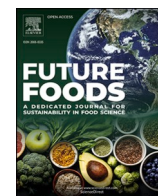


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From bean to batter: Enzymatic tailoring of faba bean protein for improved pound cakes

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ABSTRACT

The poor solubility, emulsification, and gelling properties of faba bean protein isolate (FPI) limit its use as an egg replacement. This study enhanced FPI functionality through enzymatic modification using transglutaminase (TG) and Alcalase (AC), evaluating their performance in a pound cake model system. FPI dispersions were treated with TG (1–10 U/g protein) or AC (0.1–1.0 U/g protein) under optimised conditions and lyophilised. Morphology, structure, and degree of hydrolysis were analysed via SEM, SDS-PAGE, and free amino acid profiling. Functional properties including solubility, foaming, emulsifying stability, water/oil-holding capacity, and gelation were correlated with cake quality parameters. AC treatments markedly improved solubility (up to 61.58%) and foam capacity (108.84%), with ACH showing the highest DH (9.00%) but near-complete loss of gelation ($G' < 1.5$ Pa). TG induced protein crosslinking, enhancing gelation (G' up to 6693 Pa for TGL), although solubility decreased substantially (3.93–5.48%). WHC and OHC were highest in heat-treated blanks, reflecting heat-induced denaturation and freeze-drying effects on protein microstructure. TGH (10 U/g protein) achieved the highest specific volume among FPI-derived samples (1.80 ± 0.02 mL/g) and ACL (0.1 U/g protein) produced the softest crumb (hardness: 10.16 ± 0.52 N) and lowest separation rate ($1.75 \pm 0.21\%$ /min), though none reached the CEP control (2.33 ± 0.03 mL/g). Emulsifying stability and gelation emerged as key determinants of cake quality. Enzymatic modification can selectively enhance FPI functional traits, though balancing enzyme type and concentration remains essential for optimal performance in complex food systems.

1. Introduction

The U.S. egg industry is in crisis due to widespread avian flu outbreaks, skyrocketing prices and mounting regulatory pressure. With over 166 million birds culled and retail prices still well above average these developments underscore the fragility of the current supply system. Similar challenges are reported worldwide, highlighting the urgent need for reliable, sustainable egg alternatives (Carman, 2025). A previous study identified faba bean protein concentrate as a suitable egg replacement in pound cake, emphasising the role of techno-functional properties in determining cake quality (Halm et al., 2025). However, gaps in the functionality of legume proteins have hindered the replication of the cakes' nutritional and structural composition. A potential solution is to modify these proteins to improve their functional properties, such as solubility, emulsifying behaviour, and gelling capacity, thereby yielding an improved final product.

Faba bean protein isolate (FPI), which contains approximately 90% protein (Dhull et al., 2022), exhibits poor solubility due to its compact structure, resulting from alkaline extraction conditions. These conditions cause irreversible protein denaturation and aggregation, leading to reduced functionality at neutral pH and limiting the protein's applicability in various food matrices (Eckert et al., 2019). Hydrolysis has been shown to improve protein solubility, which can enhance techno-functional properties (Liu et al., 2019a). Alcalase (AC) is a commercial enzyme preparation derived from *Bacillus licheniformis*, consisting primarily of subtilisin A, an endopeptidase with broad specificity that preferentially cleaves peptide bonds adjacent to terminal hydrophobic amino acids (Ellaiah et al., 2002). Several studies have used AC to modify faba bean proteins. For instance, Liu et al. (2019a) used AC to improve the physical and oxidative stability of oil-in-water emulsions prepared from FPI, finding that a degree of hydrolysis (DH) of 4% showed the best performance. This optimal behaviour was

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attributed to a balanced molecular weight, enhanced surface charge, and appropriate hydrophobicity. Similarly, Nawaz et al. (2022) applied moderate, targeted enzymatic hydrolysis to improve the physical stability of faba bean-based milk alternatives. A DH between 9% and 16% significantly enhanced colloidal stability, highlighting the importance of optimising the level of hydrolysis.

Another enzymatic approach to improving the techno-functional properties of proteins involves intermolecular crosslinking. Microbial transglutaminase (TG) (γ -glutamyl-peptide, amine γ -glutamyl-transferase, EC 2.3.2.13) catalyses acyl transfer reactions between the γ -carboxamide group of glutamine residues in proteins and primary amines. When lysine residues serve as acceptors, TG forms ϵ -(γ -glutamyl)-lysine isopeptide covalent bonds, leading to protein polymerisation (Gaspar and de Góes-Favoni, 2015). Previous studies have investigated the effects of TG on the physicochemical properties of faba bean protein. Liu et al. (2019b) found that TG treatment for 60 min rendered FPI a promising emulsifier capable of maintaining physical stability. Another study confirmed that TG reduced the excessive hydrophobicity caused by heat treatment and enhanced gel strength and water retention. These modifications suggest that TG-treated FPI has strong potential as both a stable emulsifier and an effective gelling agent for food applications (Nivala et al., 2021). In an earlier study, TG was also applied to improve the colloidal stability and foaming properties of FPI. However, excessive TG concentrations reduced solubility and negatively impacted foaming capacity due to inter-particle aggregation, while lower concentrations had an opposite, more favourable effect (Nivala et al., 2017).

While previous studies have demonstrated that both TG and AC can individually improve specific functional properties of FPI, such as emulsification, colloidal stability, or gel strength, these investigations were conducted at single enzyme concentrations and without evaluating performance in a real baking application. As the multi-functional demands of a baking matrix require simultaneous emulsification, aeration, gelation, and water retention, the optimal enzyme type and concentration cannot be predicted from single-property measurements alone. This highlights the need for a systematic, multi-concentration comparison of both enzymatic strategies, directly linked to baking performance, and leads to the hypothesis that TG crosslinking and AC hydrolysis will differentially tune FPI functionality, with trade-offs between enzyme type and concentration determining the optimal cake quality outcome.

To tailor FPI towards egg-like performance, we selected two mechanistically complementary enzymes that have already been validated on faba bean proteins: microbial transglutaminase to strengthen protein network formation and Alcalase to improve solubility and interfacial functionality (Liu et al., 2019b, 2019a; Nivala et al., 2021, 2017). In preliminary screening, other commercial proteases (Flavourzyme, Neutrase) did not provide comparable improvements in FPI functionality and were therefore not pursued further.

Enzyme-assisted processes have been used to extract and tailor plant-based proteins for bakery applications, and enzymatic hydrolysis has also been applied to improve the techno-functional properties of soy-based ingredients for structured foods such as meat analogues (Bildstein et al., 2008; Mookerjee and Tanaka, 2023). However, to the best of our knowledge, the effects of enzymatic modification on the functional properties of FPI and its subsequent application in a pound cake matrix have not yet been reported. This study aims to use enzymatic treatment and enzyme concentration to tune the techno-functional properties of FPI towards egg-like behaviour, so that FPI can deliver the best possible pound cake quality when used as an egg replacer. Specifically, we aim to investigate how enzymatic modification affects the protein structure, using techniques such as scanning electron microscopy (SEM), electrophoresis, degree of hydrolysis, and free amino acid analysis, as well as key physicochemical properties, including protein solubility, water- and oil-holding capacities, foaming, emulsification, and gelation. In addition, we evaluate the potential of enzyme-treated FPI as an egg substitute in pound cake formulations,

assessing its effects on cake quality in comparison to conventional whole egg powder (CEP).

2. Materials and methods

2.1. Materials

Faba bean protein isolate (FPI) was obtained from AGT Food and Ingredients (Regina, Saskatchewan, Canada). FPI was the same batch as used in our previous study, where its proximate composition was reported as 80.60 ± 0.37 g protein/100 g, 7.14 ± 0.84 g fat/100 g, 5.30 ± 0.36 g ash/100 g and 8.60 ± 0.70 g moisture/100 g. A detailed composition is provided in Halm et al. (2026). Transglutaminase (TG) (ACTIVA®-FV, with maltodextrin) with approximately 100 U/g activity was kindly provided by Ajinomoto Food Europe S.A.S. (Mesnil-Saint-Nicaise, France). Alcalase® 2.4 L (AC) (protease form *Bacillus licheniformis*) with an activity of ≥ 2.4 U/g was purchased from Sigma-Aldrich (St. Louis, MO, USA). The control pound cake was produced using biscuit flour (Odlums Group, Dublin, Ireland), sucrose (Siucra, Dublin, Ireland), salt (Glacia, UK), sunflower oil (The King, Svitlovodsk, Ukraine), whole egg powder (CEP) (Igrecia, Seiches sur le Loir, France), baking powder (Dr. Oetker, Bielefeld, Germany), and filtered tap water. According to data reported previously for CEP (Halm et al., 2026), it contains 53.40 ± 2.10 g protein/100 g, 34.20 ± 2.00 g fat/100 g, 3.76 ± 0.25 g ash/100 g and 5.07 ± 0.46 g moisture/100 g. CEP was replaced with FPI to obtain a protein content (13 %) comparable to the control cake.

