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Research Paper

Quality and staling characteristics of white bread fortified with lysozyme-hydrolyzed mealworm powder (*Tenebrio molitor* L.)

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ABSTRACT

Edible insects have a low environmental impact but are rich in nutrients and have been promoted as alternative protein sources. However, adding insect flour to bread negatively affects the overall quality, especially loaf volume and textural properties. Furthermore, relevant studies on chitin are limited. Therefore, this study examined chitin hydrolysis using lysozymes to enhance the quality characteristics in defatted mealworm (*Tenebrio molitor* L.) powder (DF-M)-supplemented bread. The chitin hydrolysis degree by lysozymes was evaluated using the 3,5-dinitrosalicylic acid assay and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. The amount of chitin oligomers increased with time, and no significant difference in the hydrolysis efficiency between water and 400 mM acetate buffer was observed. Enzymatic hydrolysis improved the DF-M water- and oil-binding and antioxidant capacities. In addition, chitin hydrolysis increased the volume and softened the texture of white bread. In particular, bread supplemented with DF-M hydrolyzed for 4 h at 10 % had the highest moisture content among the mealworm-added bread groups during storage for 5 days. Moreover, sensory evaluation showed a positive effect of chitin hydrolysis on acceptability. Our findings indicate that chitin hydrolysis can improve the quality of bread containing insect additives. In conclusion, this study provides novel insights into producing high-quality and functional bakery products from edible insects by the enzymatic hydrolysis of edible insect powders and could expand the applications of edible insects as food ingredients.

1. Introduction

The global population is growing at an unprecedented rate and is expected to reach approximately 9.7 billion by 2050 (United Nations, 2022). Consequently, the demand for food will increase, accompanied by decreased land resource availability for food production. Furthermore, fully using the available land area can jeopardize the global population (Premalatha et al., 2011). In addition, increase in livestock production has contributed to some environmental challenges (Steinfeld et al., 2006), and attention is being directed at eco-friendly alternative protein foods (Marquis et al., 2023). Insect farming can conserve the environment in various aspects, including decreasing deforestation, land and water use, and greenhouse gas emissions (Halloran et al., 2016).

Insects are rich in protein and offer numerous potential health benefits through diverse bioactive compounds with anti-oxidative, -microbial, -inflammatory, and other properties (Belluco et al., 2013; Borges, da Costa, Trombete, and Câmara, 2022). Despite such advantages, the psychological aversion of consumers renders it challenging to use insects as food resources (Megido et al., 2016). Therefore, removing their semblance by processing them into powder or protein concentrate has improved consumer acceptance considerably (Borges et al., 2022; Queiroz et al., 2023). However, using insect powder in food processing often deteriorates rheological properties of food. For example, decline in overall quality characteristics, including elevated hardness and decreased volume of breads containing insects have been reported, in addition to decreased consumer acceptance (de Oliveira et al., 2017; González et al., 2019). Protein hydrolysis has been attempted in insect powder owing to the high protein proportions, to improve rheological quality (Samantha Rossi et al., 2021; S Rossi et al., 2022); however, the influence of chitin has not been investigated.

Chitin is a polysaccharide in with β(1 → 4)-linked N-acetyl-D-glucosamine as the repeating structural unit, forming long linear chains,

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Table 1
Proximate composition and chitin content of wheat flour, defatted mealworm powder, and lysozymes.

Samples	Moisture	Fat	Ash	Protein	Crude fiber	Chitin
	(g/100 g)					(g/100 g)
Defatted mealworm flour	3.18 ± 0.02	9.34 ± 0.01	6.51 ± 0.08	68.17 ± 0.42	12.82 ± 0.53	4.75 ± 0.15
Wheat flour	12.81 ± 0.08	0.88 ± 0.01	0.51 ± 0.01	15.39 ± 0.19	70.43 ± 0.25	–
Lysozyme	5.51 ± 0.22	0.06 ± 0.04	0.29 ± 0.00	93.92 ± 0.17	0.23 ± 0.01	–

Values are presented as mean ± standard deviation (n = 3).

and it is the second most abundant natural polymer after cellulose (Rolandi and Rolandi, 2014). Three crystalline forms, α-, β-, and, rarely, γ-chitins, are generally found in nature. α-Chitin has the most stable structure and is primarily present in the cell walls of crustaceans, fungi, and the epidermis of insects (Zhang et al., 2015). α-Chitin molecules are aligned tightly in an antiparallel fashion by strong intermolecular hydrogen bonds between the amide and carbonyl groups (Kurita, 2001). Such strong hydrogen bonds between chitin chains make them insoluble in water and most organic and inorganic solvents (Khan et al., 2017). The unique structure of chitin can impede its availability in insects for food processing. For example, high molecular weight chitin (HMWC) in insect powder negatively affects bread quality, influenced by interactions with starch, gluten, or both, due to strong intermolecular hydrogen bonding (Kerch et al., 2010).

Chitinase (EC 3.2.1.14), cellulase (EC 3.2.1.4), and lysozymes (EC 3.2.1.17) are popular enzymes used for hydrolyzing chitin (Lin et al., 2009). The primary role of lysozymes involves breaking the β-glycosidic bond between the C-1 of N-acetylmuramate and the C-4 of N-acetylglucosamine of the bacterial peptidoglycan (Bokma et al., 1997), leading to strong antibacterial activity. Therefore, lysozymes are often used as antibacterial agents in the food industry (Nawaz et al., 2022). Owing to the chemical similarity between the two polysaccharide substrates, lysozymes can hydrolyze chitin less efficiently (Wohlkönig et al., 2010). Lysozymes have been used to produce low-molecular-weight chitins (LMWCs) (Lin et al., 2009), and lysozyme-hydrolyzed LMWCs exhibit beneficial physiological activities, such as antibacterial, anti-hypertensive, and immune-enhancing properties (Jeon et al., 2000; Kurita, 2001). Furthermore, the lysozyme is an industrial enzyme available commercially in large quantities at low costs and have a wide range of reaction conditions (Masselin et al., 2021). Therefore, using lysozymes as chitinolytic enzymes provides several benefits for industrial application. Although some studies have revealed that chitin can decrease bread quality (Kerch et al., 2010), its relationship with mealworm constituents have not yet been verified.

Therefore, in the present study, we attempted enzymatic hydrolysis of chitin using lysozymes to ameliorate the quality and staling characteristics of insect-fortified white bread. This is the first study to attempt chitin hydrolysis in mealworm powder and not in isolated chitin. The aim of the present study was to elucidate the effects of hydrolyzed chitin on other constituents in the mealworm powder and thus promote the industrial application of edible insects.

2. Material and methods

2.1. Proximate compositions of raw materials

All chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). The proximate compositions of the flour (Cheil Jedang, Seoul, Korea), defatted mealworm powder (DF-M; Hanmi, Gyeonggi-do, Korea), and food-grade lysozymes from hen egg whites (EC 3.2.1.17; Creative Enzymes, Shirley, NY, USA) were determined according to the AACC standard analytical methods (AACC, 2000). Moisture (Method 44-15A), ash (Method 08-01), fat (Method 30-25), and crude protein (Method 46-13) were quantified, and the carbohydrate content was calculated by subtracting the sum of the other components.

Chitin was extracted as previously described to determine the amount of chitin in DF-M (Son et al., 2021). After deproteinization and acetylation, the samples were filtered through an ash-free Whatman no. 5 filter paper (Whatman, Buckinghamshire, UK), and the remaining powder was freeze-dried. The proximate compositions of the flour, DF-M, lysozyme, and the chitin yield extracted from DF-M are shown in Table 1.

2.2. Chitinolytic activity of lysozymes

DF-M was used as the substrate to measure the chitinolytic activity of the lysozymes. DF-M (1 g) was incubated for 30 min at 35 °C with 10 mL of 0.1 % lysozyme solution (w/v). After centrifugation at 10,000 rpm for 10 min, the amount of reducing sugars in the supernatant was determined using N-acetylglucosamine as the standard (Lin et al., 2009). One unit of enzymatic activity is defined as the amount of enzyme that produces 1 μmol equivalent of N-acetylglucosamine per minute.

2.3. Determination of the enzymatic hydrolysis degree of mealworm chitin

2.3.1. 3,5-Dinitrosalicylic acid assay

The degree of chitin hydrolysis in DF-M by lysozymes was assessed by measuring the enzyme-released reducing sugar content. The reducing sugar content was analyzed using the 3,5-dinitrosalicylic acid assay (DNS) (Miller, 1959). Enzymatic hydrolysis was performed in water and 400 mM acetate buffer (pH 4.5). DF-M (10 g) was mixed with 100 mL water or acetate buffer containing 0.1 % lysozymes (140 U/g). Hydrolysis was conducted in a shaking water bath at 35 °C. The supernatant (2 mL) was collected at 0, 1, 2, 3, 4, and 6 h and boiled for 10 min at 95 °C to stop the enzymatic reaction. The supernatant was centrifuged at 10,000 rpm for 10 min, mixed with the same volume of DNS reagent, and boiled for 15 min to measure the reducing sugar content in the enzymatically decomposed defatted mealworm (ED-M) powder. The solution was cooled on ice immediately and centrifuged at 10,000 rpm at 5 °C. The absorbance was measured at 570 nm using a SpectraMax M2e spectrophotometer (Molecular Devices, San Jose, CA, USA). The analyses were performed in six repetitions.

2.3.2. Determination of molecular weight distribution of water-soluble chitin oligomers using matrix assisted laser desorption ionization-time-of-flight mass spectrometry

To extract the water-soluble fraction from the ED-M, 1 g of lyophilized ED-M was mixed with 10 mL of distilled water containing 0.5 % acetic acid and extracted overnight in a shaking incubator at 20 °C. After centrifugation at 3,000×*g* for 20 min, the supernatant was filtered using a 0.45 μm syringe filter, and the filtrate was ultrafiltered through a 10 kDa cut-off membrane (Amicon Ultra-4, Merck Millipore, Burlington, MA, USA). Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry analysis was performed as previously described (Rojas-Osnaya et al., 2020). The lyophilized filtrate was dissolved in 0.1 % trifluoroacetic acid (10 mg/mL), and the solution was mixed with a matrix solution (sinapinic acid, 25 g/L in acetonitrile; 1:1, v/v). Mass spectra were obtained using a MALDI-TOF/TOF (Autoflex Speed LRF, Bruker Biosciences Co., Billerica, MA, USA) equipped with a laser emitting at 355 nm and operating in linear mode.

2.3.3. Preparation of enzymatically hydrolyzed DF-M

To prepare ED-M for other experiments and for making bread, 400 g

of DF-M was mixed with 4 L of 0.1 % lysozyme solution (140 U/g) dissolved in water (w/v) and incubated in water at 35 °C while stirring. Each solution was immediately boiled for 10 min after hydrolysis to stop the enzymatic reaction and then freeze-dried. The samples were stored at −20 °C. The ED-M prepared for a specified condition was designated ED#-M (where # represents the enzymatic decomposition time). The enzymatic hydrolysis time for ED-M was set to 0, 1, and 4 h based on the DNS, MALDI-TOF, and microbial safety tests (data not shown).

