = \frac{73.53 \times A \times D}{C} \quad (3)$$

where:

A is the sample absorbance;
D is dilution factor, and;
C is the concentration of proteins (mg/mL) in the reaction mixture.

2.7. Wafer batter preparation

For wafer batter preparation, each reaction mixture consisted of wheat flour and distilled water mixed at a 1:1 ratio (water: flour). The distilled water was preheated to 35 °C before mixing. Enzyme-treated samples contained enzyme activities of 0.228, 0.403, 0.555, and 0.790 U/mL, corresponding to enzyme concentrations of 2, 4, 8, and 12 mg/mL, respectively, prepared in distilled water adjusted to pH 6.5. The solutions were mixed for 5 min at 2000 g using a Silverson L4R rotor–stator high shear mixer. The batters incubated at 35 °C for 30 min to allow the enzyme reaction before further analysis. All samples were prepared in triplicate.

2.8. Rheological measurements of wafer batter

Rheological properties of wafer batters were studied using an oscillatory rheometer (MCR 302 Anton Paar, St Albans, UK) with parallel serrated plate-plate (50 mm diameter; Profiled 1 mm x0.5 mm). Batter samples were loaded in the rheometer and allowed a resting period of 10 min at 35 °C (optimum temperature of enzyme cocktail) for temperature equilibration. A gap size of 1 mm was set, and a constant temperature of 35 °C was applied. To assess changes in batter apparent viscosity during enzymatic treatment, batters with different enzyme concentrations were loaded in the rheometer and apparent viscosity was measured at constant shear rate (2 s^{-1}) during 28 min (representing the enzymatic reaction time in the batter prior to baking), at 35 °C (optimum temperature of enzyme cocktail). To avoid water evaporation during the measurement, the edges of the samples were coated with silicone oil. All measurements were made in duplicate.

The viscoelastic properties of control and enzymatically treated batters were measured using serrated parallel plates (50 mm diameter, 1 mm × 0.5 mm profile) with a 1 mm gap. After loading, samples were allowed to rest for 10 min to reach the testing temperature (35 °C). Oscillatory frequency sweeps ranging from 0.1 to 100 Hz (0.63–628 rad/s) were performed at a constant strain amplitude of 0.08 %, which was within the linear viscoelastic (LVE) region as determined previously by stress sweeps at 1 Hz. The dynamic rheological behaviour was evaluated by recording the storage modulus (G′) and loss modulus (G″) followed by the calculation of loss factor (tan δ =G″/G′). Measurements were carried out on two batter replicates for each formulation.

2.9. Confocal laser scanning microscopy (CLSM)

The microstructure of control and enzyme treated batters was observed using a Nikon A1-R confocal microscope (Nikon, Tokyo, Japan) following a previously established method (Tao et al., 2023). An Ar laser line (488 nm and 638) was employed as light source to excite fluorescent dyes Fluorescein 5-isothiocyanate (FITC) and Rhodamine B (Fluka, Sigma-Aldrich, St. Louis, Mo., U.S.A.). Rhodamine B with λex max 638 nm and λem max 738 nm was solubilized in distilled water at 0.02 % (w/v) and used to stain proteins in red. FITC with λex max 488 nm and λem max 550 nm was solubilized in distilled water at 0.15 % (w/v) and used to stain starch in green.

For sample visualisation, 10 μL of batter (control or enzyme treated) was mixed with 10 μL of Rhodamine B followed by 10 min incubation period in the dark for diffusion of the dye into the sample. Then 10 μL of FITC was added and another 10 min of incubation in dark were allowed. The samples were observed using the ×100/1.4NA/ Plan Apo oil immersion lens (Zeiss, Oberkochen, Germany) and images were captured

(1024×1024-pixel resolution) using the microscope software (Carl Zeiss, Jena, Germany). At least three images were obtained per sample per replicate.

2.10. Wafer preparation and properties

10 g of control and enzyme treated wafer batters (0.228, 0.403, 0.555, and 0.790 U/mL) were baked at 100 °C for 3 min using a wafer baking machine fitted with two iron plates (32×25×53 cm, SILULCM, China). After baking, wafer sheets were cooled to room temperature and sealed in polypropylene bags to prevent moisture absorption. All wafer samples (control and enzyme treated) were baked in triplicate.

Colour measurements were performed with a Chroma Meter CR-400 colorimeter (Konica Minolta, Warrington, UK). The results were expressed in accordance with the CIELAB system (illuminate C and 10° viewing angle). The measurements were made with an 8 mm diameter diaphragm inset with optical glass. The parameters measured were L* (L* = 0 [black], L* = 100 [white]), a* ((–a* = greenness and +a^* = red) and b* ((–b* = blueness and +b^* = yellow). The colour of each wafer sheet was measured in four different points. The total colour difference (ΔE*) between the control wafer and each of the enzyme treated samples was calculated using the following equation (Francis and Clydesdale, 1975):

$$\Delta E^* = \left[(\Delta L^*)^2 + (\Delta b^*)^2 + (\Delta a^*)^2)\right]^{1/2} \quad (4)$$

The values used to determine whether the total colour difference was visually obvious were the following (Bodart et al., 2008):

ΔE* ⟨ 1 colour differences are not obvious for the human eye. 1 < ΔE* < 3 minor colour differences could be appreciated by the human eye depending on the hue, and Chroma. ΔE* ⟩ 3 colour differences are obvious for the human eye.

Browning index (BI) was calculated as follows:

$$BI = \frac{100(x - 0.31)}{0.172} \quad (5)$$

Where:

$$x = (a^* + 1.75L^*)/(5.645L^* + a^* - 0.3012b^*) \quad (6)$$

Texture analysis was performed using a TAXT-Plus Texture Analyzer (Stable Micro System, Godalming, UK). The three-point bending test (HDP/3 BP) was performed to measure the hardness and fracturability of wafer sheets (6 × 6 cm). The test parameters were: trigger force 0.049 N; pretest speed 1.0 mm/s; test speed 1.0 mm/s; post-test speed 10.0 mm/s; load cell 30 N; and rupture distance 20 mm. For each sample type, the test was performed using three different wafer sheets per batch, resulting in a total of six measurements per sample. The moisture content of wafers was analysed using a Sartorius M-Pact moisture analyser (Sartorius Lab Instruments, Goettingen, Germany). 3 g of grinded wafer was placed on an aluminium plate and heated at 100 °C until constant weight was reached. Water activity was determined using a HygroLab instrument (Rotronic Instruments, Crawley, UK) with 1 g of grinded sample.

2.11. Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) using XLSTAT software (version 2023.1.4, Addinsoft, France). Multiple pairwise comparisons using Duncan test were done to evaluate mean values differences ± ($p < 0.05$).

3. Results and discussion

3.1. Kinetic parameters of the enzyme cocktail

The kinetic parameters of the enzyme cocktail were measured using wheat gluten (WG), beechwood xylan and arabinoxylan as substrates. The kinetic behaviour of both enzymes provides a predictive framework for understanding how targeted enzymatic hydrolysis can be used to modulate batter functionality and final product quality. Partial degradation of arabinoxylans may have weakened the polysaccharide network in the batter, thereby influencing its viscosity and contributing to the softer, more open texture observed in the final wafers. Substrates that were more accessible and structurally simple facilitated stronger enzyme-substrate interactions, resulting in lower K_m values and higher V_{max} . In contrast, more complex or recalcitrant substrates, could increase K_m and decrease V_{max} due to steric hindrance and limited access to reactive sites. These differences show the influence of substrate structure on enzyme efficiency, affecting both binding affinity and catalytic performance (Mustefa Beyan et al., 2021).