2.2. Chemicals and reagents

Unless stated otherwise, all chemicals were sourced from Sigma-Aldrich (St. Louis, MO, USA). The following analytical reagents were used: urea (CAS 57–13–6), ethylenediaminetetraacetic acid (EDTA, CAS 60–00–4), sodium dodecyl sulphate (SDS, CAS 151–21–3), Tris base (CAS 77–86–1), 2-mercaptoethanol (BME, CAS 60–24–2), o-phthalaldehyde (OPA, CAS 643–79–8), di-sodium tetraborate decahydrate (CAS 1303–96–4), dithiothreitol (DTT, CAS 3483–12–3), L-serine (CAS 56–45–1), L-norvaline (CAS 6600–40–4), 5-methyl-L-tryptophan (CAS 1149–99–1), acetone (CAS 67–64–1), methanol (CAS 67–56–1), acetic acid (CAS 64–19–7), hydrochloric acid (HCl, CAS 7647–01–0), and sodium hydroxide (NaOH, CAS 1310–73–2).

2.3. Enzymatic treatment

Enzymatic treatments were conducted using a 13% (w/v) protein solution in Milli-Q water. This concentration was selected to match the protein content used in the cake batter formulation (Table 1), ensuring that the enzymatic treatments were conducted under conditions directly representative of the intended application. The native pH of the FPI dispersion (pH=6.28) was not adjusted prior to incubation, as the enzymatic treatments were deliberately performed under these food-relevant conditions to preserve a food-grade process. The FPI dispersions were pre-heated to the optimal temperature for each enzyme (37 °C for TG and 50 °C for AC) for 1 h while shaking at 180 rpm. Subsequently, the dispersions were incubated at their respective optimal temperatures with continuous shaking (180 rpm) for 1 h and treated with low, medium, and high concentrations of each enzyme: TG at 1.0 (TGL), 5.0 (TGM), and 10.0 (TGH) U/g of protein substrate; and AC at 0.1 (ACL), 0.5 (ACM), and 1.0 (ACH) U/g of protein substrate. The concentration ranges for TG (1.0–10.0 U/g protein) and AC (0.1–1.0 U/g protein) were selected based on a combination of the published literature and preliminary screening. For TG, published studies on FPI report effects at concentrations between 1 U/g and 60 U/g (Nivala et al., 2021, 2017), and our pilot experiments indicated that the 1–10 U/g protein range captures the transition from limited crosslinking to extensive network formation. For AC, Liu et al. (2019a) demonstrated optimal

Table 1
Pound cake formulations.

	Biscuit flour	Sucrose	Baking powder	Salt	Sunflower Oil	CEP	Faba bean isolate	Water
CEP	100	61.7	4.6	0.3	34.3	24.3	-	75.7
FPI	100	61.7	4.6	0.3	34.3	-	16.1	83.9
Enzyme-treated FPI	100	61.7	4.6	0.3	34.3	-	14.7	85.3

CEP = whole egg powder; FPI = faba bean protein isolate; All values are given based on flour (%).

emulsification at a DH of ~4%; accordingly, our range was designed to encompass DH values below, at, and above this optimum, starting from the lowest concentration tested in comparable studies (0.1 U/g protein). Blanks of the FPI dispersions (TGB/ ACB) were prepared for each temperature and processed identically, except without the addition of the respective enzyme. Enzymatic reactions were terminated by heating the mixtures at 100 °C for 15 min. The samples were then cooled, frozen, lyophilised (Telstar LyoQuest-85 Plus, Barcelona, Spain), and stored at −20 °C until further use. Moisture content of the lyophilised samples was not explicitly measured in this study. All samples underwent identical lyophilisation conditions, and the resulting powders were visually uniform. Future work should include moisture determination as a standard quality parameter. In preliminary screening, additional protease preparations (Flavourzyme, Neutrase) were also tested, but they did not improve FPI solubility, foaming, emulsifying properties and performance in the cake system to the same extent as Alcalase and were therefore not included in the main experimental design.

2.4. Enzyme-treated FPI characterisation

2.4.1. Scanning electron microscopy (SEM)

The ultrastructure of the samples was examined using scanning electron microscopy (SEM) (JSM-5510, Jeol Ltd., Tokyo, Japan). To prepare the samples, FPI and the enzyme-treated FPI were lyophilised (SP Scientific BTP-SEL-00X, Warminster, USA) and mounted on aluminium stubs using double-sided carbon tape. Subsequently, a palladium-gold coating (5 nm thickness, Au: Pd = 80:20) was applied via sputter coating using a Polaron E5150 sputter coater (Polaron, East Grinstead, UK). Imaging was performed using a field emission scanning electron microscope with a working distance of 20 mm. SEM images were captured using the SEM Control User Interface software, version 5.21 (JEOL Technics Ltd, Tokyo, Japan), at an accelerating voltage of 5 kV. SEM micrographs were evaluated qualitatively to compare relative differences in aggregate morphology and apparent porosity between samples. No quantitative image analysis of pore size or porosity was performed.

2.4.2. Electrophoresis

The protein profile of all samples was analysed by SDS–PAGE using a method by Gautheron et al. (2024) with modifications. Under reducing conditions using NuPAGE™ Bis-Tris mini gels (4–12%, 1 mm, 12-well) (Invitrogen, ThermoFisher Scientific, CA, USA). Proteins were extracted at 1% (w/v) from each sample using an extraction buffer containing 5 M urea, 1 mM EDTA, 2% SDS, and 0.1 M Tris base (adjusted to pH 8.8 with 3 M HCl), along with 1.75% 2-mercaptoethanol (BME). The samples were shaken at 500 rpm for 16 h at room temperature, followed by centrifugation (Centrifuge 5417R, Eppendorf, Hamburg, Germany) at 14,680 rpm (~36,150 × g) for 20 min. An aliquot of the supernatant was then mixed with sample buffer (4X) (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) and the NuPAGE™ Sample Reducing Agent (10X) and heated at 70 °C for 10 min. A Precision Plus Protein™ Dual Xtra Prestained Standard (Bio-Rad, CA, USA) with a molecular weight range of 2–250 kDa was prepared. A volume of 20 µL of both the diluted standard and each sample was loaded onto the gel and electrophoresed for 35 min at a constant voltage of 200 V in the diluted NuPAGE™ MES SDS Running Buffer (20X) (Invitrogen) with 1 ml NuPAGE™ antioxidant added to the chamber. Upon completion, gels were rinsed and fixed

(40% methanol, 10% acetic acid) for 30 min with shaking, stained with InstantBlue® Coomassie Protein Stain (Abcam, Cambridge, UK) for 20 min, and destained using MilliQ water until the background colour was sufficiently removed.