2.4. Determination of physical ED-M properties

2.4.1. Microstructure of ED-M

Surface images of mealworm chitin extracted from DF-M, shrimp α-chitin (Sigma-Aldrich, St. Louis, MO, USA), and ED-Ms were analyzed using field emission scanning electron microscopy (FE-SEM; ZEISS GeminiSEM 560, ZEISS, Oberkochen, Germany). Chitin and ED-M samples were fixed on a stub and coated with platinum (BAL-TEC/SCD 005 sputter coater; BAL-TEC AG, Pfäffikon Zürich, Switzerland). Sample images were captured at three magnifications (× 1,000, × 5,000, and × 10,000).

2.4.2. Intrinsic viscosity

The intrinsic viscosities of the ED-M samples were measured using a Cannon-Fenske glass capillary viscometer (Cannon Instrument Co., State College, PA, USA; size 150) at 25 °C. ED-M (1 g) was dissolved in 10 mL N,N-dimethylacetamide containing 5 % LiCl (w/v) for 4 h at 20 °C. The solution was filtered through a 0.45 μm syringe filter, and the viscosity measurement was conducted as previously described (Hou et al., 2020), with some modifications. The intrinsic viscosity was calculated using Eqs. (1) and (2):

$$\eta_r = \frac{\eta}{\eta_s} \tag{1}$$

$$\eta_r = 1 + [\eta]C \tag{2}$$

in Eq. (1), η_r, η, and η_s represent the relative viscosity, viscosity of the sample solution, and the solvent. In Eq. (2), [η] represents the intrinsic viscosity of the sample solution, and C represents the sample concentration.

2.4.3. Water and oil binding capacity

The water binding capacity (WBC) and oil binding capacity (OBC) of ED-M were determined as previously described (Wu et al., 2009). One gram of the sample was mixed with 10 mL of distilled water or rapeseed oil in a 15 mL centrifuge tube. The solution was vortexed (Fisher Gene II vortex, Waltham, MA, USA) for 2 min. The mixtures were allowed to stand at room temperature for 30 min and centrifuged using a LaboGene 1248R (LaboGene, Lillerød, Denmark) at 3,000×*g* for 20 min. After decanting the supernatant, the centrifuge tube was inverted on absorbent paper and allowed to stand for 60 min to remove any remaining liquid. The remaining sediment was weighed, and the amount of water or oil adsorbed onto the powders was calculated.

2.4.4. Foaming properties

Foaming capacity (FC) and foaming stability (FS) were measured as previously described (Guo et al., 2015). To develop the foam, ED-M was dispersed in distilled water (1 %, w/v), and 100 mL of the suspension was homogenized at 20,000 rpm for 2 min using a disperser homogenizer (HG-15A, DAIHAN Science Co., Gangwon-do, Korea). The foam volume was measured immediately after foam formation (V_0) and 30 min later (V_{30}). FC and FS were calculated using Eqs. (3) and (4).

$$\text{Foaming capacity } (\%) = \frac{V_0 - 100}{100} \times 100\% \tag{3}$$

$$\text{Foaming stability } (\%) = \frac{V_{30} - 100}{V_0 - 100} \times 100\% \tag{4}$$

2.4.5. Emulsifying activity and stability

To evaluate emulsifying activity (EA), 0.15 g of ED-M was dispersed in 15 mL of distilled water. Rapeseed oil (5 mL) was then added and homogenized for 2 min at 13,500 rpm using a homogenizer (HG-15A; DAIHAN Science Co.). The emulsified solution was immediately diluted with 0.1 % sodium dodecyl-sulfate (1:250, v/v), and the absorbance (A_0) was measured at 500 nm using a SpectraMax M2e spectrophotometer (Molecular Devices). The emulsified solution was stored at room temperature for 30 min, and the absorbance was determined similarly (A_{30}). The EA and emulsifying stability (ES) were calculated based on Eqs. (5) and (6) (Guo et al., 2015).

$$\text{EA}\left(\frac{m^2}{g}\right) = \frac{4.606 \times A_0 \times N}{C \times \varphi \times 10^4} \tag{5}$$

$$\text{ES}(\%) = \frac{A_{30}}{A_0} \times 100\% \tag{6}$$

in Eq. (5), N is the dilution factor (N = 250), C is the concentration (g/mL), and φ is the volume fraction of the oil (φ = 0.25).

2.5. Determination of ED-M antioxidant activity

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) radical scavenging activities and ferric reducing antioxidant power (FRAP) were examined to determine the antioxidant activity of ED-M (Benzie and Strain, 1996; Brand-Williams et al., 1995; Re et al., 1999). The ED-M extract was prepared as described previously (Bolat et al., 2021). Briefly, ED-Ms were placed in ethanol:acetic acid:water (50:8:42) solution and extracted in a shaking incubator at room temperature for 1 h. After centrifugation at 3,000×*g* for 10 min, the supernatant was filtered with a 0.45 μm syringe filter and the antioxidant activity was determined.

To analyze FRAP, 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl, and 20 mM FeCl3•6H2O mixed solution was prepared (10:1:1, v/v). Sample extracts (160 μL) were added to 1200 μL of the FRAP reagent and reacted for 30 min at 20 °C. The absorbance was measured at 593 nm using a UV/Vis spectrophotometer (SpectraMax M2e, Molecular Devices).

To evaluate ABTS radical scavenging activity, 1 mM 2,2′-Azobis(2-amidinopropane) dihydrochloride in 100 mM phosphate-buffered saline (PBS, pH 7.4) and 2.5 mM ABTS in 100 mM PBS buffer (pH 7.4) were prepared and mixed in the same volume. The solution was heated for an hour in a water bath at 70 °C and cooled to 37 °C to generate the ABTS + radical. Then, 980 μL of prepared solution was mixed with 20 μL of sample extracts. After 10 min, absorbance was measured at 730 nm using a UV/Vis spectrophotometer (SpectraMax M2e, Molecular Devices). To determine DPPH radical scavenging activity, 800 μL of 0.2 mM (DPPH in 80 % methanol solution was mixed with 200 μL of sample extracts. The solution was placed in a dark room for 30 min, and the absorbance was measured using a microplate reader (SpectraMax M2e, Molecular Devices) at 517 nm. Trolox was used as a standard, and the antioxidant activity of the samples is presented as mg Trolox equivalent antioxidant capacity per gram of sample weight (mg TEAC/g).

2.6. Mixolab2 behavior

The dough mixing and pasting behavior was determined using Mixolab2 (Chopin Technologies, Vileneuve-la-Garenne, France). For the experiment, white flour and flour replaced partially with DF-M hydrolysate (10 % and 20 %) without other ingredients were prepared. The Chopin + protocol was used to analyze rheological properties. The total

dough weight was set to 75 g, and optimal water absorption was determined when the torque of the dough reached 1.11 Nm.

2.7. Preparation and characterization of bread

The proportions of ingredients for bread production were set to 100 % white flour, 2 % sugar, 2 % canola oil, 1.7 % table salt, 1.5 % yeast (Saf, Mumbai, India), and 1.1 % bread improver (Sullivan et al., 2017). The amount of water in each sample group was determined according to the optimal water absorption percentage analyzed using Mixolab2. Wheat flour was replaced in 10 % or 20 % quantities with ED0-M, ED1-M, and ED4-M to prepare ED-M-supplemented bread. The sample group names of the bread were designated as E#-##MB (where # represents the enzymatically hydrolyzed time and ## represents the replacement percentage of flour with ED-M). The dough was prepared using a spiral mixer NVM-SN12 (Daeyung, Seoul, Korea). After the initial proofing (1 h at 27 °C and 75 % relative humidity), the dough was divided into 200 g, shaped into a circle, and placed in a mold (9.5 cm cube). After the secondary proofing (45 min at 35 °C and 85 % relative humidity), the bread was baked in a 180 °C oven for 40 min. After cooling for 2 h at 25 °C, the samples were immediately packed in low-density polyethylene bags and stored at 25 °C for 0, 1, 3, and 5 days, and used in experiments.

2.8. Characterization of white bread supplemented with ED-M

2.8.1. Evaluation of physical properties of breads

The height of each bread was measured 15 times at its highest point. Specific volume was calculated as the ratio of bread weight (g) to bread volume (mL) using the rapeseed displacement method (Method 10-05.01) (AACC, 2010) (n = 4). The total aerobic plate count and the prevalence of *Escherichia coli* and mold were measured to determine the microbiological safety of the bread. The center of the bread was sliced (1 cm), mashed with a sterile saline solution (1:10, w/v), and tenfold serially diluted. Microbial counts were performed according to the official AACC method (AACC, 2010) using 3M Patrifilms (3M, St. Paul, MN, USA). The limit of detection (LOD) was <10 CFU/mL.

2.8.2. Crumb structure image analysis

A flatbed scanner (M283fdw, HP, Palo Alto, CA, USA) captured 1200 dpi images of bread slices (n = 10). Partial images of bread slices (40 × 40 mm) were converted to 8-bit grayscale. Image analysis was performed as previously described (Scheuer et al., 2015) using the ImageJ-based Fiji 1.46 software package. An OTUS thresholding algorithm was used for image segmentation. The mean cell area (mm^2), density (cells/cm^2), and size distribution (very small size (0.2 $mm^2 \leq$ cell area), as well as small (0.2 mm^2 < cell area $\leq$3.0 mm^2), medium (3.0 mm^2 < cell area $\leq$10.0 mm^2), and large sizes (cell area >10.0 mm^2) of bread samples were determined as previously described (Martins et al., 2018).

2.8.3. Bread color analysis

The crust and crumb colors of bread were determined using the CIE Lab (L*, a*, and b*) system. The light source and measurement angle were set at D65 and 10°, respectively. The color was measured using a Color Difference Meter (KONICA MINOLTA, CM-36dG, Japan). Twelve measurements were taken of the crust and crumbs. The total color difference (ΔE) and browning index (BI) of the bread were determined according to Eqs. (7)–(9) (Kowalski et al., 2022).

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \tag{7}$$

$$BI = \frac{[100(X - 0.31)]}{0.71} \tag{8}$$

in Eq. (8), where X is

$$X = \frac{(a^* + 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)} \tag{9}$$

2.9. Quality parameters of bread shelf-life

2.9.1. Bread texture and moisture analysis

Texture profile analysis (TPA) was performed using a texture analyzer (Stable Micro Systems Ltd., TA.XTplus, Godalming, UK) at the BT Research Facility Center, Chung-Ang University. A 36-mm P/36R probe was used to determine the hardness, springiness, cohesiveness, chewiness, gumminess, and resilience properties of the bread (Kowalski et al., 2022). The test speed was 1 mm/s, and the compression rate was 60 % of the sample height. The interval between the first and second compressions was 5 s, and the trigger force was 5 g. Bread crumbs stored for 0, 1, 3, and 5 days were cut into cubes (2 × 2 × 2 cm) for texture analysis.

The moisture content of the crust and crumb of bread stored for 0, 1, 3, and 5 days was determined according to the AACC method 62-05.01 (AACC, 2010).