When WG was used as substrate, the protease activity exhibited K_m of 25.85±0.40 mg/mL, V_{max} of 0.11±0.08 U/mL, K_{cat} of 0.02±0.001 1/min and K_{cat}/K_m of 0.001±0.002 mL/min per mg. For the xylanase side activity of the enzyme cocktail, using beechwood xylan as substrate, K_m was 10.48±0.87 mg/mL, V_{max} was 0.68±0.12 U/mL, K_{cat} was 0.17 ±0.04 1/min, K_{cat}/K_m was 0.016±0.04 mL/min.mg. When arabinoxylan was used as the substrate, K_m was 202.57±1.33 mg/mL, V_{max} was 3.07 ±0.97 U/mL, K_{cat} was 0.76±0.26 1/min, and K_{cat}/K_m was 0.003±0.01 mL/min per mg.

Based on these results, the lower K_m of the proteolytic activity denotes a higher affinity of the particular enzyme for wheat gluten. Enzyme activity is influenced by substrate type, assay conditions and the purity of enzyme, making direct comparisons of their kinetic constants often challenging (Alamnie et al., 2023). K_m values vary across enzymes; for instance, K_m values for proteases from *Bacillus* sp. can range from 28 mg/mL to 100 mg/mL (Ahmed et al., 2021; Shad et al., 2024), denoting the influence of the type of substrate and its structural variations.

With regards to the kinetic parameters of the enzyme's xylanase activity, it exhibited a lower K_m value toward beechwood xylan compared to arabinoxylan. This higher affinity may be attributed to the ability of xylanase to easily recognize and bind to cleavage sites on beechwood xylan, facilitating its hydrolysis (Sepulchro et al., 2020). The catalytic efficiency, represented by the specificity constant (K_{cat}/K_m) was also higher for beechwood xylan compared to arabinoxylan. The specificity constant is a critical measure of enzyme efficiency and reflects how effectively the enzyme converts a substrate into a product. Moreover, the maximum velocity (V_{max}) of xylanase was higher when acting on arabinoxylan, indicating a greater potential of xylanase to hydrolyse arabinoxylan at saturation levels. Furthermore, the turnover number (K_{cat}), which represents the number of substrate molecules converted into product per enzyme molecule per min, was approximately four times higher for arabinoxylan compared to beechwood xylan. This suggested that while the enzyme has a higher affinity and efficiency for beechwood xylan, it exhibited a faster catalytic rate for arabinoxylan under optimal conditions. This difference could be attributed to structural variations between beechwood xylan and arabinoxylan, particularly in branching and linkage patterns. Beechwood xylan is predominantly linear, whereas arabinoxylan is highly branched with arabinose substitutions (Mendonça et al., 2023). The kinetic results of substrate hydrolysis provided information into the endo- and exo-activities of the enzyme cocktail: the stronger affinity for beechwood xylan suggested that the enzyme possesses exo-activity, which is better suited for linear substrates with fewer steric hindrances (Moreira and Filho, 2016). The observed differences in turnover rates, indicated that the enzyme exhibits both endo- and exo-activities. The higher affinity of xylanase toward beechwood xylan compared to arabinoxylan has also been previously reported for xylanase from *Beauveria bassiana*, (Amobonye et al., 2021) and xylanase from *Fusarium graminearum* (Zhang et al., 2024). The kinetic parameters obtained for the enzyme cocktail indicated that both protease and xylanase exhibited efficient catalytic activity toward their respective substrates, which provided a predictive framework for understanding how targeted enzymatic

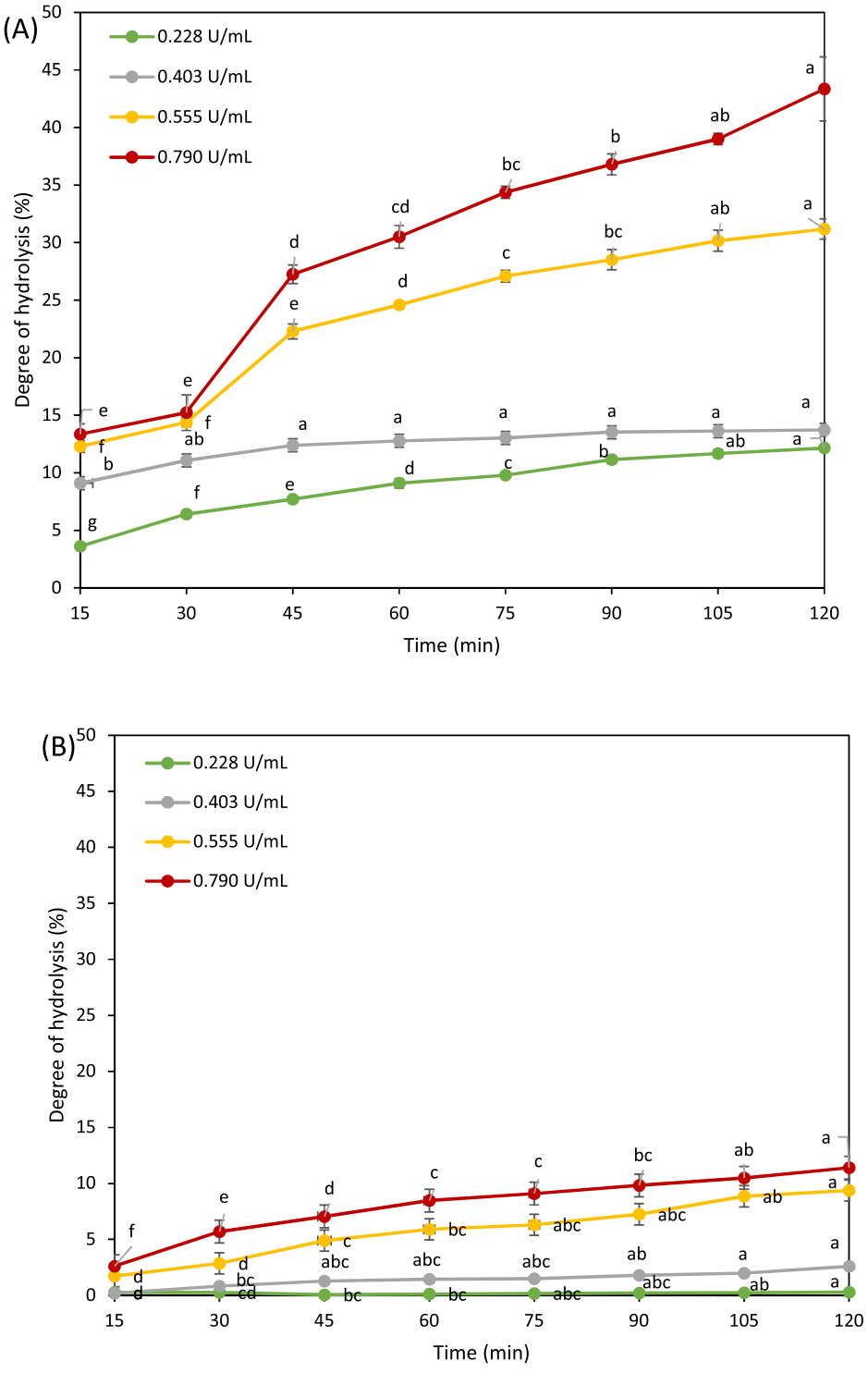

Fig. 1. Effect of enzyme concentration (0.228, 0.403, 0.555, and 0.790 U/mL) on the degree of hydrolysis of (A) wheat gluten and (B) wheat flour, within 120 min. Different superscript letters in each curve indicate statistical significance at $p < 0.05$.

hydrolysis influences the rheology and physical properties of the final wafers.