2.4.3. Degree of hydrolysis

The degree of hydrolysis (DH) was determined using the o-phthalaldehyde (OPA) method of Nielsen et al. (2001). The OPA reagent was prepared by dissolving 7.62 g of di-sodium tetraborate decahydrate and 200 mg of SDS in 150 mL deionised water, followed by the addition of 160 mg of OPA (dissolved in 4 mL ethanol) and 176 mg of DTT. The final volume was adjusted to 200 mL with deionised water. A serine standard (0.9516 meqv/L) was prepared by dissolving 50 mg of serine in 500 mL of deionised water. Samples (0.1 g) were dissolved in 10 mL of deionised water. For analysis, 400 µL of sample, standard, or blank (deionised water) was mixed with 3 mL of OPA reagent in test tubes and incubated for exactly 2 min at room temperature. Absorbance was measured at 340 nm using a spectrophotometer (Genesys 50, Thermo Fisher Scientific, Waltham, MA, USA). DH was calculated based on absorbance values using a serine calibration curve and the following formula:

$$\text{Serine} - \text{NH}_2 = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{standard}} - \text{Abs}_{\text{blank}}} \cdot 0.9516 \frac{\text{meqv}}{\text{L}} \cdot 0.01 \cdot 100 / X \cdot P \quad (1)$$

$$\text{DH} = \frac{h}{h_{\text{tot}}} \cdot 100\% \quad (2)$$

Where serine-NH₂ = meqv serine NH₂/g protein; X = g sample; P = protein % in sample; 0.01 is the sample volume in liter (L); h = (serine-NH₂ − β) / α meqv/g protein, where α = 1.00 and β = 0.40 for legume proteins; h_{tot} = 8.00 (legume proteins).

2.4.4. Free amino acid analysis

For free amino acid analysis, 150 mg of sample was mixed with 30 µL of Norvaline standard (5 mM), 30 µL of 5-Methyl-Tryptophan standard (10 mM), and 1440 µL of Milli-Q water. The samples were shaken at 1000 rpm for 1 h at room temperature, then centrifuged (Centrifuge 5417R, Eppendorf, Hamburg, Germany) at 10,000 × g for 5 min. An aliquot of the supernatant was filtered using a 0.45 µm syringe filter (Filtropur S, Sarstedt), followed by a second filtration using a 0.2 µm syringe filter (Chromafil, Fisher Scientific). The final filtrate was used for separate tryptophan measurement and derivatisation, followed by total amino acid analysis. Samples were derivatised using the AccQ-Tag Ultra Derivatization Kit (Waters Corp., Milford, MA, USA), and the amino acid profile was analysed using a Thermo Scientific Dionex Ultimate 3000 RS Series HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Waters AccQ-Tag™ Ultra C18 column (150 × 4.6 mm, 2.5 µm) and connected to an RS Diode Array Detector (Thermo Fisher Scientific, Waltham, MA, USA). Eluent A (AccQ-Tag™ Ultra Eluent A/ultra-pure water, 5:95 v/v) and Eluent B (acetonitrile) were used at a flow rate of 1.7 mL/min in gradient mode (0 min, 0% B; 0.36 min, 0% B; 15.54 min, 5.5% B; 19.67 min, 9.1% B; 25.07 min, 17.6% B; 25.74 min, 19.6% B; 27.05 min, 21.3% B; 28.2 min, 59.7% B; 30.08 min, 59.7% B; 30.38 min, 0% B; 35.48 min, 0% B). After separation, amino acids were detected at a wavelength of λ = 260 nm. External calibration was performed using an amino acid standard (AccQ-Tag, Pico-Tag, AccQ-Tag Ultra), with Norvaline as the internal standard (Waters Corp., Milford, MA, USA). Tryptophan was quantified using fluorescence

detection (excitation wavelength: 280 nm; emission wavelength: 350 nm) on the same HPLC system.

2.5. Techno-functional properties of enzyme-treated FPI

2.5.1. Foam capacity and stability

Foam capacity and stability were assessed following the method described by Halm et al. (2025). Dispersions were prepared at a concentration of 2% (w/w) in distilled water. The pH was adjusted to 7 using HCl or NaOH, and the samples were hydrated overnight at 4 °C. After bringing the samples to room temperature, the sediment was carefully redispersed, and the pH was rechecked and readjusted if necessary. The initial sample height was recorded before foaming. Samples were then frothed using an Ultra-Turrax T18 equipped with an S10N-10 G dispersing element (IKA Labortechnik, Janke & Kunkel GmbH, Staufen, Germany) at 25,000 rpm for 30 s. Foam height was measured immediately (0 min) and again after 1 h. Foam capacity was calculated as the percentage increase in volume immediately after mixing, while foam stability was expressed as the percentage of foam retained after 1 h, relative to the initial foam height. The following equations were used:

$$\text{Foaming capacity (\%)} = \left(\frac{\text{Foam height immediately after foaming}}{\text{Initial sample height}} \right) \cdot 100 \quad (3)$$

$$\text{Foam stability (\%)} = \left(\frac{\text{Foam height after 1 h}}{\text{Foam height immediately after foaming}} \right) \cdot 100 \quad (4)$$

2.5.2. Protein solubility

Protein solubility was determined by extracting protein from the samples following a standardised protocol. Samples containing approximately 1% (w/v) protein were weighed into tubes, and 35 mL of distilled water was added. The pH was adjusted to 7 using HCl or NaOH, and the volume was then brought up to 40 mL with distilled water. Tubes were extracted overnight (~16 h) at 4 °C and 500 rpm. After extraction, samples were equilibrated at room temperature for 1 h, briefly vortexed, and the pH was rechecked and adjusted if necessary. Approximately 10 mL of the well-mixed extract was transferred to a 15 mL tube (whole extract), while the remaining contents were centrifuged (Rotina 380R, Hettich, Tuttlingen, Germany) at 5000 rpm (~4000 × g) for 10 min. The supernatant was carefully collected without disturbing the sediment. Nitrogen content of both the whole extract and the supernatant was determined using the Kjeldahl method (N × 6.25). Protein solubility (%) was calculated as:

$$\text{Protein solubility (\%)} = \left(\frac{\text{Protein content in supernatant}}{\text{Protein content in whole extract}} \right) \cdot 100 \quad (5)$$

2.5.3. Emulsifying characteristics

Emulsifying properties were evaluated according to the method described by Halm et al. (2025). Aqueous sample dispersions containing 1% protein (w/w) were prepared using distilled water, with the pH adjusted to 7 using HCl or NaOH. Samples were hydrated by shaking overnight at 4 °C. Emulsions were then formed by mixing the protein dispersions with sunflower oil at a 90:10 ratio and shearing using an Ultra-Turrax T18 fitted with a S10N-10 G dispersing element (IKA Labortechnik, Janke and Kunkel GmbH, Staufen, Germany) at 25,000 rpm for 2 min. Emulsion stability was assessed using an analytical centrifuge (LUMiSizer, LUM GmbH, Berlin, Germany) at 100 × g and 15 °C over 15 min. The instrument measures transmission of near-infrared light through the sample as a function of time and position. Results were reported as the separation rate (%/min), which was derived from the rate of change in the transmission profile across the sample height and represents the rate of phase separation, a lower value indicates a more

stable emulsion.

2.5.4. pH and total titratable acidity (TTA)

The pH and TTA were determined following the method of Halm et al. (2025). 10 g of sample was mixed with 95 mL of distilled water and 5 mL of acetone, then stirred thoroughly. The pH was measured using a pH meter (Mettler Toledo, Columbus, OH, USA). Titration with 0.1 M NaOH was carried out until pH 8.5 was reached. After a 3-min waiting period, the pH was rechecked and adjusted if necessary. TTA is expressed as mL of 0.1 M NaOH per 10 g of sample required to reach the endpoint.

2.5.5. Water- and oil-holding capacity

Water- (WHC) and oil-holding capacity (OHC) were evaluated using the method described by Halm et al. (2025). WHC and OHC were assessed by weighing 1 g of sample into pre-weighed tubes, followed by the addition of 6 g of water (for WHC) or sunflower oil (for OHC). Samples were vortexed for 3 min, left at room temperature for 1 h, and then centrifuged (Rotina 380R, Hettich, Tuttlingen, Germany) at 4000 × g for 30 min at 20 °C. The supernatant was discarded, and tubes were inverted on a paper towel for 30 min to drain excess liquid. The final weight was recorded, and WHC/OHC were calculated using the following equation:

$$\text{WHC / OHC (\%)} = \left(\frac{\text{Weight}_{\text{tube+pellet}} - \text{Weight}_{\text{tube}} - \text{Weight}_{\text{initial sample}}}{\text{Weight}_{\text{initial sample}}} \right) \cdot 100 \quad (6)$$

2.5.6. Gelling properties

The gelling properties were analysed using a method described by Nivala et al. (2021) with slight modifications. Heat-induced gelation was analysed using whole FPI protein suspensions, monitored by small-deformation oscillatory rheology. Samples of 13% (w/w) protein were prepared by dispersing FPI in distilled water shaking for 30 min at room temperature (1500 rpm). Rheological measurements were carried out on a Rheometer Physica MCR 301 (Anton Paar Group AG, Graz, Austria) with a Peltier plate, 50 mm plate-plate geometry, and a 1 mm gap. A solvent trap with mineral oil was used to minimise evaporation. A sample aliquot (2 mL) was loaded and subjected to a temperature ramp from 25 °C to 90 °C (2 °C/min), held for 5 min at 90 °C, then cooled to 25 °C (2 °C/min), followed by a 15-min conditioning period at 25 °C. Measurements were conducted at 0.1 Hz frequency and 0.1% strain, within the linear viscoelastic region of the final gels.