2.9.2. Starch gelatinization and retrogradation

The starch retrogradation characteristics during bread storage were measured using differential scanning calorimetry (DSC). ED-M-supplemented breads were stored for 0, 1, 3, and 5 days at 25 °C and analyzed using DSC 204 F1 Phoenix (NETZSCH, Selb, Germany). Two loaves were prepared, and crumbs were collected from the center of each bread. The crumbs were then freeze-dried and ground. The lyophilized crumb sample was mixed with distilled water (3:7, w/v), and the slurry was sealed in a DSC aluminum crucible (Kotsiou et al., 2022). The slurries were maintained at room temperature for 2 h before DSC analysis to hydrate the sample particles. Then, the sample crucible was heated from 5 °C to 120 °C (heating rate: 5 °C/min). The onset temperature (T_o), the peak temperature (T_p), the conclusion temperature (T_c), and the melting enthalpy ($\Delta H_{retrogradation}$) of the retrograded starch were obtained from the respective thermograms (endothermic transition). Wheat flour and DF-M replacement flour were measured using the same method to obtain the melting enthalpy ($\Delta H_{gelatinization}$) of starch gelatinization (endothermic transition). The retrogradation index (RI) of starch was calculated using Eq. (10):

$$RI\ (\%) = \left(\Delta H_{retrogradation} \times 100\right) / \Delta H_{gelatinization} \tag{10}$$

2.10. Sensory analysis

The sensory characteristics of the bread were analyzed using quantitative descriptive analysis (QDA). The 12 trained panelists developed 10 lexicons (porosity, mealworm odor, mealworm flavor, bitter taste, salty taste, firmness, chewiness, moistness, graininess, and residual taste) for bread samples and evaluated each characteristic on a 15-cm line scale. In addition, the overall acceptability of the bread samples was scored using a nine-point scale (1 = extreme dislike, 9 = extreme like) by 30 untrained panelists. All the panelists were recruited from Chung-Ang University. Sensory evaluation complied with an Institutional Review Board (IRB) document approved by the Chung-Ang University IRB Committee (1041078-20230130-HR-021).

2.11. Statistical analyses

Student's t-test and one-way analysis of variance (ANOVA) were performed using IBM SPSS Statistics software version 28 (IBM, Armonk, NY, USA) to compare the mean values of all the analyzed parameters. Duncan's test was used to describe statistical differences within groups at a significance level of $p < 0.05$.

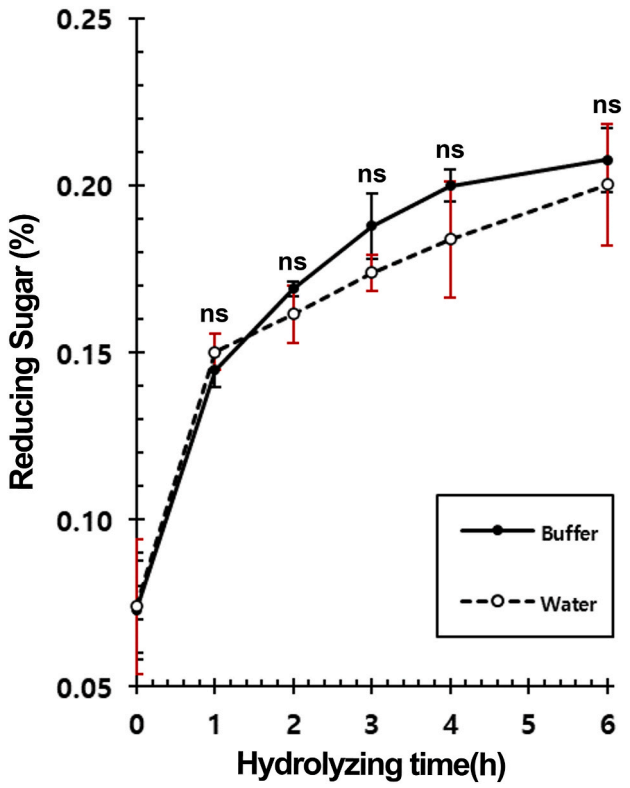

Fig. 1. Changes in reducing sugar content during defatted mealworm hydrolysis using lysozymes for 6 h. Enzymatic hydrolysis was performed in a 400 mM acetate buffer (pH 4.5) and distilled water. Student's t-tests were conducted for statistical analysis. ns, not significant ($p > 0.05$).

3. Results and discussion

3.1. The enzymatic hydrolysis rate of mealworm chitin

3.1.1. Reducing sugar contents during hydrolysis in water and acetate buffer

Fig. 1 shows the changes in reducing sugars during hydrolysis by lysozymes with 10 % DF-M as the substrate in water and acetate buffer. A progressive increase in the amount of reducing sugars was observed, with a 202.97 % increase in water and 198.50 % increase in the buffer at 1 h, and further increases were observed at 4 h (248.51 % in water and 274.37 % in acetate buffer compared to 0 h). Notably, no statistically significant difference ($p > 0.05$) was observed between the quantity of reducing sugars released in water and the acetate buffer during hydrolysis by lysozymes for up to 6 h. Therefore, this study selected water as the solvent, considering its universal availability in the food industry.

3.1.2. Analysis of chitin oligomers produced by lysozyme hydrolysis

The DF-M lysozyme hydrolysate was prepared using a 10 kDa cut-off filter and lyophilized prior to MALDI-TOF analysis. The size distribution of molecules <2,000 Da in the sample was measured by MALDI-TOF. Fig. 2 illustrates the MALDI-TOF spectrum of the chitin oligomers (COs) after hydrolysis. A relative increase in COs was observed in the MALDI-TOF spectrum from degree of polymerization (DP) 2 to DP 4 by lysozyme hydrolysis; however, DP 5 CO showed a faint peak. Lysozyme is an endotype chitinase used for chitin hydrolysis (Tegl et al., 2016). Thus, the increase in CO indicated that lysozyme degraded HMWC into smaller units, consistent with the results obtained by DNS analysis. Hydrolysis led to a noticeable increase in CO, corresponding to DP 2–4 instead of DP 5. Lysozyme predominantly degrades hexametric substrates containing three, four, or more acetylation units (Nordtveit et al., 1994). Consequently, DP 6 CO was rapidly degraded compared with the other COs, owing to its higher lysozyme binding specificity. This phenomenon may explain the higher proportion of DP 2–4 COs in our study (Tegl et al., 2016). In summary, lysozymes function well in water and are suitable for commercial use with safe and economic merits.

3.2. Physicochemical properties of ED-Ms

3.2.1. FE-SEM images of mealworm chitin and ED-Ms

FE-SEM analysis was performed to compare the morphology of commercial shrimp and mealworm chitin isolated from the DF-M used in this study. Nanofibers and pore structures were observed on the surfaces of the shrimp and mealworm chitin (Fig. 3). The nanopores on the chitin surface are pore tubes that transport ions and nutrients from the subcutaneous layer to the inner epidermis and interconnect structures. Their arrangement and shape are irregularly determined by the orientation of the chitin fibers that form the lamellae of the endocuticle (Erdogan and Kaya, 2016). Chitin can have various morphologies, from fibrous or non-fibrous surfaces to smooth surfaces without nanopores, depending on the origin (da Silva et al., 2021). The shrimp chitin surface was relatively smooth with randomly distributed nanopores (Fig. 3A). In contrast, chitin extracted from DF-Ms showed more porous structures arranged tightly along the fiber structures (Fig. 3B). The morphology of DF-M-derived chitin was similar to that reported in previous studies (Erdogan and Kaya, 2016; Kaya et al., 2014; Son et al., 2021).

The FE-SEM images of the ED-Ms are shown in Fig. 4. A hexagonal array structure was observed on the surface of the DF-M. This structure is comparable to the shape of the insect surface cuticle (Ureña et al., 2016). The hexagonal array structure of the DF-M cuticle changed over time. Compared with ED0-M, the chitin surfaces observed in ED1-M and ED4-M had more bumpy and irregular morphologies, which was likely due to the modification of the bond structure of the surface chitin by hydrolysis.

3.2.2. Physical properties of ED-M powder

The intrinsic viscosities of ED-M at different hydrolysis times ranged from 199.12 ± 0.43 to 203.73 ± 0.09 mL/g (Table 2). WBC and OBC are crucial properties of food ingredients in food processing and associated applications. Particularly in bread, WBC and OBC can directly affect product texture and flavor (Wang et al., 2021). The increase in WBC can be explained by the weakening of hydrogen bonds within the chitin structures as hydrophobic HMWC hydrolysis occurs. In addition, the increased accessibility of functional groups such as –OH and -NH_3 by hydrolysis is also expected to increase hydrogen bonding with water molecules (Sampath et al., 2022). With respect to OBC, the longer polymer chains of HMWCs show lower fat-binding capacity owing to stronger intramolecular interactions and the formation of particle aggregates, whereas the medium-length polymer chains are more flexible and can retain higher proportions of fat molecules inside the particles (F. B. Silva et al., 2021). The results indicate that the improved OBC is based on conversion of long polymer chains in the HWMC to medium-length polymers via hydrolysis.

Emulsions are another crucial physicochemical property of ingredients used in the food industry. Colloidal particles such as chitin also increase EA in foods (Tzoumaki et al., 2011). The present study demonstrated a slight increase in EA over hydrolysis time, reaching 137.5 % and 147.2 % in ED1-M and ED4-M, respectively, compared with that in ED0-M. Reduced chitin particle size can decrease particle surface tension between the oil and water interphases. Moreover, chitin decomposition increases the exposure of retained functional groups, such as –OH and -NH_3, making chitin more amphiphilic, which could have contributed to the increase in EA in ED4-M. Similarly, Sampath et al. (2022) reported increased EA with hydrolyzed chitin and increased acid hydrolysis rates.

The FC of ED-M ranged from 0.73 ± 0.06 % to 0.77 ± 0.06 %, but the difference was insignificant ($p > 0.05$). The FC and FS of the present study were lower than those reported in previous studies (Zielińska

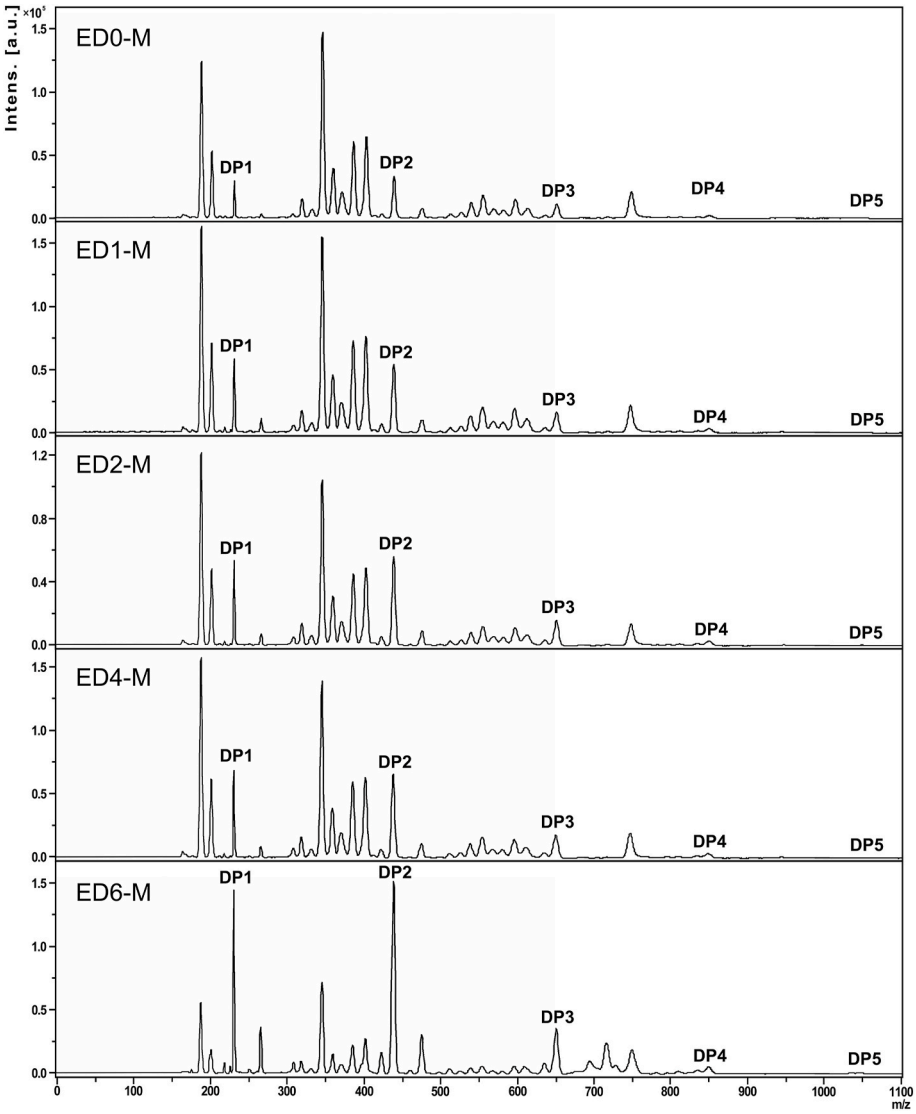

Fig. 2. Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) spectrum of the lysozyme-hydrolyzed defatted mealworm powder.

et al., 2018). A decrease in FC was reported due to heat treatment denaturation of mealworm protein extracts (Lee et al., 2019). Heat treatment used to terminate lysozyme activity could have denatured the proteins and contributed to the decrease in the FC of ED-M.