3.2. Enzymatic hydrolysis of gluten and wheat flour

The effect of varying concentrations of the enzyme cocktail on WG and flour was investigated, and results are shown in Fig. 1. In gluten, at the lowest proteolytic activity (0.228 U/mL), the DH after 120 min of hydrolysis was 12.15 %. When the proteolytic activity increased to 0.403 U/mL, the DH reached 12.7 % after 60 min and plateaued at 13.7 % at 120 min. A notable increase in DH was observed at higher proteolytic activities (0.555 U/mL and 0.790 U/mL), whereby after 120 min, the DH reached 31.17 % and 43.35 %, respectively. These findings suggest that although the increase rate of DH varied, it was more pronounced at higher proteolytic activities.

Furthermore, the effect of enzymatic hydrolysis of wheat flour was

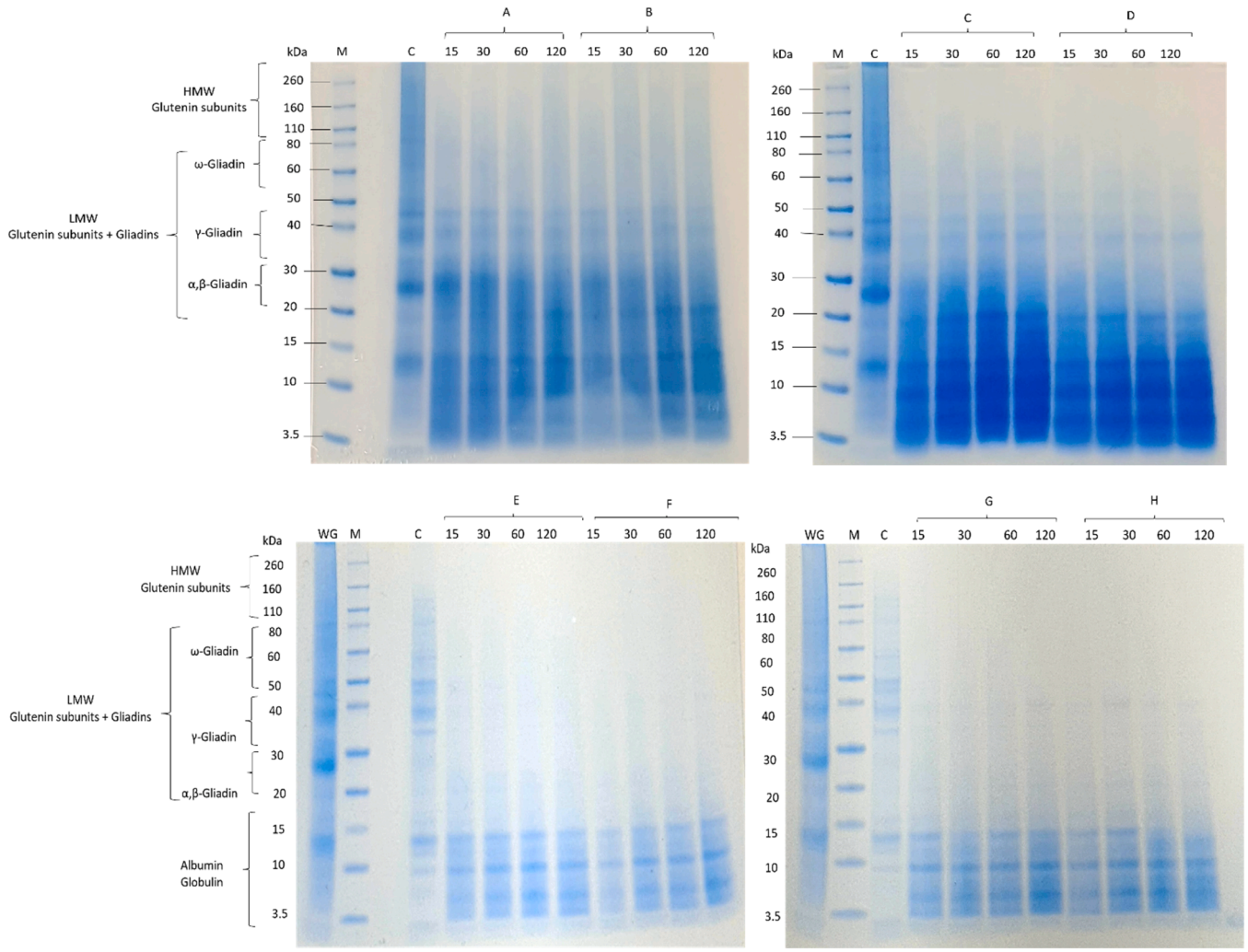

Fig. 2. SDS-PAGE of wheat gluten (A-D) and wheat flour (E-H) hydrolysed by enzyme cocktail within 120 min. (A) (E): 0.228 U/mL protease, (B) (F): 0.403 U/mL protease 0, (C) (G): 0.555 U/mL protease, and (D) (H): 0.790 U/mL protease. M represents the molecular marker, WG stands for wheat gluten and C represents control.

examined. As depicted in Fig. 1B, low proteolytic activity (0.228 U/mL) was associated with low DH values. Higher DH values were observed with increasing proteolytic activity, reaching up to 11.40 % (0.790 U/mL protease). Due to the presence of diverse components such as starch, lipids, and non-starch polysaccharides, the accessibility of proteins to proteases was limited, resulting in lower DH compared to purified gluten preparations.

Additionally, the xylanase activity in the enzyme cocktail targets arabinoxylans, reducing their molecular weight and disrupting arabinoxylan-protein interactions. This breakdown weakens the structural barriers formed by arabinoxylans, increasing protein accessibility to proteases and enhancing overall proteolytic efficiency. This synergistic effect is important for understanding the enzymatic breakdown of proteins and polysaccharides in wafer batter. These differences in DH can directly impact the textural and structural properties of the final wafers. While proteolysis alone weakens the gluten network, the concurrent breakdown of arabinoxylans further modifies water-binding capacity and batter rheology, contributing to softer, more manageable wafers (Pourmohammadi and Abedi, 2021).

The protease used in this study exhibited affinity toward WG, as demonstrated by the kinetic analysis, and showed substantial endopeptidase activity based on OPA assay results for hydrolysed WG and wheat flour proteins. The progressive breakdown of proteins into smaller peptides and free amino acids, as observed through DH measurements can be indicative of exopeptidase activity of the enzyme cocktail suggesting that the enzyme employed in this research exhibited exopeptidase capabilities in addition to its known endopeptidase activity.