2.6. Cake analysis

Cake preparation and analysis were performed according to Halm et al. (2025) with some modifications.

2.6.1. Cake preparation

The cake formulations are shown in Table 1. For the control cake, water was added to the CEP, and the mixture was pre-mixed using a Kenwood Chef mixer (Kenwood, Havant, UK) with a whisk attachment for 2 min at maximum speed. For the cakes containing the various FPI derivatives, dispersions (13% protein) were prepared by mixing the FPI samples with water in the same manner as for the CEP cake. After combining the dry ingredients (flour, baking powder, sucrose, and salt), the liquid ingredients (CEP mix or FPI dispersion and sunflower oil) were added and mixed for 120 s at the lowest speed setting using the same mixer. A portion of 250 g batter was transferred into a greased baking tin (height: 58.14 mm; top length: 149.62 mm; bottom length: 138.46 mm; top width: 85.16 mm; bottom width: 75.22 mm). Cakes were baked in a preheated deck oven (MIWE condo, Arnstein, Germany) at 180 °C for 45 min, then cooled at room temperature for 2 h before further analysis.

All liquid ingredients (CEP dispersion, FPI dispersion, and enzyme-

treated FPI dispersion) were standardised to a protein content of 13% (w/v) prior to incorporation into the batter, and all values in Table 1 are expressed on a flour basis (%). The higher inclusion of CEP (24.3%) reflects its lower protein content relative to FPI (~54% vs ~90% protein, dry weight basis), requiring a greater quantity to achieve equivalent protein delivery. The lower inclusion of enzyme-treated FPI (14.7%) compared to untreated FPI (16.1%) is due to the lyophilisation step: freeze-drying removes water from the dispersion, concentrating the protein and thereby reducing the mass of powder required to achieve the same 13% protein concentration in the batter. This replacement strategy was based solely on protein content equivalence; functional equivalence was not assumed but evaluated as the primary outcome of the study.

2.6.2. Bake loss

The bake loss (%) during baking was determined by weight difference before and after baking.

2.6.3. Specific volume

The specific volume was determined using a VolScan Profiler (Stable Micro Systems, Godalming, UK).

2.6.4. Textural analysis

Crumb texture was assessed using texture profile analysis (TPA) with a TA-XT2i texture analyser (Stable Micro Systems, Godalming, UK) fitted with a flat-ended cylindrical aluminium probe (50 mm diameter) and a TA-90 platform. The probe diameter was slightly smaller than the cake slice cross-section so that only the crumb was compressed, avoiding interference from the crust. Cakes were sliced into 25 mm-thick sections, excluding the end slices, resulting in four slices per cake for analysis. A two-cycle compression test was performed with a pre-test speed of 1.0 mm/s, test speed of 5.0 mm/s, post-test speed of 10 mm/s, and a compression distance of 10 mm.

2.6.5. Crumb structure

The cake slices prepared for the textural analysis were used for the crumb structure analysis. Crumb structure was evaluated using a C-Cell imaging system (CC.400.01, Calibre Control International Ltd, Warrington, UK), which measured the number of cells, number of holes, cell elongation, and cell diameter. The C-Cell system distinguishes between 'cells' (gas cells fully enclosed within the crumb matrix) and 'holes' (larger, irregularly-shaped voids that are not fully enclosed or exceed a defined size threshold, as determined by the manufacturer's algorithm). Cell elongation is a dimensionless shape descriptor calculated as the ratio of the major to minor axis of the fitted ellipse for each cell, where a value of 1.0 indicates a perfectly circular cell and higher values indicate increasingly elongated cells. All crumb structure parameters were calculated based on the entire cross-sectional area of the analysed cake slice.

2.6.6. Crust and crumb colour

Crust and crumb colour were measured using a Colorimeter CR-400 (Konica Minolta, Osaka, Japan) based on the CIE L* a* b* colour system. Crust colour was assessed by taking ten measurements across different areas on the top of each cake. Crumb colour was determined from five measurements per slice across four slices per cake. Colour differences compared to CEP were calculated using the Scofield equation:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad (7)$$

2.7. Statistical analysis

All analyses were performed in triplicate. Each batch of cakes consisted of two cakes, and baking was also conducted in triplicate. For data with a normal distribution, one-way analysis of variance (ANOVA) was carried out ($p \leq 0.05$), followed by Tukey's post hoc test using

OriginPro© 2025 (Version 10.2.0.196, Learning Edition; OriginLab Corporation, Northampton, MA, USA). When the data did not meet the assumptions of normality, a Kruskal–Wallis test was applied ($p < 0.05$). Regression and correlation analyses were conducted using Microsoft Excel 2021 (Microsoft Corporation, Redmond, WA, USA) to examine the relationships between the functional properties of the ingredient and the cake analysis results.

3. Results

3.1. Enzyme-treated FPI characterisation

3.1.1. Scanning electron microscopy (SEM)

Scanning electron micrographs of the different samples are shown in Fig. 1. The image of FPI shows the heterogeneous surface morphology typical of plant protein isolates. Visible particles appear as irregularly shaped aggregates and fragments, with some exhibiting a more compact and globular structure. In comparison, TGB is characterised by larger and more rounded aggregates. Similar features are observed in ACB, which presents an even more compact and fused structure, including gel-like areas. The application of transglutaminase in TGL resulted in irregular, roughly spherical to elongated shapes. These structures appear aggregated into clusters, with a collapsed and deflated morphology. A higher concentration of transglutaminase in TGM produced a more fused and interconnected structure, showing clear evidence of aggregation. As a result, a dense, aggregated network with smooth, folded surfaces and visually reduced porosity was formed. In TGH, an even more pronounced aggregation is visible, with protein clusters forming large, fused masses and indistinct boundaries between individual particles. Additionally, numerous pore-like openings and hollow, tunnel-like features suggest significant internal crosslinking within the protein matrix. In contrast, the lowest concentration of Alcalase (ACL) produced highly irregular, rough, and rugged protein particles. Numerous protrusions and cracks are present across the surfaces. The particles remain relatively large and compact, with partial disintegration at the edges. In ACM, the field is densely populated with small, roughly spherical to irregular protein particles, along with numerous flat, angular, and plate-like fragments. Similarly, ACH displays small, porous-like and irregular particles with rough, eroded surfaces. Fine particles and residual flakes coexist, and the particles appear smaller and more open, indicating an overall shift to smaller peptide fragments.

3.1.2. Electrophoresis

SDS-PAGE profiles of CEP, as well as FPI and its enzymatically treated derivatives, are presented in Fig. 2. The CEP lane displays a characteristic multi-band pattern, with major bands corresponding to ovalbumin (~45 kDa), ovotransferrin (~76 kDa), and other egg proteins. The untreated FPI sample exhibits bands at approximately 50 kDa and 37 kDa, corresponding to the major storage proteins, along with additional bands in the 20–25 kDa range, indicating the presence of various subunits. TGB shows an identical pattern to FPI. With increasing concentrations of transglutaminase, a progressive reduction in the intensity of bands in the 37–50 kDa region is observed, while the lower molecular weight bands (<25 kDa) remain relatively unchanged. Increasing Alcalase concentrations result in a marked reduction or complete disappearance of bands above 25 kDa, accompanied by the appearance of intense bands in the 10–15 kDa region. At the highest Alcalase concentrations (ACM, ACH), high molecular weight bands are almost entirely absent, and the gel is dominated by low molecular weight peptides. Additionally, progressive smearing is clearly visible.