3.2.3. Antioxidant properties of ED-M

Chitin can scavenge free radicals owing to its hydroxyl and free amino groups (NH_2). These functional groups can stabilize macromolecular chitin radicals after radical scavenging, and the NH_2 groups form ammonium groups (NH_3^+) after hydrogen ion absorption (NGO et al., 2010). DPPH, ABTS, and FRAP assays were performed to evaluate the antioxidant capacity of ED-M at different hydrolysis times. The antioxidant activities against DPPH radicals were 3.40 ± 0.32, 3.53 ± 0.07, and 3.73 ± 0.32 mg TEAC/g sample for each hydrolysis time (0, 1, and 4 h, respectively) (Fig. 5A). The ABTS radical scavenging activities of ED0-M, ED1-M, and ED4-M were 4.69 ± 0.20, 5.00 ± 0.16, and 5.01 ± 0.14 mg TEAC/g sample, respectively (Fig. 5B), and ED4-M showed the highest antioxidant activity ($p < 0.05$). An increase in the antioxidant activity of ED-M with increased hydrolysis time was also observed in the FRAP (Fig. 5C). Higher-molecular-weight chitosan has stronger intramolecular hydrogen bonds and a more compact molecular structure than lower-molecular-weight chitosan, resulting in a weaker ability of the hydroxyl and amine groups to react with free radicals (Huang et al., 2012). Our results suggest that the antioxidant activity of DF-M was improved by HMWC hydrolysis in DF-M into medium molecular weight chitin and LMWC.

3.3. Fundamental dough rheological properties

Mixolab2 data for dough prepared by replacing wheat flour with ED-M powder (10 % or 20 %) at different hydrolysis times (0, 1, and 4 h) are presented in Table 3 and Fig. 6. The water absorption rate was determined using Mixolab2 by gauging the water amount that can reach the torque of 1.1 ± 0.05 Nm in dough kneading. The water absorption rates were 59.1 %, 58.4 %, and 59.6 % for ED-M replacement levels of 0 %, 10 %, and 20 %, respectively, and the hydrolysis time did not affect the water absorption rate at any replacement level. Dough development time, stability, and C1 Mixolab2 parameters can evaluate dough development during mixing, gluten network, and stability at constant mechanical shear (Schmiele et al., 2017). Replacing dough with ED-M weakened the gluten network and decreased dough stability. These results are consistent with those of previous studies that reported reduced dough stability when wheat flour was substituted with insect powder (Pérez-Rodríguez et al., 2023; Plustea et al., 2022). C2 and C2–C1 (protein network strength during heating) also decreased with an increase in ED-M replacement rate, consistent with the results of the

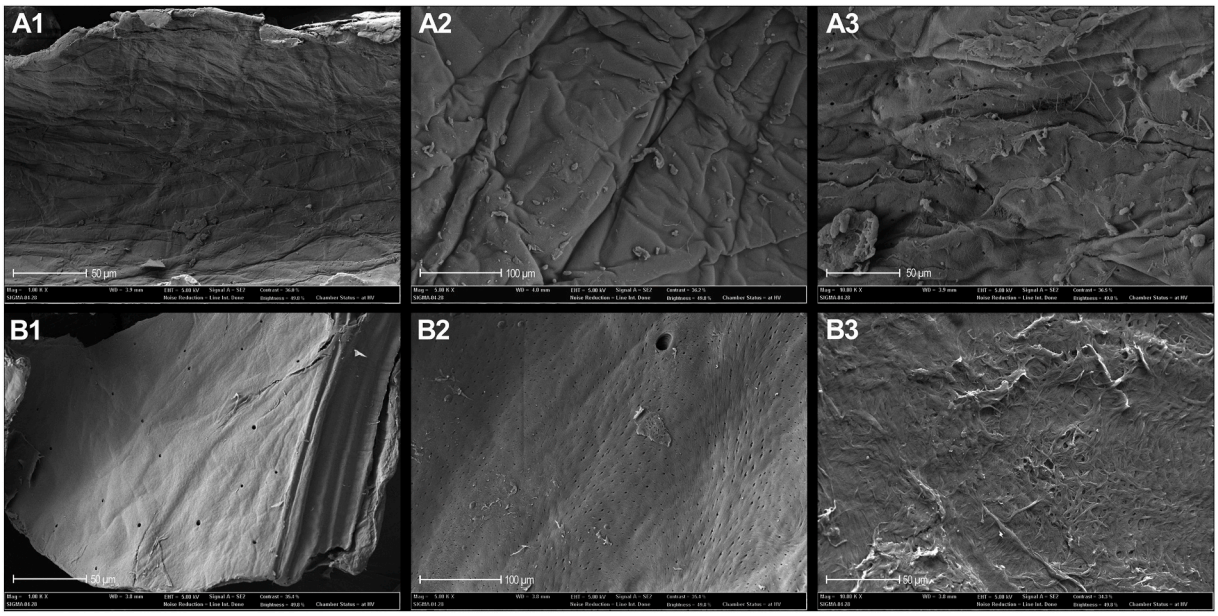

Fig. 3. Field emission scanning electron microscopy (FE-SEM) images of mealworm and shrimp chitin. Images of (A) mealworm chitin and (B) shrimp chitin were obtained at × 1,000 (A1, B1), × 5,000 (A2, B2), and × 10,000 (A3, B3) magnifications.

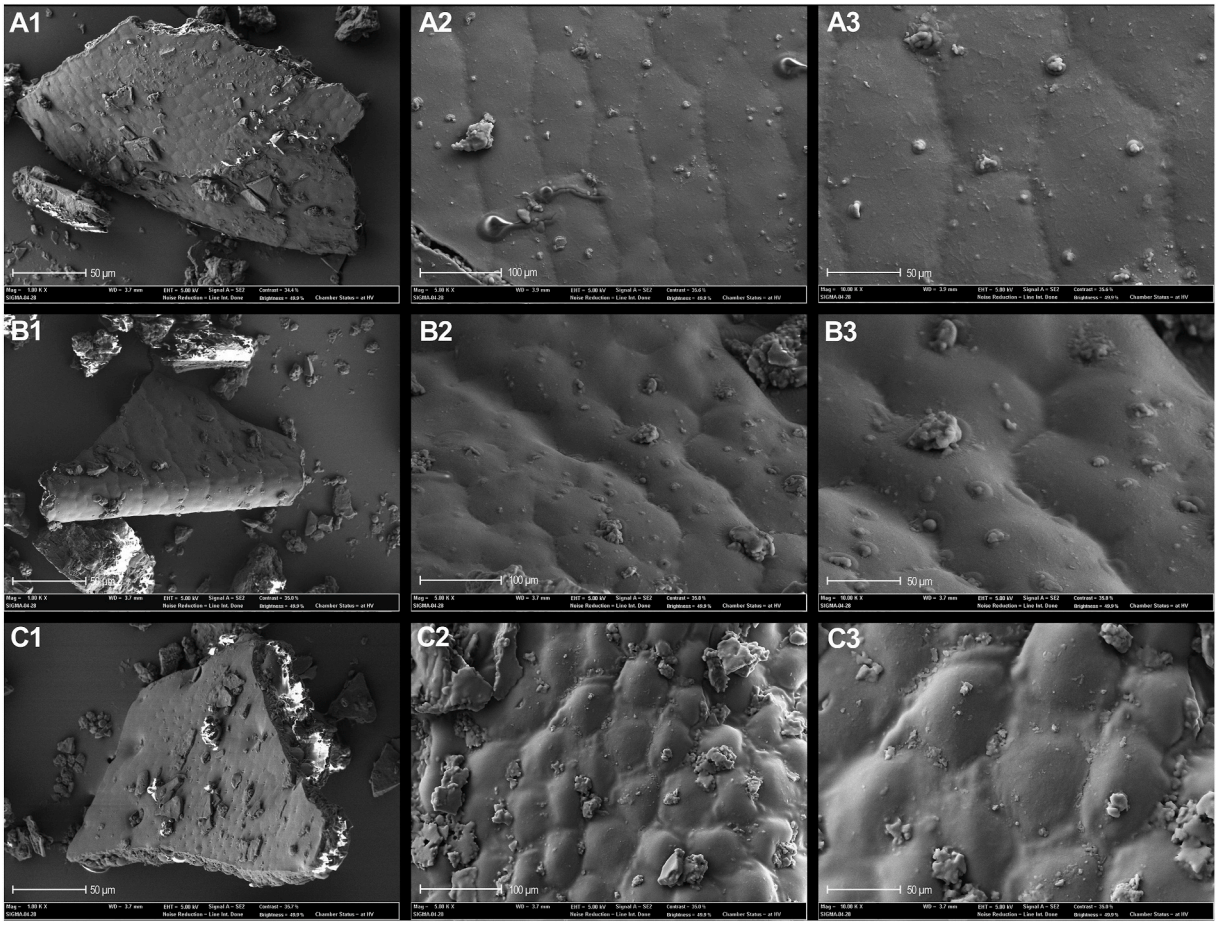

Fig. 4. Field emission scanning electron microscopy (FE-SEM) images of lysozyme-hydrolyzed defatted mealworm powder. Images of (A) ED-0M, (B) ED-1M, and (C) ED-4M were obtained at × 1,000 (A-C1), × 5,000 (A-C2), and × 10,000 (A-C3) magnifications. ED0-M, enzymatically decomposed defatted mealworms at 0 h; ED1-M, enzymatically decomposed defatted mealworms at 1 h; and ED4-M, enzymatically decomposed defatted mealworms at 4 h.

Table 2
Physical properties of defatted mealworm hydrolysates.