In this study, maximum DH value of 43.35 % was achieved within 120 min at the highest enzyme concentration (0.790 U/mL). This result is notably higher than the maximum DH reported by previous studies, showing the high catalytic efficiency of the enzyme used in this study. Specifically, in a previous study, the hydrolysis of wheat gluten by *Aspergillus oryzae* protease resulted in up to 60 % gluten hydrolysis, yielding a DH up to 10 % over 420 min (Hardt et al., 2013). Similar DH values have been reported using other commercial proteases (Alcalase) (Nordqvist et al., 2012), whereas higher DH values (~20 %) have been reported for much more extended hydrolysis time (36 h) (Cui et al., 2011).

3.3. SDS-PAGE of wheat gluten and flour hydrolysates

Changes in the molecular size distribution of WG and flour hydrolysates were monitored under reduced conditions using SDS-PAGE. Gluten primarily comprises glutenin and gliadin. Glutenin has high molecular weight subunits (HMW-GS) ranging between 100 and 140 kDa, and low molecular weight subunits (LMW-GS) ranging from 30 kDa to 55 kDa (Bietz and Simpson, 1992). Moreover, gliadin consists of

Table 1
Analysis of secondary structure in (A) wheat gluten and (B) wheat flour hydrolysates after 120 min using 0.790 U/mL protease.

(A)			
Time (min)	**β-sheet (%)**	**Random coil and α-helix (%)**	**β-turn (%)**
Control	38.89±1.49[a]	47.70±1.54 [a]	13.69±1.34 [c]
0	35.66±1.13 [ab]	53.38±1.19 [b]	10.77±0.85 [b]
15	38.35±0.87 [ab]	36.81±1.76 [c]	24.91±1.18 [a]
30	34.77±0.86 [ab]	38.47±1.08 [c]	26.32±0.96 [a]
60	35.20±2.18 [ab]	38.25±0.91 [c]	26.51±−0.82 [a]
120	37.20±0.63 [b]	36.77±0.95 [c]	26.09±0.83 [a]
(B)			
Time (min)	**β-sheet (%)**	**Random structure and α-helix (%)**	**β-turn (%)**
Control	44.24±1.59[c]	46.46±0.84 [a]	8.36±1.05 [c]
0	40.56±1.35 [b]	41.95±01.44 [b]	17.94±1.45 [b]
15	47.42±0.99 [b]	35.52±0.52 [c]	17.25±0.79 [b]
30	52.54±0.83 [a]	35.47±0.97 [c]	14.99±0.70 [ab]
60	52.75±1.94 [a]	35.58±1.08 [c]	15.51±0.77 [a]
120	52.13±0.86 [a]	28.57±1.17 [c]	19.09±0.79 [a]

Different superscript letters in each column indicate statistical significance at $p < 0.05$.

ω-gliadins (46 kDa to 74 kDa), and γ-gliadins and α/β-gliadins with lower molecular weights, ranging between 30 and 45 kDa (Kasarda et al., 1983). According to Fig. 2A-D, the SDS-PAGE patterns of gluten hydrolysates showed a gradual reduction in the intensity of bands greater than 90 kDa which correspond to HMW-GS. Furthermore, clear and thick bands with molecular weight of around 28 to 42 kDa were detectable in the control sample (non-hydrolysed gluten) which correspond to α, β, γ and ω gliadins and LMW-GS. These bands disappeared after enzymatic hydrolysis. Following the hydrolysis of gluten by enzyme cocktail, new bands appeared below 10 kDa (Fig. 2). Moreover, higher concentrations of protease (0.555 U/mL and 0.790 U/mL) led to the appearance of new bands with higher intensity compared with lower enzyme concentrations (0.228 U/mL and 0.403 U/mL) showing that more enzyme molecules were able to interact with the protein and break down substrate molecules. These findings suggest that the enzyme could break down the amorphous or less-ordered regions of gliadins in gluten as well as polymeric proteins of gluten, such as glutenin with crystalline structure.

The SDS-PAGE patterns of hydrolysed wheat flour by varying concentrations of enzyme cocktail under reducing conditions are presented in Fig. 2E–H. The SDS-PAGE results of the control sample (C) showed band patterns within the range of 110–160 kDa and 35 to 80 kDa. These ranges are representative of HMW-GS and a combination of LMW-GS and gliadins, respectively. These bands disappeared after hydrolysis across all enzyme cocktail concentrations utilized. Bands between 10 and 15 kDa were observed although with a diminished intensity after hydrolysis, suggesting a partial degradation of proteins or polypeptides within this molecular weight range. Additionally, some low-intensity polypeptide chain bands in range of 3.5 to 10 kDa appeared. The intensity of these bands displayed a noticeable increment correlating with extended hydrolysis duration across all enzyme concentrations.

3.4. Impact of enzymatic hydrolysis on secondary structure and free SH content of wheat gluten and flour

ATR-FTIR analysis was performed to investigate alterations in the secondary structure of wheat flour proteins as well as gluten. As shown in Table 1, notable changes were observed after the enzymatic hydrolysis of gluten and wheat flour. There was a consistent decrease in total α-helix and random coils with increasing hydrolysis time. The amide I band, typically observed in the 1600–1700 cm^{-1} range in the FT-IR spectrum, is primarily due to the C = O stretching vibration and a minor contribution from the C–N stretching. The bands in 1648–1667 cm^{-1} region is indicative of α-helix structures, known for their coiled configuration (Deng et al., 2016). Moreover, bands at 1640–1650 cm^{-1} were attributed to random coils (Gantumur et al., 2023). The decrease in total α-helix and random coils suggested that the protease activity was effectively disrupting the hydrogen bonds that stabilize these structures, leading to a loss of organized secondary structures. Hydrogen bonds play a key role in stabilizing protein secondary structures, and their strength depends on bond length and direction (Jackson and Mantsch, 1995). Shorter and more linear hydrogen bonds are generally stronger than longer or more angular ones. These stronger hydrogen bonds increase electron sharing with the carbonyl (C = O) group in peptide bonds, reducing its electron density. As a result, the amide I band shifts to a lower wavenumber, reflecting structural changes within the protein (Jackson and Mantsch, 1995).

Similarly, the amount of β-sheets was reduced as hydrolysis time increased. The β-sheets, essential for protein stability, are identified by bands in two ranges: 1618–1640 cm^{-1} and 1670–1690 cm^{-1} (Wang et al., 2016). This reduction further indicated the disassembly of the secondary structure of the protein, impacting the overall protein conformation. Previous studies have reported that the decrease in β-sheet content in the secondary structure of WG was closely associated with increased solubility (Li et al., 2024a; Liu et al., 2021). Similarly, the use of endopeptidases for gluten hydrolysis altered the solubility of gluten hydrolysates by breaking down the secondary structure and generating smaller polypeptide units through enzymatic action (Deng et al., 2016).