3.1.3. Degree of hydrolysis

No degree of hydrolysis was detectable for FPI, the blanks (TGB, ACB), or the samples treated with transglutaminase. In contrast, samples treated with Alcalase showed significantly different values depending on

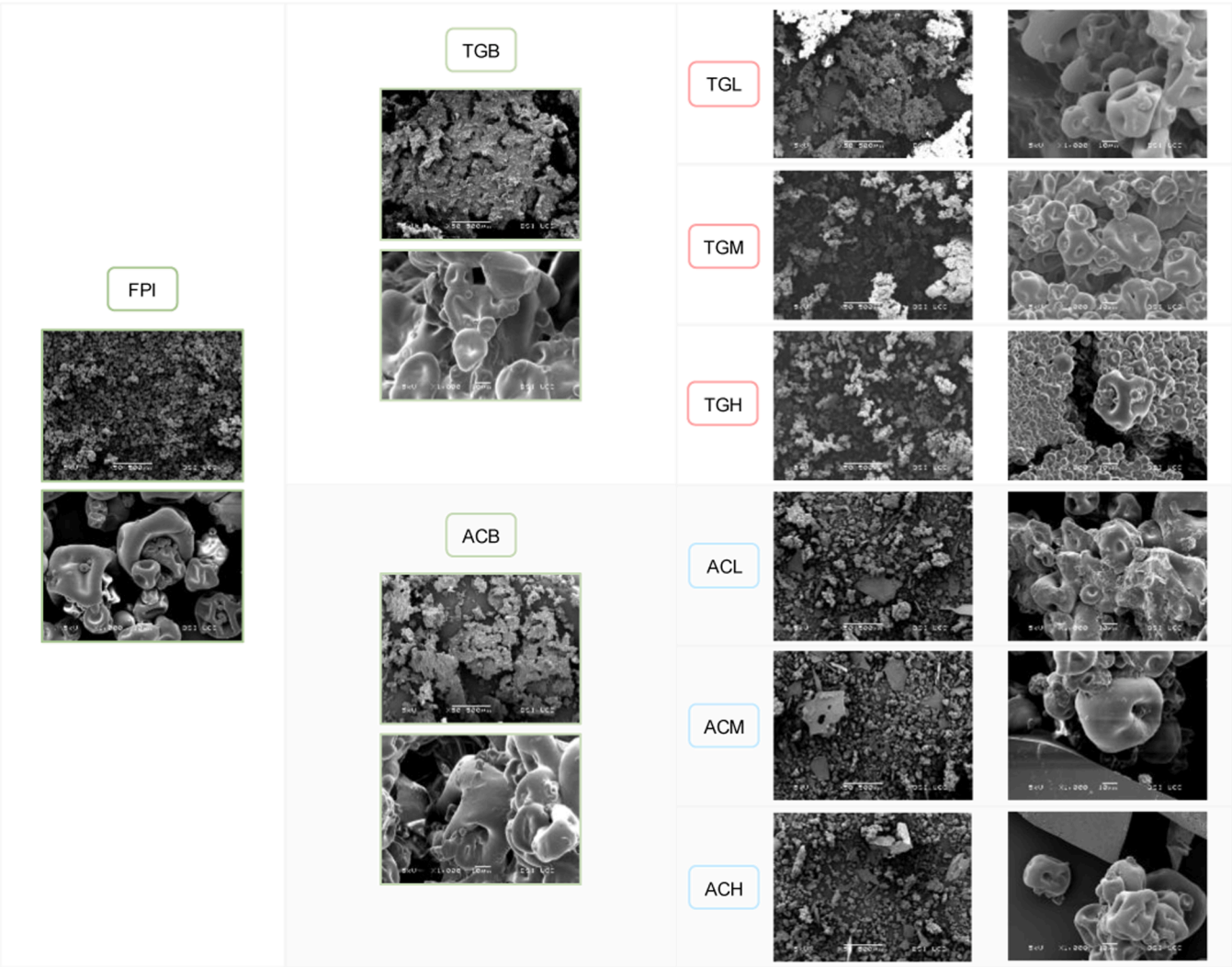


Fig. 1. SEM micrographs of faba bean protein isolate (FPI) and enzyme-treated FPI; magnifications of x50 and x1000. TG = Transglutaminase treatment, AC = Alcalase treatment, B = Blank, L = Low concentration, M = Medium concentration, H = High concentration.

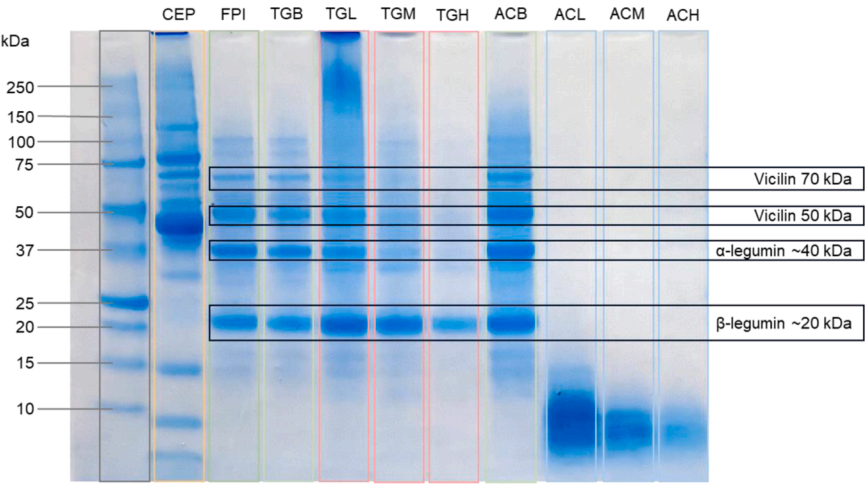


Fig. 2. SDS-PAGE patterns of the whole egg powder (CEP), native (FPI), blank (TGB, ACB) and enzyme-treated faba bean protein isolates. TG = Transglutaminase treatment, AC = Alcalase treatment, B = Blank, L = Low concentration, M = Medium concentration, H = High concentration. Bands in the FPI lane are annotated to indicate the positions of vicilin (~50–70 kDa), α-legumin (~40 kDa), and β-legumin (~20 kDa), as previously reported by [Nivala et al. \(2017\)](#).

the enzyme concentration. ACL exhibited the lowest degree of hydrolysis at $2.97 \pm 0.17\%$, followed by ACM at $6.74 \pm 0.14\%$, and

ACH at $9.00 \pm 0.18\%$. These results indicate that a higher concentration of Alcalase resulted in a higher degree of hydrolysis.

3.1.4. Free amino acids analysis

The free amino acid composition of FPI and its enzymatically treated derivatives is presented in supplementary (Table 1-S). CEP served as the reference and exhibited the highest overall amino acid levels. Transglutaminase treatments resulted in reduced free amino acid concentrations across all levels, with no significant differences between the low (TGL), medium (TGM), and high (TGH) enzyme doses. In contrast, Alcalase treatments led to more pronounced reductions, particularly at higher concentrations (ACM, ACH), where most amino acids reached their lowest levels. Exceptions included histidine (2.54 ± 0.26 mg/100 g in ACL), methionine (5.79 ± 0.74 mg/100 g in ACL), serine (1.84 ± 0.10 mg/100 g in ACH), threonine (3.62 ± 0.04 mg/100 g in ACL), and tryptophan (2.08 ± 0.05 mg/100 g in ACH), which increased in Alcalase-treated samples compared to FPI (His: 1.90 ± 0.40 ; Met: n.d.; Ser: n.d.; Thr: 1.10 ± 0.19 ; Trp: 1.61 ± 0.02 mg/100 g).