Samples	Intrinsic viscosity (mL/g)	Water binding capacity (g/g)	Oil binding capacity (g/g)	Emulsion activity (m^2/g)	Emulsion stability (%)	Foaming capacity (%)	Foaming stability (%)
ED0-M	200.73 ± 0.48^{b}	1.68 ± 0.01^{a}	1.55 ± 0.02^{a}	0.72 ± 0.07^{a}	43.05 ± 2.08^{b}	0.73 ± 0.06^{a}	77.98 ± 13.40^{a}
ED1-M	199.12 ± 0.43^{a}	1.69 ± 0.02^{a}	1.57 ± 0.01^{ab}	0.99 ± 0.00^{b}	29.45 ± 6.54^{a}	0.77 ± 0.06^{a}	60.71 ± 12.88^{a}
ED4-M	203.73 ± 0.09^{c}	1.72 ± 0.01^{b}	1.60 ± 0.03^{b}	1.06 ± 0.20^{b}	39.90 ± 1.64^{b}	0.77 ± 0.06^{a}	65.48 ± 5.15^{a}

ED0-M, enzymatically decomposed defatted mealworm at 0 h; ED1-M, enzymatically decomposed defatted mealworm at 1 h; ED4-M, enzymatically decomposed defatted mealworm at 4 h.
Values are presented as mean ± standard deviation (n = 3). Different superscript letters within columns (a-c) represent significant differences ($p < 0.05$).

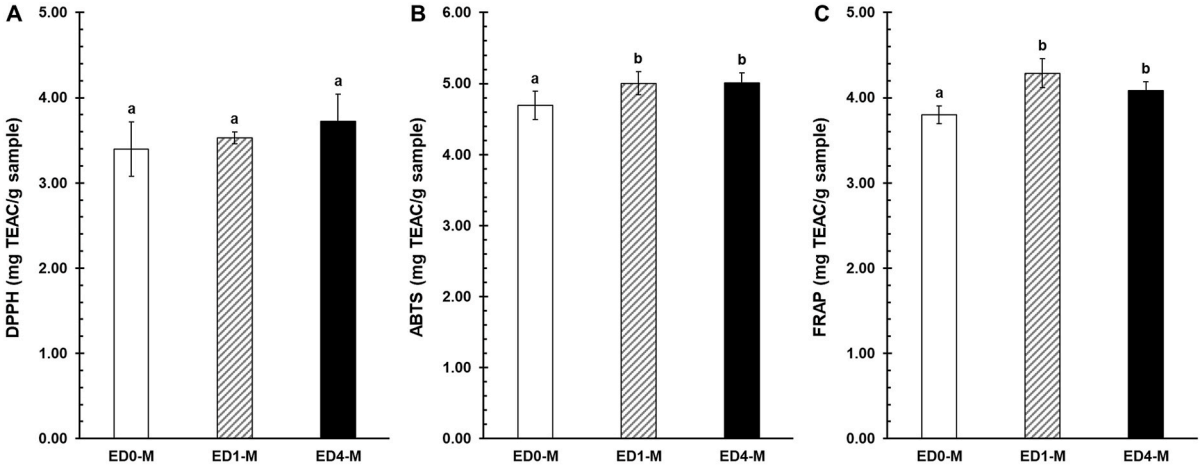

Fig. 5. Antioxidative activity of defatted mealworm hydrolysate extracts. (A) 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazin-1-yl (DPPH) radical-scavenging activity. (B) 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging activity. (C) Ferric-reducing antioxidant power (FRAP). Differences in antioxidative activities among the samples were analyzed using one-way analysis of variance (ANOVA), and Duncan's multiple range test was used for post-hoc analysis. Different superscripts represent statistically significant differences at $p < 0.05$. ED0-M, enzymatically decomposed defatted mealworm at 0 h; ED1-M, enzymatically decomposed defatted mealworm at 1 h; ED4-M, enzymatically decomposed defatted mealworm at 4 h. TEAC, Trolox equivalent antioxidant capacity.

Table 3
Mixolab2 parameters of wheat flour and defatted mealworm hydrolysate replacement flours.

Samples	WA (%)	DDT (min)	Stability (min)	Torque (Nm)				
				C1	C2	C3	C4	C5
Control	59.10	8.06^{ab}	9.63^{c}	1.11^{a}	0.53^{b}	1.49^{c}	1.35^{c}	2.41^{b}
E0-10MB	58.40	7.16^{a}	7.53^{b}	1.11^{a}	0.53^{b}	1.36^{b}	1.26^{b}	2.21^{ab}
E1-10MB	58.40	6.88^{a}	7.48^{b}	1.08^{a}	0.50^{b}	1.33^{b}	1.22^{b}	2.2^{ab}
E4-10MB	58.40	6.98^{a}	7.63^{b}	1.09^{a}	0.51^{b}	1.34^{b}	1.22^{b}	2.2^{ab}
E0-20MB	59.60	9.21^{b}	5.98^{a}	1.12^{a}	0.46^{a}	1.21^{a}	1.05^{a}	2.04^{a}
E1-20MB	59.60	8.55^{ab}	6.38^{a}	1.10^{a}	0.46^{a}	1.22^{a}	1.09^{a}	2.03^{a}
E4-20MB	59.60	9.30^{b}	6.00^{a}	1.11^{a}	0.46^{a}	1.22^{a}	1.07^{a}	2.05^{a}

E#, # indicate time (h) for enzymatic hydrolysis; **MB, ** represents the proportion of wheat flour replaced with defatted mealworm hydrolysates.
WA, water absorption; DDT, dough development time; C1, maximum torque during mixing; C2, protein weakening as a function of mechanical work and temperature; C3, Starch gelatinization, maximum torque during the heating phase; C4, hot gel stability, minimum torque during the heating phase; C5, starch retrogradation in the cooling phase.
Values are presented as mean (n = 3). Different superscript letters within columns (a-e) represent significant differences ($p < 0.05$).

parameters for the dough kneading phase. Negative effects of DF-M on dough quality were also observed for other Mixolab2 parameters, including C3 and C3–C2, related to the starch gelatinization rate, and C4, related to hot gel stability. Chitin hydrolysis did not cause significant changes in the rheological properties of the dough within the same replacement level groups. This result is likely due to the greater effect of proteins, with a higher proportion of DF-M (68.17 %) than chitin (4.75 %). Therefore, the proportions of starch and gluten proteins in dough strongly affect dough characteristics.

3.4. Characteristics of ED-M-supplemented white bread

3.4.1. Physical properties of white bread partially supplemented with ED-M

Chitin is a dietary fiber, and adding dietary fiber to bread weakens the gluten network and can disrupt the starch-gluten matrix (Kerch et al., 2008). Chitosan adsorbs bread gluten and reduces the interaction between gluten and water and lipids, impeding gluten structure development (Rakcejeva et al., 2011).

The appearance and pore images of the bread supplemented with ED-M are shown in Fig. 7, and the physical properties of the bread, including height, specific volume, and microbiological evaluation, are shown in Table 4. The height and specific volume of the bread decreased after adding mealworm powder (Fig. 7). Significant specific volume and

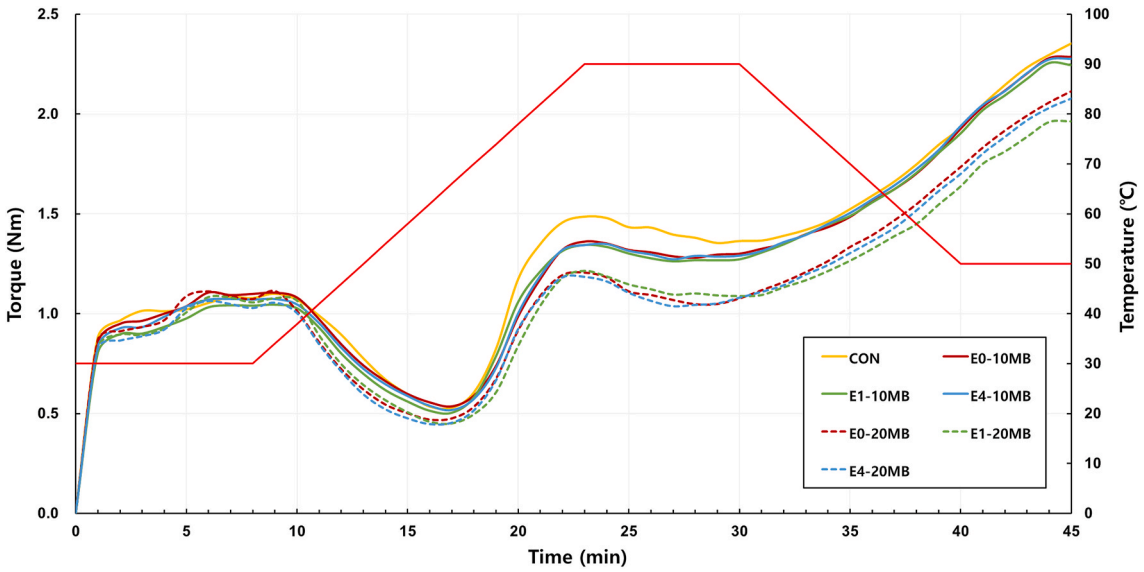

Fig. 6. Mixolab2 curves of defatted mealworm hydrolysate replacement flours. ED0-M, enzymatically decomposed defatted mealworms at 0 h; ED1-M, enzymatically decomposed defatted mealworms at 1 h; and ED4-M, enzymatically decomposed defatted mealworms at 4 h.

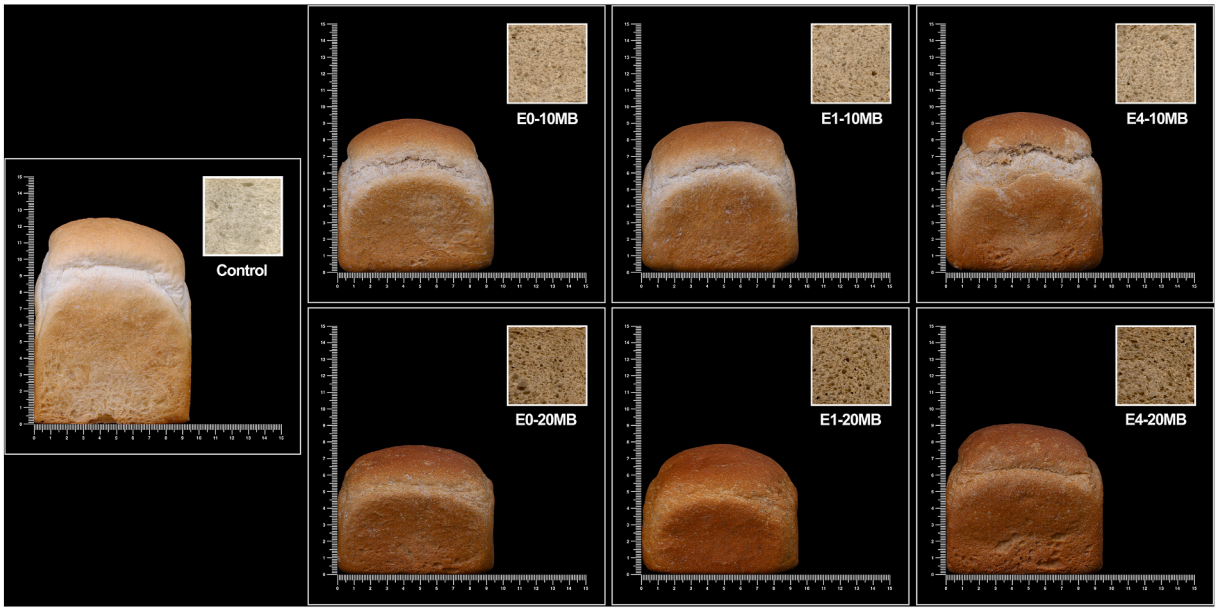

Fig. 7. Images of the external appearance and cross-sectional slices of loaves. E#, # indicate time (h) for enzymatic hydrolysis; **MB, ** represents the proportion of wheat flour replaced with defatted mealworm hydrolysates.

height increases were observed in ED4-M when the ED-M content was identical. This tendency was more prominent in the 20 % replacement group; therefore, chitin hydrolysis patently improved loaf volume. Addition of 30–120 kDa chitosan, corresponding to a medium molecular weight, at a 0.1–0.3 % concentration increased the loaf volume (H.-Y. Lee et al., 2002). Therefore, medium-molecular-weight chitin generated from HMWC hydrolysis in DF-M could have contributed to the increase in loaf volume.