Furthermore, hydrolysis with the enzyme cocktail led to an increase in β-turn structures in both wheat flour and gluten (Table 1). β-turns, which are essential for the compact folding of proteins, have been associated with bands at 1660–1670 cm^{-1} and 1690–1700 cm^{-1} (Wang et al., 2016). As β-turns are mainly located on the surface of protein molecules, they influence the direction of the peptide chain, promoting bending, folding, and reorientation (Jiang et al., 2021). Therefore, the rise in β-turn content may indicate modifications in the molecular morphology of WG (Li et al., 2024a). These results agree with previous studies that reported a reduction in α-helix and β-sheet structures, along with an increase in β-turns of WG when hydrolysed by the combination of Neutrase and Flavourzyme (Dai and An, 2024). This suggested that enzymatic treatment disrupted the ordered structure of the protein, leading to disorder after hydrolysis (Dai and An, 2024). Similarly, the breakdown of WG by Alcalase led to a reduction in both α-helix and β-sheet structures, suggesting increased flexibility and susceptibility of the Alcalase treated samples to other enzymatic action compared to the untreated ones (Wang et al., 2016).

The DH values of WG and flour hydrolysis are presented in Fig. 3. According to Fig. 3A, after 15 min of hydrolysis, a DH of 23.87 % was observed for WG. This value increased to 32.24 % at the 30-min mark. Notably, the DH remained nearly constant between 30 and 120 min, reaching 33.62 % at the end of hydrolysis. This pattern indicated a rapid initial hydrolysis phase, where significant breakdown of the protein structure occurs, followed by a plateau phase where further hydrolysis did not alter the DH. This suggested that the majority of hydrolysable bonds in the gluten are cleaved within the first 30 min, after which the protein structure becomes more resistant to further hydrolysis. As shown in Fig. 3B, the hydrolysis of wheat flour resulted in a lower DH compared to WG. The DH of wheat flour increased over time but plateaued after 90 min and reached a maximum of 19.21 % within 120 min.

The changes of free SH during the hydrolysis of gluten and flour by protease are shown in Fig. 3. The free SH content in gluten showed a notable increase, reaching 3.06 μmol/g within the initial 15 min of hydrolysis. Extending the hydrolysis time up to 120 min increased the free SH levels up to 4.99 μmol/g (Fig. 3A). The free SH levels in wheat flour were lower than those observed in WG. After 90 min, the free SH levels in wheat flour reached 3.55 μmol/g and remained almost stable

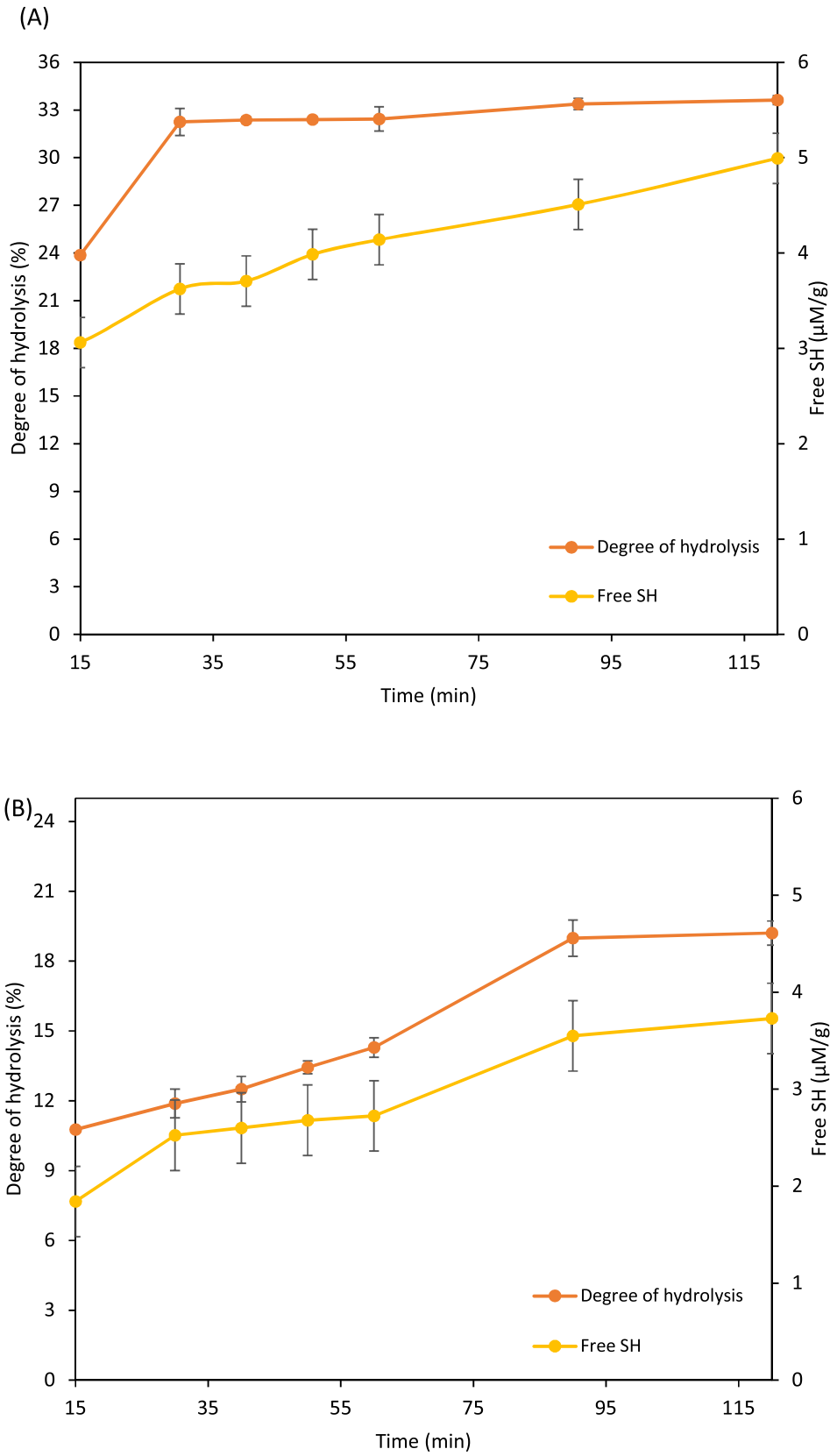

Fig. 3. Degree of hydrolysis and free SH content of (A) gluten protein, and (B) wheat flour, within 120 min hydrolysed by 0.790 U/mL protease activity.