3.2. Techno-functional properties of enzyme-treated FPI

3.2.1. Foam capacity and stability

FPI showed low foam capacity ($24.99 \pm 2.22\%$) with moderate foam stability ($62.24 \pm 7.79\%$) (Table 2). Similar values were observed for CEP ($26.14 \pm 5.06\%$; $74.92 \pm 4.50\%$). TG treatment did not significantly modify the foaming properties of FPI, all TG-treated samples (TGB, TGL, TGM, TGH) exhibited comparably low foam capacities (19.05 – 23.06%) while maintaining relatively high foam stability (68.15 – 75.91%). In contrast, AC hydrolysis strongly enhanced foam capacity in a degree-dependent manner. The low-hydrolysis sample (ACL) reached more than fourfold higher capacity than FPI ($108.84 \pm 5.89\%$), with a gradual decline at intermediate (ACM: $92.01 \pm 9.19\%$) and high hydrolysis (ACH: $72.12 \pm 0.75\%$). However, this improvement came at the expense of foam stability, AC-treated samples showed sharp reductions compared to FPI, with ACL displaying the lowest stability ($10.73 \pm 2.83\%$), and only partial recovery at higher hydrolysis levels (ACM: $24.34 \pm 4.29\%$; ACH: $27.35 \pm 1.47\%$).

3.2.2. Protein solubility

The protein solubility at pH 7 of the protein ingredients is shown in Table 2. FPI displayed very low solubility at pH 7 ($8.24 \pm 0.55\%$). Similar values were observed for the process blanks, with TGB ($9.59 \pm 0.44\%$) and ACB ($7.43 \pm 0.39\%$) confirming that processing alone did not improve solubility. In fact, TG treatment further reduced solubility, with TGL, TGM and TGH showing the lowest values among all samples ($5.48 \pm 0.60\%$, $4.34 \pm 0.09\%$, and $3.93 \pm 0.01\%$, respectively). In contrast, AC hydrolysis markedly enhanced solubility. ACL reached $42.61 \pm 0.46\%$, while further hydrolysis led to even greater improvements, with ACM and ACH showing the highest solubility across the FPI-derived samples ($57.55 \pm 0.54\%$ and $61.58 \pm 0.55\%$, respectively). These values approached the solubility of CEP ($71.44 \pm 2.33\%$), which served as the benchmark.

3.2.3. Emulsifying characteristics

Emulsion stability, as measured by separation rate, varied significantly across samples (Table 2). The highest separation rates, indicating poorest stability, were observed in ACM (4.09 ± 0.80 %/min) and ACH (4.04 ± 0.04 %/min). These were closely followed by TGL (3.27 ± 0.09 %/min) and TGM (3.21 ± 0.04 %/min). Intermediate separation rates occurred in TGH (2.47 ± 0.10 %/min), ACB (2.67 ± 0.04 %/min), TGB (2.39 ± 0.04 %/min), and FPI (1.96 ± 0.04 %/min). The most stable emulsions were ACL (1.75 ± 0.21 %/min) and CEP (0.17 ± 0.03 %/min). ACL and FPI (1.96 ± 0.04 %/min) did not differ significantly from each other, whereas both showed significantly lower separation rates than TGL, TGM, ACM, and ACH.

While the LUMiSizer separation rate provides a robust macroscopic measure of emulsion stability, we acknowledge that particle size and size distribution (e.g., by laser diffraction) would provide complementary information on droplet formation and coalescence. This represents a limitation of the current study and should be addressed in future work.

3.2.4. pH and total titratable acids (TTA)

The pH and titratable acids (TTA) values are presented in Table 2. The pH values ranged from 5.74 ± 0.03 (TGH) to 8.68 ± 0.01 (CEP), with CEP exhibiting a significantly higher pH compared to all other samples. The remaining samples had pH values grouped between 5.93 ± 0.00 (ACL) and 6.29 ± 0.02 (ACB), except for ACM (5.78 ± 0.01) and

Table 2
Techno-functional properties.

	Foam capacity [%]	Foam stability [%]	Protein solubility [%] [pH 7]	Separation rate [%/min]	pH	TTA [mL 0.1 M NaOH/10 g]	Water-holding capacity [%]	Oil-holding capacity [%]
CEP	26.14 ± 5.06^d	74.92 ± 4.50^a	71.44 ± 2.33^a	0.17 ± 0.03 g	8.68 ± 0.01^a	n.d.	162.48 ± 6.17^f	82.07 ± 0.60 g
FPI	24.99 ± 2.22^d	62.24 ± 7.79^a	8.24 ± 0.55^e	$1.96 \pm 0.04^{e,f}$	6.28 ± 0.01^a	29.90 ± 0.10^b	$280.06 \pm 2.14^{c,d}$	74.50 ± 2.73 g
TGB	23.06 ± 1.87^d	75.91 ± 3.72^a	9.59 ± 0.44^e	$2.39 \pm 0.04^{e,f}$	6.13 ± 0.01^a	35.23 ± 0.35^d	342.68 ± 12.11^a	250.03 ± 3.02^a
TGL	21.53 ± 1.20^d	72.73 ± 6.36^a	$5.48 \pm 0.60^{f,g}$	$3.27 \pm 0.09^{b,c}$	6.12 ± 0.01^a	35.50 ± 0.50^d	300.70 ± 1.22^b	224.44 ± 1.89^b
TGM	20.03 ± 3.37^d	75.91 ± 3.72^a	4.34 ± 0.09 g	$3.21 \pm 0.04^{c,d}$	6.13 ± 0.00^a	34.33 ± 0.15^e	$293.30 \pm 3.55^{b,c}$	179.27 ± 3.07^d
TGH	19.05 ± 1.18^d	68.15 ± 8.98^a	3.93 ± 0.01 g	$2.47 \pm 0.10^{d,e,f}$	6.16 ± 0.01^a	33.20 ± 0.10^f	270.79 ± 4.08^d	148.18 ± 1.82^e
ACB	32.36 ± 7.40^d	66.89 ± 6.06^a	$7.43 \pm 0.39^{e,f}$	$2.67 \pm 0.04^{c,d,e}$	6.29 ± 0.02^a	32.03 ± 0.15 g	359.05 ± 4.70^a	212.90 ± 3.87^c
ACL	108.84 ± 5.89^a	10.73 ± 2.83^c	42.61 ± 0.46^d	1.75 ± 0.21^f	5.93 ± 0.00^a	56.40 ± 0.13^c	184.88 ± 5.00^e	133.13 ± 2.00^f
ACM	92.01 ± 9.19^b	$24.34 \pm 4.29^{b,c}$	57.55 ± 0.54^c	4.09 ± 0.80^a	5.78 ± 0.01^b	78.92 ± 0.19^b	162.74 ± 7.15^f	136.70 ± 3.33^f
ACH	72.12 ± 0.75^c	27.35 ± 1.47^b	61.58 ± 0.55^b	$4.04 \pm 0.04^{a,b}$	5.74 ± 0.03^b	90.55 ± 0.44^a	149.79 ± 6.67^f	128.67 ± 5.67^f

CEP = whole egg powder; FPI = faba bean protein isolate; TG = Transglutaminase treatment, AC = Alcalase treatment, B = Blank, L = Low concentration, M = Medium concentration, H = High concentration.

Values are presented as the average $\pm$ standard deviation. No significant difference occurred between values in the same column with the same lowercase letter ($p < 0.05$).

ACH (5.74 ± 0.03), which were statistically distinct from the rest. Titratable acidity, expressed as mL of 0.1 M NaOH per 10 g, also varied among the samples. ACH showed the highest TTA (90.55 ± 0.44), followed by ACM (78.92 ± 0.19) and ACL (56.40 ± 0.13). To compare, FPI had the lowest TTA (29.90 ± 0.10), with TGB, TGL, TGM, TGH and ACB displaying intermediate values ranging from 32.03 ± 0.15 (ACB) to 35.50 ± 0.50 (TGL). TTA for CEP was not determined.

3.2.5. Water- and oil-holding capacity

As shown in Table 2, FPI displayed moderate water-holding capacity (WHC: $280.06 \pm 2.14\%$) but very low oil-holding capacity (OHC: $74.50 \pm 2.73\%$). CEP showed an even lower value for WHC ($162.48 \pm 6.17\%$) and a value of $82.07 \pm 0.60\%$ for OHC. Processing without enzyme addition already had a marked impact. TGB exhibited significantly enhanced WHC ($342.68 \pm 12.11\%$) and the highest OHC across all samples ($250.03 \pm 3.02\%$). Similarly, ACB showed the strongest improvement in WHC ($359.05 \pm 4.70\%$) and a moderate increase in OHC ($212.90 \pm 3.87\%$). When enzyme was applied, divergent effects were observed. TG treatment maintained the improvements seen in the blank, with TGL and TGM showing consistently high WHC ($300.70 \pm 1.22\%$ and $293.30 \pm 3.55\%$) and OHC ($224.44 \pm 1.89\%$ and $179.27 \pm 3.07\%$). TGH, however, produced only a modest increase (WHC: $270.79 \pm 4.08\%$; OHC: $148.18 \pm 1.82\%$). In contrast, AC hydrolysis reduced both properties compared to ACB. Hydrolysed samples (ACL, ACM, ACH) all showed significantly lower WHC (184.88 – 149.79%) and OHC (133.13 – 128.67%) than both FPI and ACB, with the decline becoming more pronounced at higher degrees of hydrolysis.