Edible insect materials are vulnerable to microbial contamination, posing a potential risk of bacterial transfer to the food matrix from insects, particularly bacteria with heat-resistant spores (Roncolini et al., 2019, 2020). We conducted a microbiological analysis 2 h after bread baking to determine the presence of aerobic bacteria, *E. coli*, and mold in the bread. Viable counts of aerobic bacteria and molds on the bread were lower than the LOD (10 CFU/g per plate), and *E. coli* was not detected in any sample groups. Heat treatment was used for blanching, and blanched mealworms were dried in a hot-air drying oven. High-temperature conditions have also been developed for pressure defatting and lysozyme inactivation. Moreover, baking requires high thermal energy (180 °C, 40 min). Thus, a chain of heat treatments could have contributed to inactivation of spore-forming bacteria in mealworms (Dopazo et al., 2023).

3.4.2. *Effect of ED-M supplementation on white bread pores*

A decrease in the specific volume of the bread implies an increase in the density of the bread. Higher levels of ED-M replacement resulted in smaller loaves with tighter pores (Table 5 and Fig. 7). Even though adding chitosan can create a network capable of retaining gas, excessive cross-linking can make the dough overly strong, resulting in bread with a lowered specific volume (H. A. Silva et al., 2020). Using ED4-M in

Table 4
Changes in bread quality characteristics for defatted mealworm hydrolysate replaced bread.

Bread Type	Bread height (cm)	Specific volume (cm^3/g)	Aerobic bacterial counts (CFU/g)	Mold (CFU/g)	*Escherichia coli* (CFU/g)
Control	12.43 ± 0.39^{d}	6.10 ± 0.16^{d}	n.d.	n.d.	n.d.
E0-10MB	9.23 ± 0.11^{b}	4.79 ± 0.16^{b}	n.d.	n.d.	n.d.
E1-10MB	9.14 ± 0.19^{b}	4.92 ± 0.11^{b}	n.d.	n.d.	n.d.
E4-10MB	9.52 ± 0.20^{c}	5.27 ± 0.13^{c}	n.d.	n.d.	n.d.
E0-20MB	7.95 ± 0.38^{a}	4.28 ± 0.14^{a}	n.d.	n.d.	n.d.
E1-20MB	7.96 ± 0.34^{a}	4.43 ± 0.15^{a}	n.d.	n.d.	n.d.
E4-20MB	9.18 ± 0.33^{b}	4.80 ± 0.09^{b}	n.d.	n.d.	n.d.

E#, # indicate time (h) for enzymatic hydrolysis; **MB, ** represents the proportion of wheat flour replaced with defatted mealworm hydrolysates. N.d., not detected.
Limit of detection for microbial counts was 10 CFU/g per plate.
Values are presented as mean ± standard deviation. Different superscript letters within columns (a-d) represent significant differences ($p < 0.05$).

replacement levels of 10 % and 20 % caused a remarkable loaf volume increase and increased the ratio of large-size pores; cell density was similar to that of the control bread. This porous structure can be caused by reducing strong intermolecular hydrogen bonds and gluten interactions by decomposing HMWC.

3.4.3. Effect of DF-M hydrolysates on white bread color

Color is one of the factors that can influence consumer choice and acceptance of bakery products (Kowalski et al., 2022). Differences in bread crust color were observed at different levels of ED-M replacement and hydrolysis times (Table 6). In contrast, no significant differences in color within the groups with the same ED-M replacement levels were observed in the crumbs. The BI of bread crust decreased by approximately 81 % in ED4-M compared with that in ED0-M, particularly at the 20 % substitution level. The increase in the brown color is primarily based on the intrinsic color of the mealworm; however, the Maillard reaction may also mediate the intensity. Chitosan and chitin are conceivable participants in the Maillard reaction that reacts with reducing sugars (Agulló et al., 2003). Another possible modulation mechanism of the Maillard reaction by ED-M is the change in the water migration ratio during baking. HMWC has cationic properties that form electrostatic interactions with gluten and other anionic polysaccharides. Addition of HMWC to bread expedites gluten dehydration but prevents water migration into starch granules (Kerch et al., 2010). Therefore, surplus water easily migrates to the crust during baking, and the higher moisture content in the crust could have intensified the degree of the Maillard reaction. The results suggest that the hydrolysis of chitin in DF-M can lighten the color of the product, in turn enhancing consumer acceptance.

3.5. Textural characteristics and staling kinetics of white bread supplemented with ED-M

3.5.1. Moisture changes during bread storage

Water loss during storage, gluten dehydration, and recrystallization of extragranular amylose and amylopectin networks contribute to bread staling. Furthermore, moisture change is an essential parameter in

Table 5
Pore characteristic analysis of bread crumbs replaced with defatted mealworm hydrolysates.

Bread type	Number of cells (40 mm^2)	Mean cell area (mm^2)	Cell density (cells/mm^2)	Very small size (CA≤0.2 mm^2)	Small size (0.2<CA≤3.0 mm^2)	Medium size (3.0<CA≤10.0 mm^2)	Large size (10.0 mm^2≤CA)
Control	292.33 ± 43.06^{a}	1.16 ± 0.25^{b}	0.18 ± 0.03^{a}	29.93 ± 1.88^{b}	62.48 ± 2.51^{bc}	5.80 ± 1.52^{a}	1.79 ± 1.27^{cd}
E0-10MB	388.11 ± 36.43^{c}	1.04 ± 0.08^{ab}	0.24 ± 0.02^{bc}	29.61 ± 2.70^{b}	63.13 ± 3.24^{bc}	6.07 ± 0.86^{a}	1.19 ± 0.35^{abc}
E1-10MB	418.56 ± 63.95^{cd}	1.05 ± 0.18^{ab}	0.26 ± 0.04^{cd}	33.62 ± 2.12^{d}	59.43 ± 2.07^{a}	5.89 ± 1.19^{a}	1.05 ± 0.66^{ab}
E4-10MB	342.56 ± 58.39^{b}	1.17 ± 0.15^{b}	0.21 ± 0.04^{b}	32.55 ± 1.83^{cd}	58.97 ± 1.89^{a}	6.80 ± 1.38^{a}	1.67 ± 0.38^{cd}
E0-20MB	403.11 ± 48.53^{c}	1.01 ± 0.1^{ab}	0.25 ± 0.03^{c}	28.97 ± 2.77^{b}	63.42 ± 2.59^{c}	6.73 ± 1.34^{a}	0.88 ± 0.45^{a}
E1-20MB	451.89 ± 33.28^{d}	0.96 ± 0.11^{ab}	0.28 ± 0.02^{d}	31.09 ± 1.82^{bc}	62.41 ± 1.21^{bc}	5.82 ± 1.44^{a}	0.68 ± 0.30^{a}
E4-20MB	276.22 ± 33.04^{a}	1.54 ± 0.27^{c}	0.17 ± 0.02^{a}	26.40 ± 2.08^{a}	60.69 ± 3.43^{ab}	10.49 ± 2.13^{b}	2.43 ± 0.86^{d}

E#, # indicate time (h) for enzymatic hydrolysis; **MB, ** represents the proportion of wheat flour replaced with defatted mealworm hydrolysates. CA, cell area.
Values are presented as mean ± standard deviation (n = 10). Different superscript letters within columns (a-d) represent significant differences ($p < 0.05$).

Table 6
Color values and browning index of bread replaced with defatted mealworm hydrolysates.

Bread Type	Crust color					Crumb color				
	L*	a*	b*	ΔE	BI	L*	a*	b*	ΔE	BI
Control	69.55 ± 2.33^{f}	6.41 ± 1.33^{a}	31.00 ± 1.53^{a}		15.42 ± 1.45^{a}	77.64 ± 3.65^{c}	−0.85 ± 0.20^{a}	13.24 ± 2.06^{a}		4.16 ± 0.62^{a}
E0-10MB	53.25 ± 2.05^{d}	15.44 ± 1.03^{c}	35.08 ± 0.81^{b}	19.08 ± 2.34^{b}	29.19 ± 2.57^{bc}	63.63 ± 1.54^{b}	3.31 ± 0.32^{b}	19.69 ± 0.74^{b}	15.99 ± 1.54^{a}	9.62 ± 0.67^{b}
E1-10MB	55.31 ± 1.33^{e}	14.19 ± 0.94^{b}	35.30 ± 0.91^{b}	16.80 ± 1.69^{a}	27.43 ± 1.93^{b}	63.95 ± 2.26^{b}	3.25 ± 0.32^{b}	18.97 ± 0.84^{b}	15.44 ± 2.11^{a}	9.17 ± 0.69^{b}
E4-10MB	50.98 ± 2.00^{c}	15.97 ± 0.95^{cd}	35.66 ± 1.40^{b}	21.44 ± 2.24^{c}	31.97 ± 3.07^{c}	64.64 ± 2.24^{b}	3.23 ± 0.29^{b}	19.05 ± 0.90^{b}	14.87 ± 1.97^{a}	9.09 ± 0.60^{b}
E0-20MB	42.26 ± 1.21^{b}	17.73 ± 0.78^{e}	38.45 ± 1.80^{c}	30.51 ± 1.51^{d}	48.51 ± 4.65^{e}	51.75 ± 1.22^{a}	5.85 ± 0.27^{c}	23.23 ± 0.44^{d}	28.55 ± 1.08^{b}	15.91 ± 0.56^{c}
E1-20MB	39.42 ± 2.16^{a}	18.01 ± 0.61^{e}	34.92 ± 2.38^{b}	32.61 ± 1.87^{e}	46.99 ± 3.51^{e}	50.55 ± 2.03^{a}	5.88 ± 0.28^{c}	23.67 ± 0.76^{d}	29.81 ± 1.93^{b}	16.80 ± 1.21^{d}
E4-20MB	42.74 ± 1.79^{b}	16.45 ± 0.78^{d}	34.11 ± 1.91^{b}	28.86 ± 1.86^{d}	39.49 ± 4.12^{d}	50.19 ± 1.25^{a}	5.48 ± 0.38^{b}	21.87 ± 0.83^{c}	29.48 ± 1.18^{b}	15.31 ± 0.85^{c}

E#, # indicate time (h) for enzymatic hydrolysis; **MB, ** represents the proportion of wheat flour replaced with defatted mealworm hydrolysates. L*, lightness; a*, redness; b*, yellowness; ΔE, color difference; BI, browning index.
Values are presented as mean ± standard deviation (n = 15). Different superscript letters within columns (a-f) represent significant differences ($p < 0.05$).