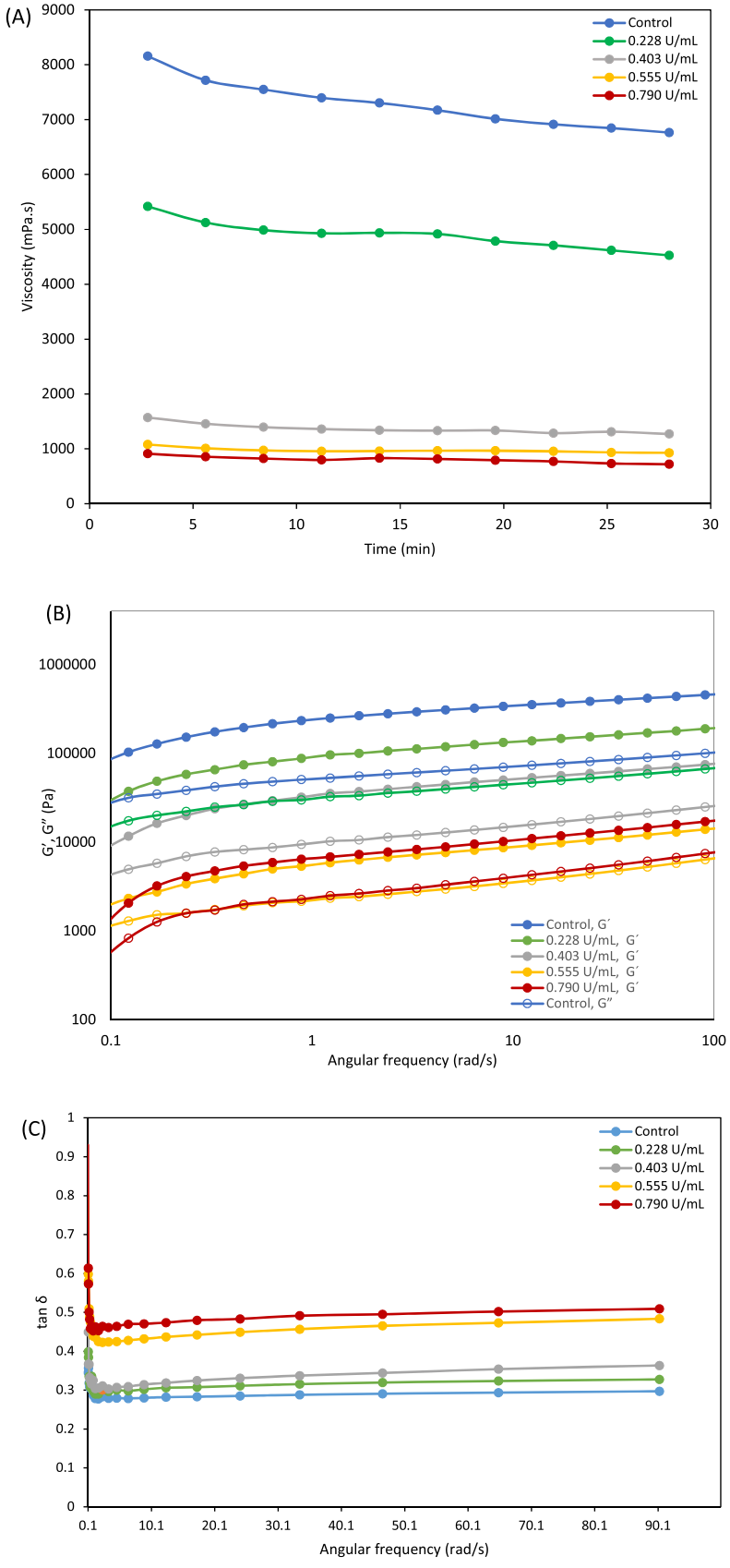

Fig. 4. (A) Viscosity of wafer batter in control and enzyme-treated samples at various enzyme concentrations (0.228, 0.403, 0.555, and 0.790 U/mL). (B) Comparison of the storage modulus (G′) and loss modulus (G″) of the control and enzyme treated batters (0.228, 0.403, 0.555, and 0.790 U/mL) as a function of frequency. (C) tanδ (G″/ G′) of control and enzyme treated batters (0.228, 0.403, 0.555, and 0.790 U/mL) as a function of frequency.

thereafter, indicating that no further pronounced hydrolysis occurred beyond this time point (Fig. 3B).

When proteases act on gluten peptides containing sulfhydryl (SH) groups, they disrupt the peptide structure, causing the proteins to unfold. This unfolding process exposes sulfhydryl groups that were previously hidden within the internal structure of protein, thereby increasing the free SH contents. This increase in free SH content showed the disruption of the native gluten structure. Additionally, the formation of new disulfide bonds may occur between newly exposed SH groups and existing unpaired cysteine residues, contributing to the stabilization of smaller peptides (Lindsay and Skerritt, 1999; Deng et al., 2016).

3.5. Rheological properties of wafer batter

The viscosity of wafer batter is crucial in wafer production; high viscosity, often due to excess gluten formation, can lead to poor processing conditions and undesirable texture and quality of baked wafers (Verma, 2019). In this study, enzymatic hydrolysis of wheat gluten (WG) and wheat flour using a protease-xylanase cocktail induced modifications in each substrate, reflected in their respective degrees of hydrolysis (DH). In the case of WG, hydrolysis disrupted the protein network, particularly affecting the secondary structures such as α-helices and β-sheets. These compositional and structural modifications are expected to influence the rheological properties of the resulting batter, including its viscosity and viscoelastic behaviour.

In this study, batter viscosity was measured at a constant shear rate over a 28 min to evaluate changes during enzyme incubation at 35 °C. As shown in Fig. 4A, all batters exhibited a decrease in viscosity over the incubation period. This reduction in viscosity increased with higher enzyme concentration. These results could be linked to the effect of protease in protein secondary structure and rearrangement of SH groups, increasing protein solubility and thus decreasing batter viscosity. However, this decrease in viscosity may also result from the hydrolysis of arabinoxylans, which reduced their molecular weight and consequently lowered their water absorption capacity. Kinetic data confirmed the xylanase activity toward arabinoxylans, supporting its role in modulating batter viscosity. These findings align with previous studies showing that xylanase treatment of wheat batter reduces water-extractable arabinoxylan content, thereby decreasing water absorption and viscosity (Petit-Benvegnen et al., 1998; Ha and Kweon, 2024).

Frequency sweeps provide information on the viscoelastic properties of samples; high-frequency oscillations mimic rapid changes occurring over short time scales, while low-frequency oscillations correspond to slower changes over extended periods or conditions of rest (Tsatsaragkou et al., 2021). The changes in storage modulus (G′) and loss modulus (G″) of control and enzyme treated wafer batters as a function of frequency are shown in Fig. 4B In all batter samples, the storage modulus (G′) consistently exceeded the loss modulus (G″) in the whole range of frequencies studied, suggesting that batters had a predominant elastic behaviour of viscoelastic matrix. The elevated G′ modulus indicated that the batter possesses a well-formed structure capable of storing energy when subjected to deformation. A higher G′ over G'' suggested a stronger and more stable batter that can better retain its shape. Furthermore, increasing the enzyme concentration led to a decrease in both G′ and G″ values (Fig. 4B), which can be due to the degradation of proteins within the wheat batter. The viscoelastic properties of wheat batter are primarily determined by the gluten protein, which forms a cohesive and elastic matrix responsible for gas retention and structure. The primary activity of the enzyme cocktail was a protease activity, which played a vital role in modifying the properties of batter by disrupting gluten. As the enzyme dosage increased in the wafer batters, the structural protein matrix was progressively weakened, leading to a reduction in both G′ and G″ values.

The frequency dependence of the samples was analysed using a Power Law model, which describes the relationship between the storage modulus and frequency (Quinchia et al., 2011):

Table 2
Mean values of the control and enzyme treated (0.228, 0.403, 0.555, and 0.790 U/mL) wafer batters obtained by fitting the storage modulus (G′) versus angular frequency data to a power-law model.