3.2.6. Gelling properties

The thermal gelation behaviour of the samples, as depicted in Fig. 3, was assessed by monitoring the evolution of storage modulus (G') during controlled heating and cooling cycle. FPI exhibited limited gelation capacity, with storage modulus (G') reaching only 1707 Pa at the end of the heating-cooling cycle. CEP served as a benchmark, showing strong gelation with the highest final modulus (9637 Pa). Processing without enzyme addition already influenced the gelation behaviour, TGB and ACB both demonstrated moderate gel formation, with maximum G' values between 3223 and 4037 Pa, reflecting a clear effect of the processing conditions alone. When enzymes were applied, distinct trends emerged. TG treatment improved gelation compared to FPI but with outcomes depending on enzyme level. TGL displayed a pronounced

increase in G' (6693 Pa), whereas TGM and TGH showed only weak gelation, with maximum values of 956 Pa and 511 Pa, respectively. In contrast, AC hydrolysis almost completely disrupted gelation. The hydrolysed samples (ACL, ACM, ACH) all exhibited extremely low storage moduli throughout the temperature cycle, with final values below 3 Pa (ACL: ~ 3 Pa, ACH: <1.5 Pa, ACM: <0.5 Pa). Loss modulus (G'') showed analogous treatment-dependent trends with lower absolute values, and the resulting $\tan \delta$ values were highly variable between replicates, so only G' is reported for clarity.

3.3. Cake analysis

3.3.1. Bake loss

The bake loss during baking is listed in Table 3, ranging from $9.21 \pm 0.37\%$ (CEP) to $12.41 \pm 0.18\%$ (ACH). FPI ($10.61 \pm 0.27\%$) and TGB ($10.79 \pm 0.38\%$) showed moderately low values, followed by intermediate bake losses observed in TGL ($10.82 \pm 0.25\%$), TGM ($10.93 \pm 0.35\%$), TGH ($11.34 \pm 0.49\%$) and ACB ($11.02 \pm 0.65\%$), all being characterised by the same statistical significance. The highest bake losses occurred in ACL ($11.65 \pm 0.22\%$), ACM ($11.99 \pm 0.40\%$) and ACH ($12.41 \pm 0.18\%$), with ACH showing significantly greater moisture loss than all other samples.

3.3.2. Specific volume

Table 3 and Fig. 4 present the analysed data for the specific volume. The FPI cake showed a moderate specific volume (1.73 ± 0.04 mL/g), which was considerably lower than the CEP cake (2.33 ± 0.03 mL/g). TGB (1.66 ± 0.03 mL/g) and TGL (1.65 ± 0.04 mL/g) demonstrated slightly reduced volumes compared to FPI, while ACB (1.68 ± 0.03 mL/g) showed no significant difference to FPI. TG treatment had a variable effect on cake volume. While TGM (1.73 ± 0.02 mL/g) remained similar to FPI, TGH produced the highest value among the FPI-derived samples (1.80 ± 0.02 mL/g). In contrast, AC hydrolysis negatively affected cake volume. The low-hydrolysis sample (ACL: 1.74 ± 0.03 mL/g) was comparable to FPI, but further hydrolysis led to significantly reduced volumes, with ACM (1.56 ± 0.05 mL/g) and ACH (1.57 ± 0.03 mL/g) showing the lowest values across all faba-based cakes.

3.3.3. Textural analysis

The results of the textural analysis are displayed in Table 3. CEP produced cakes with the highest hardness (18.01 ± 1.02 N), springiness

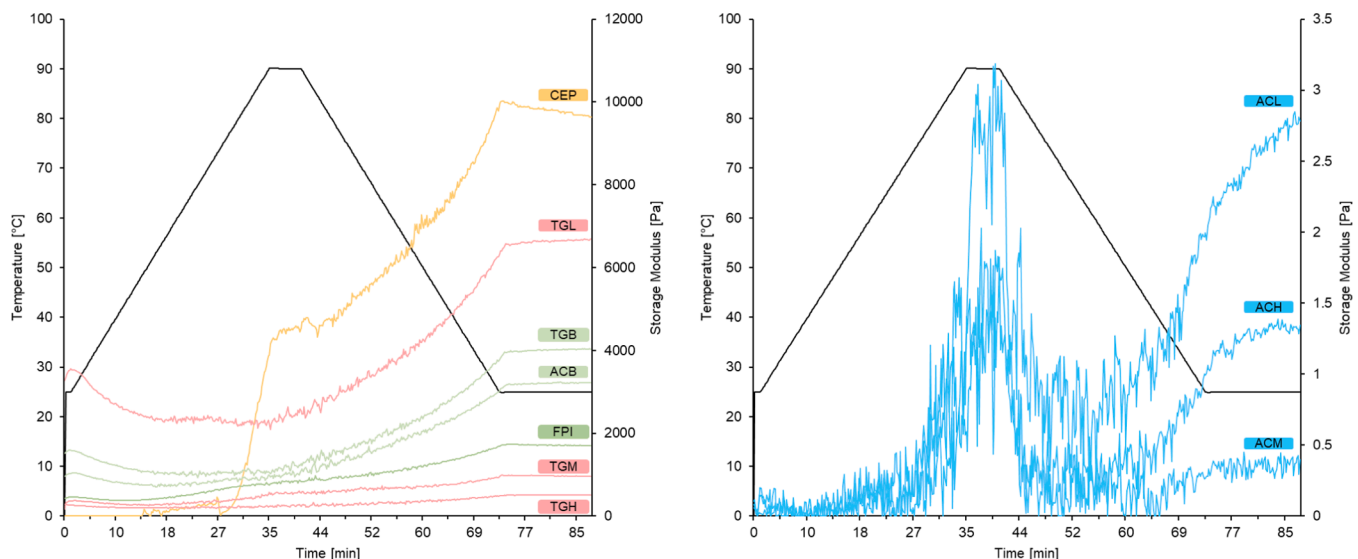


Fig. 3. Storage Modulus obtained from small deformation oscillatory measurements during temperature ramp of the whole egg powder (CEP), native (FPI), blank (TGB, ACB) and enzyme-treated faba bean protein isolates. TG = Transglutaminase treatment, AC = Alcalase treatment, B = Blank, L = Low concentration, M = Medium concentration, H = High concentration.

Table 3

Physical properties and texture of the pound cakes.