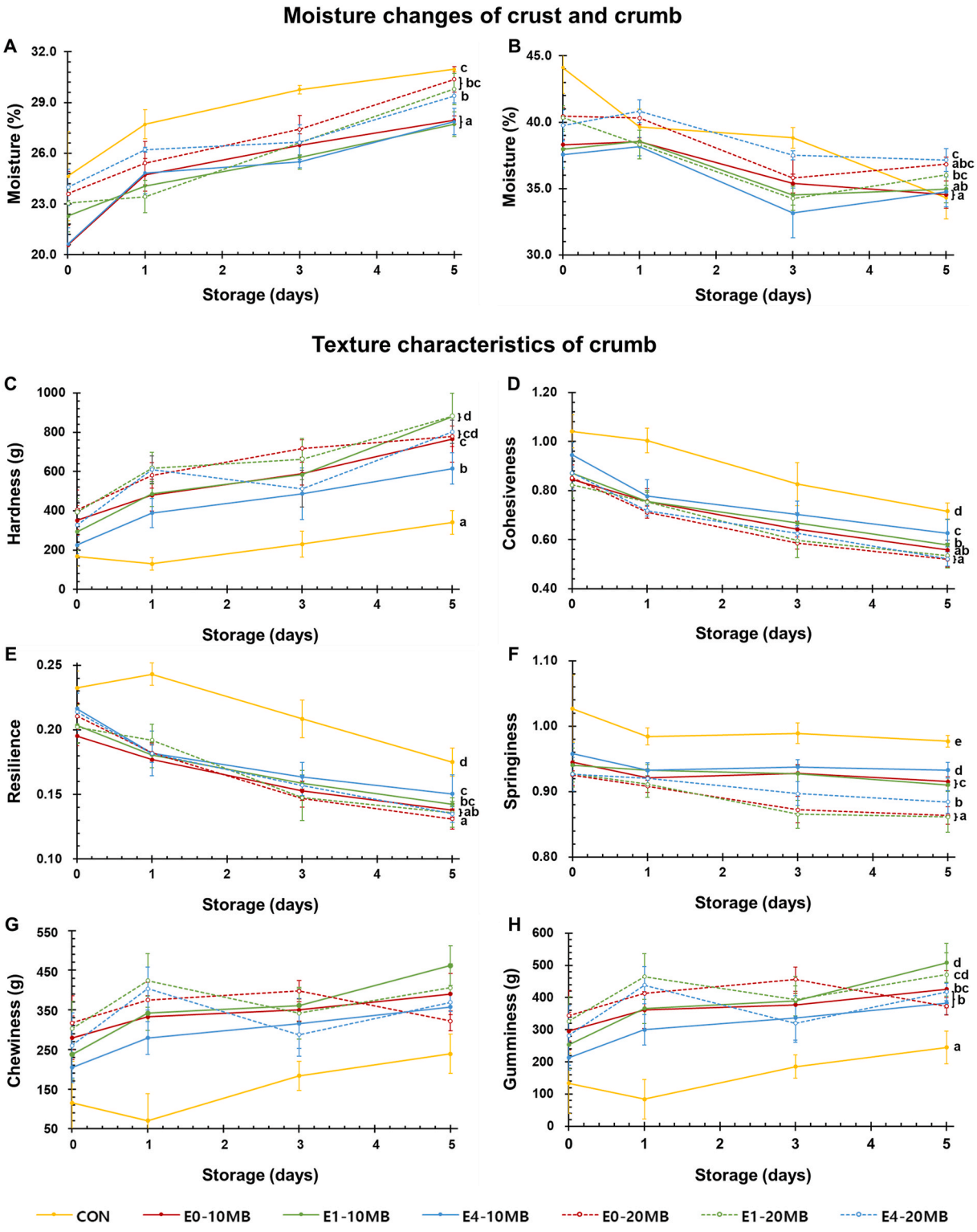

Fig. 8. Changes in moisture content and texture profiles of bread during storage for 5 days. Moisture content was determined for the (A) crust and (B) crumbs. (C) Hardness, (D) cohesiveness, (E) resilience, (F) springiness, (G) chewiness, and (H) gumminess were determined using texture profile analysis (TPA). All analyses were conducted on days 0, 1, 3, and 5. Different superscripts represent significant differences ($p < 0.05$). E#, # indicate time (h) for enzymatic hydrolysis; **MB, ** represents the proportion of wheat flour replaced with defatted mealworm hydrolysates.

staling bread products. The moisture changes in the crust and crumb during storage are shown in Fig. 8A and B. Breads supplemented with ED-4M showed the slowest moisture loss speed of the crumb during storage for 5 days (2.8 % and 2.6 %/day for E4-10MB and E4-20MB, respectively). The E4-20MB retained the highest moisture content for the crumb on day 5. In addition, ED-4M-supplemented bread showed lower moisture content in the bread crust than ED-0M and ED-1M. Addition of polymeric chitosan to bread promotes gluten and starch

Table 7
Onset temperature, peak temperature, conclusion temperature, and apparent enthalpy of defatted mealworm hydrolysates used to replace flour.

Storage time (day)	Bread type	Onset temperature, T_o (°C)	Peak temperature, T_p (°C)	Conclusion temperature, T_c (°C)	Apparent enthalpy, ΔH_{ret} (J/g)
0	**CON**	46.80 ± 0.99^{a}	48.70 ± 0.42^{a}	52.50 ± 1.70^{a}	0.07 ± 0.01^{a}
	E0-10MB	44.95 ± 0.49^{a}	50.70 ± 0.71^{a}	52.5 ± 0.570^{a}	0.07 ± 0.02^{a}
	E1-10MB	44.8 ± 0.71^{a}	50.55 ± 3.32^{a}	54.95 ± 0.21^{ab}	0.09 ± 0.00^{a}
	E4-10MB	47.55 ± 0.35^{a}	54.10 ± 1.56^{b}	56.80 ± 2.12^{b}	0.16 ± 0.07^{a}
	E0-20MB	44.8 ± 0.00^{a}	49.35 ± 1.91^{a}	54.35 ± 1.20^{ab}	0.08 ± 0.06^{a}
	E1-20MB	47.6 ± 2.26^{a}	52.15 ± 5.30^{b}	56.75 ± 2.76^{b}	0.10 ± 0.02^{a}
	E4-20MB	45.55 ± 1.34^{a}	51.95 ± 2.33^{a}	55.15 ± 0.49^{ab}	0.11 ± 0.03^{a}
5	**CON**	42.75 ± 2.47^{ab}	53.20 ± 0.00^{d}	60.80 ± 0.28^{b}	0.84 ± 0.01^{abc}
	E0-10MB	44.05 ± 1.48^{ab}	51.45 ± 0.49^{bc}	58.65 ± 2.76^{ab}	1.23 ± 0.14^{d}
	E1-10MB	43.40 ± 1.13^{ab}	50.40 ± 0.71^{ab}	58.70 ± 0.14^{ab}	1.08 ± 0.17^{bcd}
	E4-10MB	41.15 ± 1.06^{a}	49.75 ± 0.64^{a}	59.45 ± 1.06^{ab}	1.41 ± 0.11^{d}
	E0-20MB	45.65 ± 0.78^{b}	52.70 ± 0.42^{cd}	57.15 ± 0.35^{a}	0.63 ± 0.04^{abc}
	E1-20MB	45.40 ± 0.28^{b}	51.15 ± 0.07^{b}	59.25 ± 1.63^{ab}	0.74 ± 0.05^{ab}
	E4-20MB	44.00 ± 0.85^{ab}	53.00 ± 0.85^{d}	59.85 ± 0.92^{ab}	1.15 ± 0.27^{cd}

E#, # indicate time (h) for enzymatic hydrolysis; **MB, ** represents the proportion of wheat flour replaced with defatted mealworm hydrolysates. Values are presented as mean ± standard deviation (n = 3). Different superscript letters within columns (a-d) represent significant differences ($p < 0.05$).

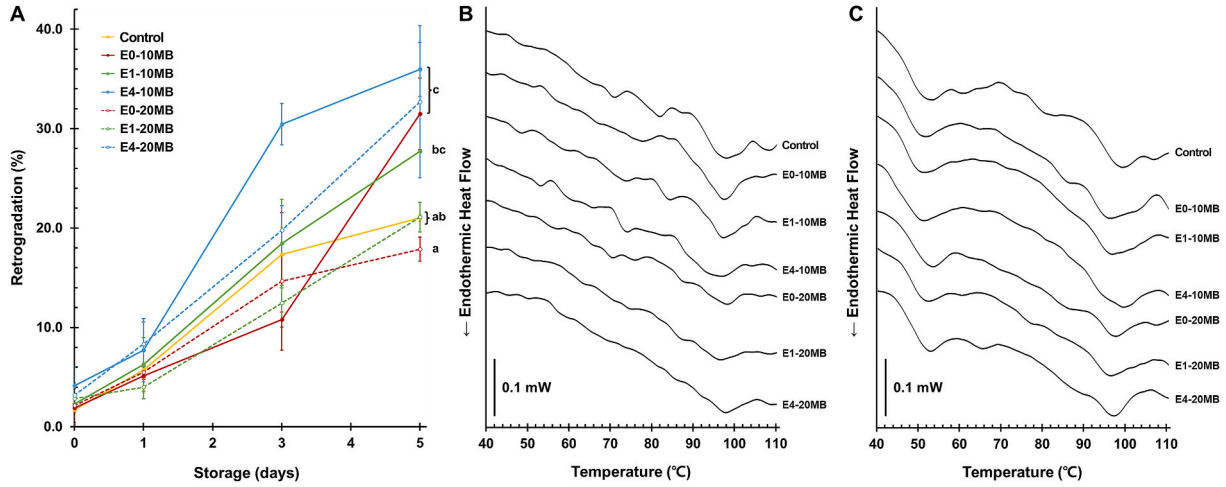

Fig. 9. Changes in starch retrogradation proportions of bread replaced by defatted mealworm lysates during storage. (A) The retrogradation index of the experimental bread was analyzed on days 0, 1, 3, and 5. (B and C) Endothermic heat flow curves of bread on days 0 and 5. Different superscripts represent significant differences ($p < 0.05$). E#, # indicate time (h) for enzymatic hydrolysis; **MB, ** represents the proportion of wheat flour replaced with defatted mealworm hydrolysates.

granule dehydration while preventing water migration and redistribution between gluten and starch, thereby promoting the free water molecule migration to the bread surface (Kerch et al., 2008). Similarly, lysozyme hydrolysis of HMWC in DF-M probably blocked water migration to the outside. Moreover, increased WBC of the ED-M could have helped the water retention in ED-M supplemented bread during storage.

3.5.2. *Changes in texture profiles of bread during storage*

The effects of flour substitution with ED-M on the bread crumb texture and staling during storage were monitored using TPA (Fig. 8). Addition of ED-M led to a significant increase in crumb hardness, chewiness, and gumminess at both replacement levels, compared with those of the control bread ($p < 0.05$) (Fig. 8C). In contrast, the resilience, cohesiveness, and springiness decreased (Fig. 8D–F). These data align with those of a previous study in which bread was prepared with mealworm powder replacement at 0–20 % concentrations (Xie et al., 2022). However, using ED4-M resulted in a softer bread texture with a low hardness similar to that of the control bread. This finding may be the same reason why the ED4-M-supplemented breads have large pores and specific volumes.