Sample	G′₁ (Pa)	n
Control	187,631.00± 203,806.38[a]	0.27±0.06 [ab]
0.228 U/mL	69,958.66±69,731.05 [ab]	0.25±0.01 [b]
0.403 U/mL	24,178.66± 12,735.89[b]	0.28±0.03 [ab]
0.555 U/mL	4522.96±1564.59[b]	0.28±0.02 [ab]
0.790 U/mL	4480.30±3542.71[b]	0.37±0.02 [a]

Different superscript letters in each column indicate statistical significance at $p < 0.05$.

$$G' = G'_1 \nu^{n'} \quad (7)$$

G′₁ represents the storage modulus at a frequency of ν = 1 Hz. The Power Law index (n′) corresponds to the slope of the fitted lines and reflects the extent of frequency dependence. The results obtained from power law model are shown in Table 2. All samples showed n′<1 (0.25–0.37) indicating that they exhibited some degree of frequency dependence, and characteristic of weak gels or structured fluids. Increasing enzyme concentration in the batter resulted in a decrease in G′ and an increase in n′. The increase in n′ suggested a higher dependence of the storage modulus on frequency, reflecting a shift toward more liquid-like, viscoelastic behaviour. This increased frequency dependence can be attributed to the reduced ability of system to maintain a stable elastic response across a range of frequencies. These findings were consistent with earlier studies that used a commercial protease in the production of wheat flour biscuit doughs (Pedersen et al., 2005), and suggested that the observed decrease in G' and G'' in protease-treated doughs could result from the reduction in molecular weight of the gluten proteins (Pedersen et al., 2005).

The variation of tan δ (tan δ = G″/G′) as a function of the oscillation frequency is shown in Fig. 4C. Enzyme treatment increased tan δ compared to the control batter, and this increase became more pronounced with higher enzyme concentration. The addition of protease made the batters exhibit more liquid-like behaviour compared with control, as indicated by the increased tan δ values. This shift suggested a reduction in the elastic component of the batter, likely due to the enzymatic breakdown of proteins. Similar results were observed in protease-treated muffins, suggesting changes in their viscoelastic properties due to the degradation of gluten proteins (Ashwini et al., 2016). Another study reported a decrease in G′ and G″ values, along with an increase in tan δ, as the degree of hydrolysis of WG by a protease increased (Dai and An, 2024). This was attributed to the degradation of proteins by the protease. The increased tan δ values were also suggested to reflect enhanced interaction of the hydrolysates with water, resulting in a more liquid-like behaviour (Dai and An, 2024).

3.6. Microstructure of wafer batters

Micrographs of control and enzyme-treated wafer batters obtained through CLSM are shown in Fig. 5. Proteins are visualized in red, while the yellow signal indicates regions where proteins and starch are associated. Control batter exhibited a heterogeneous distribution of starch granules, consisting of both small and large granules interspersed with proteins, in the form of aggregates (Fig. 5A). The addition of the enzyme cocktail revealed a progressive impact of protease concentrations on the integrity of proteins within the wafer batter matrix. As shown in Fig. 5, sample A1 (no enzyme) displayed a higher number of protein aggregates (in red), indicative of intact proteins. As enzyme concentration increased from B1 to E1, there was a consistent decline in the number of protein agglomerates. This progressive reduction could be linked to the enzymatic hydrolysis of protein structures, confirming that protease addition led to degradation of proteins within the batter. Micrographs

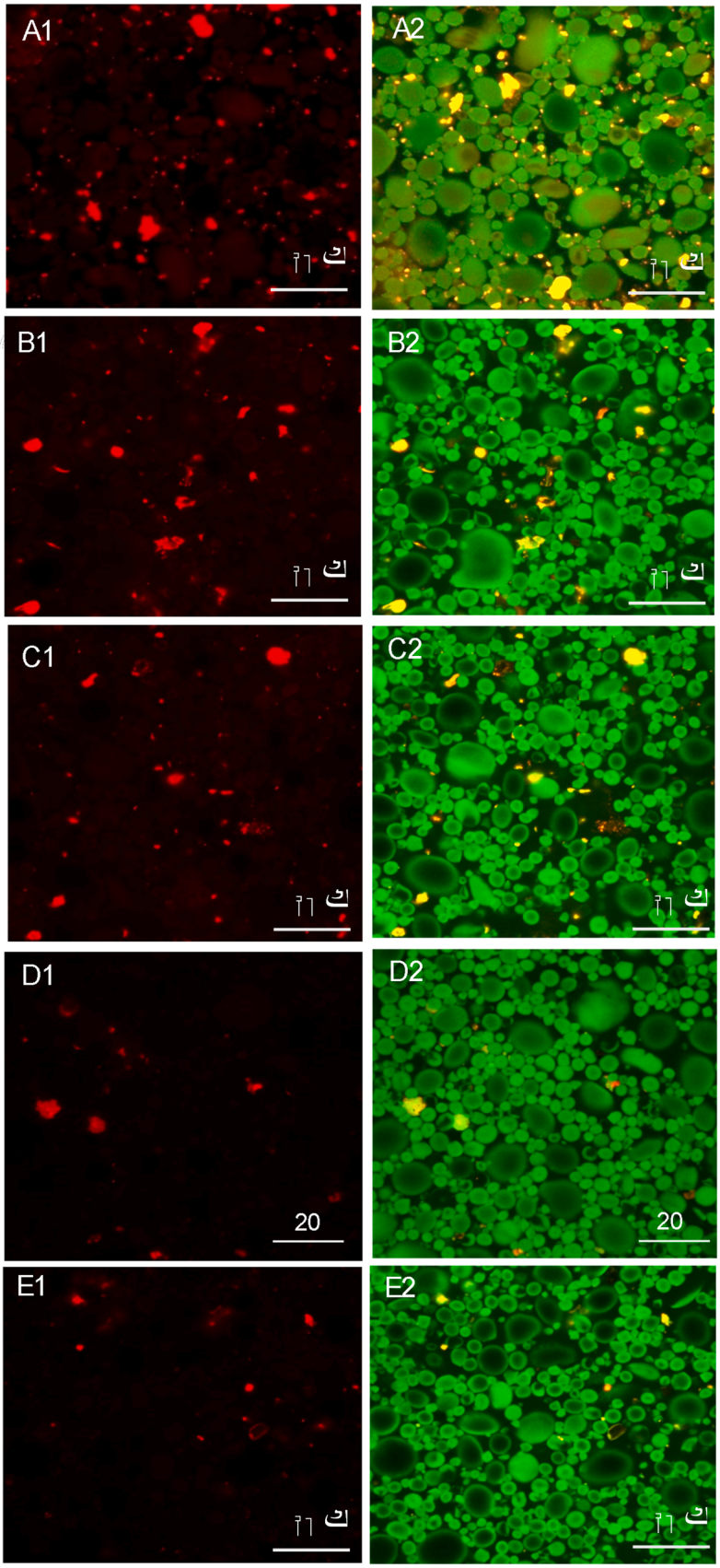

Fig. 5. CLSM images of control wafer and enzyme cocktail-treated batters, stained by (1) Rhodamine B in red showing proteins; and (2) Rhodamine B and FITC in yellow showing protein–starch associations. (A1-A2) control, (B1-B2) 0.228 U/mL protease, (C1-C2) 0.403 U/mL protease, (D1-D2) 0.555 U/mL protease and (E1-E2) 0.790 U/mL protease.