Bread staling is a complex phenomenon that occurs in bakery products due to several mechanisms, and the retrogradation of bread during storage is closely related to water loss and redistribution of water molecules (Kotsiou et al., 2021). The hardness increases as the moisture content decreases because of strong hydrogen bond formation between starch polymers and starch and proteins (Giannone et al., 2016). A sharp increase in hardness was observed on day 1 when ED-Ms were substituted, followed by a gradual increase until day 5 (Fig. 8C). The lowest hardness was observed for ED4-M groups at 10 % and 20 % replacement levels. This finding is likely because low-molecular-weight materials inhibit hydrogen bond formation between starch granules and gluten protein fibrils (Kerch et al., 2010). Cohesiveness, resilience, and springiness showed a modest decline in the 10 % replacement group, whereas the 20 % replacement group showed a sharp decline by day 3 and a gradual decline until day 5. The weakened gluten network may be grounds for flour substitution with ED-M because it attenuates starch and gluten proportions and increases chitin content in bread. Using ED4-M at both alternative levels resulted in the highest cohesiveness and resilience during storage. The chewiness and gumminess of bread supplemented with ED4-M were maintained at the lowest levels during storage. That is, the chitin hydrolysis of mealworm powder results in a softer product with a higher moisture content during storage.

3.5.3. *Amylopectin retrogradation degree of ED-M-supplemented breads*

Water migrating from gluten to starch-rich areas during storage roughens the starch retrogradation. Therefore, the prolonged retention

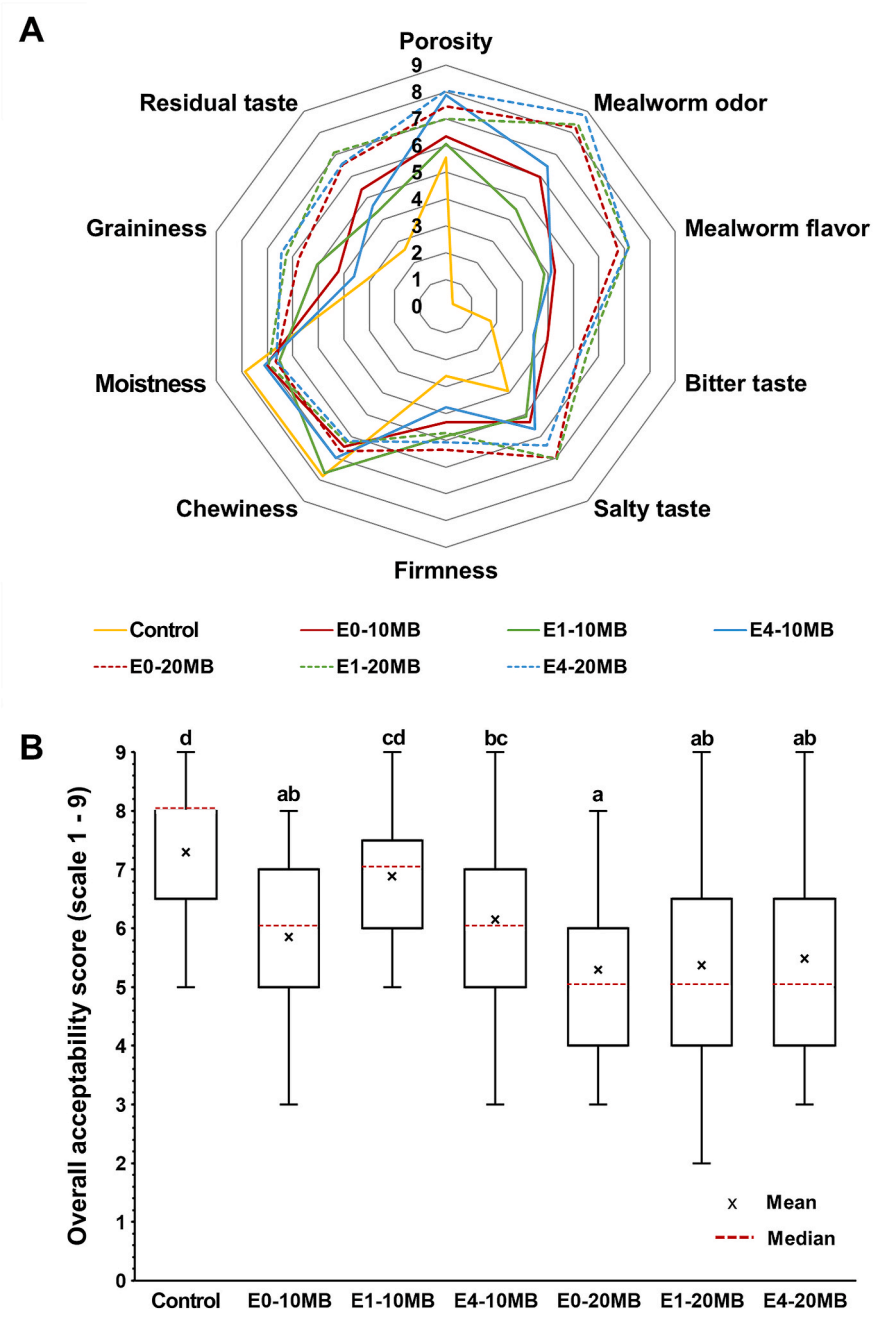

Fig. 10. Sensory characteristics of bread supplemented with mealworm hydrolysate. (A) Quantitative descriptive analysis (QDA) and (B) overall acceptance tests were conducted for the bread. Different superscripts represent significant differences ($p < 0.05$). E#, # indicates time (h) for enzymatic hydrolysis; **MB, ** represents the proportion of wheat flour replaced with defatted mealworm hydrolysates.

of water molecules in the food matrix increases the molecular mobility of amylopectin and promotes amylopectin retrogradation (Arp et al., 2020). DSC was used to monitor amylopectin retrogradation in bread crumbs during storage for up to 5 days (Table 7 and Fig. 9). All samples exhibited T_o, T_p, and T_c of approximately 42–47 °C, 48–54 °C, and 52–60 °C, respectively, and no significant differences were observed in the T_o, T_p and T_c values for all groups. In Table 7, ΔH_{ret} corresponds to the melting enthalpy of amylopectin retrogradation, reflecting the retrogradation degree during storage. After a storage period of five days, the ED4-M groups showed the highest ΔH_{ret} values (1.41 ± 0.11 and 1.15 ± 0.27 J/g for E4-10MB and E4-20MB), implying the increased starch retrogradation. The enthalpy of the starch gelatinization (ΔH_{gel}) was measured using DSC for fresh flour and flour substituted with 10 % and 20 % of ED-M to calculate the RI. The RI of the samples was calculated based on ΔH_{gel} of fresh flour and ΔH_{ret} of the samples after storage (Fig. 9A). Similarly, according to the ΔH_{ret} results, RI was also highest in ED4-M-supplemented bread. Therefore, the highest retrogradation degree was observed in samples treated with ED4-M because of low moisture loss during storage (Fig. 8A and B).

Another possible mechanism is the chitin-starch interaction in bread. Adding chitosan resulted in strong adhesion between starch and chitin (Bangyekan et al., 2006), and the attached chitins prevented water absorption into starch granules released from gluten molecules at the starch surface (Kerch et al., 2010). Chitin hydrolysis possibly diminished starch-chitin adhesion, promoting water absorption and starch retrogradation.

3.6. Sensory analysis

Using ED-M in the bread formulation intensified the "bitter taste" of the product, as well as the "graininess" and "residual taste," which are common sensory traits in whole-grain bread (Fig. 10A). The presence of "mealworm odor" and "mealworm taste" increased with an increase in the amount of ED-M, resulting in a low acceptability score. Notably, hydrolysis time also changed the mealworm odor and taste of the bread. Specifically, bread containing ED4-M exhibited the highest "mealworm odor" and "mealworm flavor" scores. The relatively high OBC properties of ED4-M could have enhanced its retention of aromatic compounds, leading to higher "mealworm odor" and "mealworm flavor" scores in the bread.

In addition, increased hydrolysis time was associated with larger apparent pore size in the bread. Regarding "firmness," the control bread had the softest texture. Among the breads supplemented with mealworm powder, E4-10M had the softest texture. The trends were consistent with the TPA results. Addition of ED-M also increased the saltiness. The -NH_3^+ groups in chitin can adsorb Cl^- ions through electrostatic interactions, increase free Na^+ ions, and enhance the salty taste (Jiang et al., 2017). This may reduce salt addition in food products when ED-M is supplemented.

In the overall acceptability test conducted with 30 participants, the overall acceptability score decreased with an increase in ED-M, irrespective of the hydrolysis time (Fig. 10B). This trend aligns with the findings reported by other researchers. For instance, García-Segovia et al. (2020) evaluated bread characteristics containing 5 % and 10 % of mealworm or buffalo worm (*Alphitobius diaperinus* P.). They found that addition of insect flour to bread impaired the sensory scores depending on the replacement proportion. Similarly, Kowalski et al. (2022) reported that sensory evaluations of bread containing 10 %, 20 %, and 30 % mealworm and buffalo worms resulted in decreased consumer acceptance scores; however, the 10 % addition-bread showed better scores than the control bread. Notably, a significant improvement in consumer acceptability of the 10 % ED-M replacement group was observed in this study, regardless of the degree of hydrolysis. E1-10MB exhibited the highest acceptance rate among them at the same mealworm proportions.

4. Conclusion

This study investigated whether enzymatic chitin hydrolysis could mitigate the potential negative effects on bread fortified with edible insects. This study is the first attempt to use chitin hydrolysis to DF-M to enhance the quality of insect bread. Lysozymes can hydrolyze chitin without isolating chitin. Moreover, owing to the various activation conditions of lysozymes, water can be used as a solvent for hydrolysis, increasing the availability of chitin hydrolysis in the food industry. The enzymatic hydrolysis of DF-M led to gradual increases in WBC and OBC, crucial characteristics of food ingredients for processing and applications. Additionally, DF-M exhibited improved antioxidant capacity when the hydrolysis time was increased.

Chitin hydrolysis significantly influenced the quality of mealworm-supplemented white bread. Although adding mealworms negatively affected bread volume and texture, chitin hydrolysis contributed to increased volume and improved the soft texture. Throughout 5-day storage, the ED4-M bread maintained a soft texture and high moisture content, compared with the ED0-M bread. In sensory analysis, a higher ED-M proportion in bread reduced acceptability; however, DF-M hydrolysis at each replacement level positively impacted acceptability, and ED1-10MB exhibited the highest acceptability among mealworm-supplemented breads.

Our findings demonstrate that the enzymatic hydrolysis of chitin in DF-M enhanced bread volume and texture. Therefore, we suggest that using lysozymes in mealworm powder to hydrolyze chitin can produce high-quality bakery products fortified with edible insects. However, further research will be necessary to enhance chitin hydrolysis efficiency, including pre-heat treatment and ultrasonic application, to break strong chitin hydrogen bonds and explore various edible chitinolytic enzymes.

CRediT authorship contribution statement

Su-Hyeon Pyo: Investigation, Validation, Conceptualization, Formal analysis, Methodology, Data curation, Writing – review & editing, Writing – original draft, Visualization. **Chae-Ryun Moon:** Investigation, Validation, Formal analysis, Methodology, Data curation. **So-Won Park:** Investigation, Validation, Formal analysis, Methodology, Data curation. **Ji-yu Choi:** Formal analysis, Methodology, Data curation. **Jong-Dae Park:** Formal analysis, Methodology, Data curation. **Jung Min Sung:** Formal analysis, Methodology, Data curation. **Eun-Ji Choi:** Formal analysis, Methodology, Data curation. **Yang-Ju Son:** Project administration, Investigation, Validation, Conceptualization, Formal analysis, Methodology, Data curation, Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Supervision.

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.

Data availability

Data will be made available on request.

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