Table 3
Textural properties, colour analysis, moisture content and water activity of the wafer sheets in control and samples treated with various protease activities.

	Control	0.228 U/mL	0.403 U/mL	0.555 U/mL	0.790 U/mL
Textural properties					
Fracturability (mm)	31.05 ± 1.46 ^{a}	29.42 ±0.77 ^{b}	28.47 ±0.45 ^{c}	27.96 ±0.64 ^{c}	27.90 ±0.58 ^{c}
Hardness (N)	40.90 ±4.90 ^{a}	38.74 ±2.09 ^{a}	35.37 ±1.80 ^{ab}	29.71 ±2.50 ^{b}	17.98 ±2.92 ^{c}
Colour analysis					
L*	77.92 ±0.65^{a}	68.76 ±3.02 ^{b}	62.20 ±1.74 ^{c}	60.47 ±1.83 ^{c}	55.37 ±1.78 ^{d}
a*	1.21 ±0.11 ^{d}	5.49 ±1.41 ^{c}	6.11 ±1.24 ^{c}	8.19 ±0.95 ^{b}	9.75 ±0.45 ^{a}
b*	18.52 ±0.92 ^{c}	24.37 ±2.31 ^{b}	23.65 ±1.83 ^{b}	27.73 ±1.27 ^{a}	28.25 ±1.24 ^{a}
ΔE*	0	11.70 ±3.99 ^{b}	17.36 ±2.02 ^{a}	20.94 ±2.28 ^{a}	26.06 ±1.46 ^{a}
BI	3.47 ±0.16 ^{d}	9.34 ±2.24 ^{c}	10.82 ±1.82 ^{c}	14.26 ±1.69 ^{b}	17.59 ±0.97 ^{a}
Water activity and moisture content					
Moisture content (%)	8.72 ±1.13 ^{a}	6.94 ±1.18 ^{b}	6.19 ±1.30 ^{b}	2.98 ±0.81 ^{c}	2.13 ±0.64 ^{c}
Water activity	0.44 ±0.03 ^{a}	0.30 ±0.05 ^{b}	0.26 ±0.04 ^{bc}	0.20 ±0.05 ^{cd}	0.15 ±0.02 ^{d}

Different letters in lines indicate statistically significant differences ($p < 0.05$).

on the second column (A2 to E2) showed a yellow signal suggesting protein–starch associations. As the enzyme dosage increased these interactions decreased. These findings showed that protein degradation by the enzyme cocktail could have limited protein-starch interactions. Disruption of the structural protein matrix was observed in previous studies where proteases were used for wheat gluten hydrolysis. In a previous study aiming to improve the solubilization of WG through protease treatment, the protein aggregates were significantly broken down, resulting in smaller and more uniformly distributed particles in CLSM images of the treated samples (Li et al., 2024b).

3.7. Wafer sheet properties

The results of the textural analysis of baked wafers are summarized in Table 3. Wafers prepared without the addition of enzymes exhibited the highest ($p < 0.05$) hardness among all samples. Increased enzymatic activities (0.666 U/mL and 0.790 U/mL) resulted in a significant reduction in hardness ($p < 0.05$). The reduction in hardness observed in wafer samples treated with the enzyme cocktail can be attributed to the combined enzymatic effects. The protease weakened the protein structure which reduced the mechanical strength of the wafers, while xylanase reduced water-binding capacity, leading to higher moisture loss, and ultimately a softer wafer texture (Naderi et al., 2022). Additionally, enzyme-treated wafer samples exhibited significantly lower ($p < 0.05$) fracturability values compared to the control. Without enzymatic modification, the protein and polysaccharide matrix remains firm, leading to increased brittleness and a greater tendency to crack or break under mechanical force. This contributes to higher fracturability values (Kim et al., 2004). In contrast, enzyme-treated wafers exhibited a more flexible structure with reduced fracturability. Higher fracturability values in the control samples could pose challenges during the cutting process, potentially leading to breakage or inconsistencies in wafer sheet dimensions (Mert et al., 2015).

Colour analysis revealed notable changes due to enzymatic treatment. The L* value, representing lightness, significantly decreased ($p < 0.05$), while the a* (redness) and b* (yellowness) values significantly increased ($p < 0.05$) in the enzyme-treated samples, indicating a darker and brownish appearance compared to the lighter control samples (Table 3). Additionally, the browning index (BI) of the samples showed a significant increase ($p < 0.05$) in enzyme-treated samples. The main cause of the observed colour changes is Maillard reaction. As the dosage of the enzyme cocktail increased in the wafer formulation, a higher level of reducing sugars could be generated, due to the xylanase activity of the enzyme cocktail. These reducing sugars serve as key substrates for non-enzymatic browning pathways, including the Maillard reaction. Additionally, protease activity led to the breakdown of proteins, releasing more free amino acids and peptides. The increased availability of these Maillard precursors accelerated the reaction during baking, resulting in greater browning index and darker wafer colour (Ben Hmad et al., 2024; Kara et al., 2005).

The moisture content and water activity of the wafers significantly decreased ($p < 0.05$) after enzymatic treatment (Table 3). Both gluten proteins and arabinoxylans are known for their high water-binding capacities due to their hydrophilic side chains and ability to form extended polymeric networks. The kinetic parameters of the enzyme cocktail suggested that both protease and xylanase were effective in breaking down these macromolecules during the hydrolysis stage. This enzymatic hydrolysis likely reduced the ability of macromolecules to retain water, resulting in lower moisture content and water activity in the wafers (Liu et al., 2017). This reduction in moisture content may contribute to a lighter, more crisp texture in the final product (Nasabi et al., 2021; Manley, 2011b).

4. Conclusion

This study showed that enzymes, and particularly proteases, can be effective in the preparation of wafer batter, affecting its viscosity, a key factor in achieving the desired texture and quality of wafers. Enzymatic hydrolysis of wheat flour and gluten resulted in the reduction of high and low molecular weight glutenin subunits, and batters from hydrolysed wheat flour were generally stable and exhibited decreased viscosity and a liquid-like nature. Furthermore, baked wafer sheets had reduced hardness and slightly lower fracturability compared to non-enzyme treated sheets. These findings indicate the feasibility of enzyme technology as a tool in wafer manufacturing, enabling the expansion of flours used for wafers, such as those with varied gluten content or non-cereal flours.

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Ethical Statement- studies in humans and animals

As a corresponding author of the manuscript entitled **"Impact of enzymatic modification on the structural and rheological properties of wafer batter and wafer quality"** under consideration for publication in the prominent *Future Foods* journal, under the SI "EFFoST 2024", I declare that:

- The work described in the manuscript did not involve the use of human subjects or animals

CRediT authorship contribution statement

Seyedeh Fatemeh Sadeghian-Motahar: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Data curation. **Julia Rodriguez- Garcia:** Writing – review & editing, Supervision, Formal analysis. **Paola Tosi:** Writing – review & editing, Supervision, Methodology. **Beatrice Kuschel:** Supervision, Funding acquisition. **Michael Merz:** Funding acquisition. **James Osborne:** Funding acquisition. **Afroditi Chatzifragkou:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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