Cake	Physical Properties		Textural properties					
	Specific Volume [mL/g]	Bake Loss [%]	Hardness [N]	Springiness [-]	Resilience [-]	Cohesiveness [-]	Chewiness [J]	Adhesiveness [J]
CEP	2.33 ± 0.03 ^a	9.21 ± 0.37 ^f	18.01 ± 1.02 ^a	0.94 ± 0.00 ^a	0.33 ± 0.01 ^a	0.72 ± 0.01 ^a	12.20 ± 0.74 ^a	-0.002 ± 0.003 ^a
FPI	1.73 ± 0.04 ^c	10.61 ± 0.27 ^e	12.16 ± 0.82 ^d	0.81 ± 0.03 ^{b,c}	0.20 ± 0.01 ^d	0.52 ± 0.02 ^{d,e,f}	5.10 ± 0.36 ^{e,f}	-0.025 ± 0.012 ^b
TGB	1.66 ± 0.03 ^d	10.79 ± 0.38 ^{d,e}	13.75 ± 0.92 ^c	0.80 ± 0.03 ^b	0.20 ± 0.01 ^d	0.52 ± 0.02 ^f	5.66 ± 0.38 ^{c,d,e}	-0.028 ± 0.014 ^b
TGL	1.65 ± 0.04 ^d	10.82 ± 0.25 ^{d,e}	14.41 ± 0.99 ^c	0.80 ± 0.03 ^b	0.20 ± 0.01 ^{c,d}	0.52 ± 0.01 ^{d,e,f}	6.02 ± 0.49 ^c	-0.033 ± 0.014 ^b
TGM	1.73 ± 0.02 ^c	10.93 ± 0.35 ^{c,d,e}	13.41 ± 0.81 ^c	0.80 ± 0.03 ^b	0.21 ± 0.01 ^b	0.54 ± 0.01 ^{c,d}	5.73 ± 0.37 ^{c,d,e}	-0.029 ± 0.013 ^b
TGH	1.80 ± 0.02 ^b	11.34 ± 0.49 ^{b,c,d}	12.15 ± 0.78 ^d	0.81 ± 0.02 ^b	0.21 ± 0.01 ^{b,c}	0.53 ± 0.01 ^{d,e}	5.25 ± 0.37 ^{d,e,f}	-0.022 ± 0.010 ^b
ACB	1.68 ± 0.03 ^{c,d}	11.02 ± 0.65 ^{c,d,e}	14.07 ± 1.22 ^c	0.80 ± 0.02 ^{b,c}	0.20 ± 0.01 ^d	0.52 ± 0.01 ^{e,f}	5.84 ± 0.52 ^{c,d}	-0.026 ± 0.014 ^b
ACL	1.74 ± 0.03 ^c	11.65 ± 0.22 ^{b,c}	10.16 ± 0.52 ^e	0.81 ± 0.02 ^{b,c}	0.22 ± 0.01 ^b	0.55 ± 0.01 ^{b,c}	4.58 ± 0.29 ^f	-0.033 ± 0.015 ^b
ACM	1.56 ± 0.05 ^e	11.99 ± 0.40 ^{a,b}	16.46 ± 2.34 ^b	0.76 ± 0.20 ^c	0.21 ± 0.02 ^b	0.56 ± 0.04 ^b	6.97 ± 1.99 ^b	-0.087 ± 0.039 ^c
ACH	1.57 ± 0.03 ^e	12.41 ± 0.18 ^a	12.20 ± 1.05 ^d	0.84 ± 0.01 ^{a,b}	0.20 ± 0.01 ^d	0.54 ± 0.03 ^{c,d}	5.55 ± 0.65 ^{c,d,e}	-0.076 ± 0.029 ^c

CEP = whole egg powder; FPI = faba bean protein isolate; TG = Transglutaminase treatment, AC = Alcalase treatment, B = Blank, L = Low concentration, M = Medium concentration, H = High concentration.

Values are presented as the average ± standard deviation. No significant difference occurred between values in the same column with the same lowercase letter ($p < 0.05$).

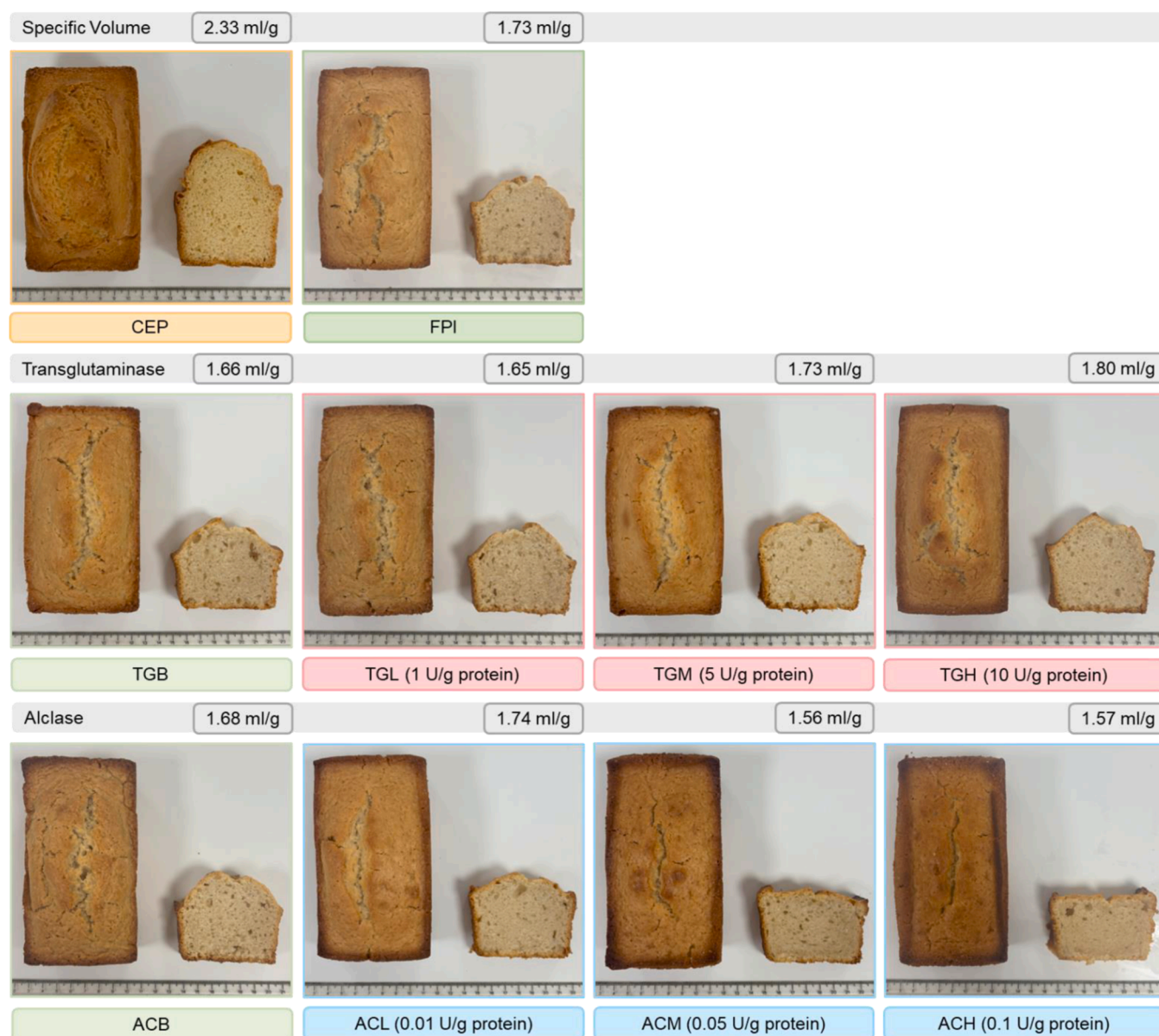


Fig. 4. Appearance and average specific volume of the pound cakes containing whole egg powder (CEP), native (FPI), blank (TGB, ACB) and enzyme-treated faba bean protein isolates. TG = Transglutaminase treatment, AC = Alcalase treatment, B = Blank, L = Low concentration, M = Medium concentration, H = High concentration.

(0.94 ± 0.00), resilience (0.33 ± 0.01), cohesiveness (0.72 ± 0.01) and chewiness (12.20 ± 0.74 J), forming a distinct statistical group across all parameters. ACM cakes showed the second-highest hardness (16.46 ± 2.34 N), though with notably higher variability in springiness (0.76 ± 0.20). TGL, TGB, TGM and ACB cakes exhibited intermediate textural properties, with hardness values ranging from 13.41 ± 0.81 (TGM) to 14.41 ± 0.99 N (TGL) and chewiness from 5.73 ± 0.37 (TGM) to 6.02 ± 0.42 (TGL). The ACL cake was among the softest (10.16 ± 0.52 N), while FPI (12.16 ± 0.82), TGH (12.15 ± 0.78) and ACH (12.20 ± 1.05) showed similar soft textures. Adhesiveness was lowest in ACM (-0.087 ± 0.039 J) and ACH (-0.076 ± 0.029 J). Springiness was highest in CEP (0.94 ± 0.00) and ACH (0.84 ± 0.01), while resilience was consistently low across all samples except CEP.

3.3.4. Crumb structure

In Table 4, the analysis of the cake crumb structure is displayed. The CEP cake showed the highest cell density (3170 ± 224) and the highest number of holes (2.55 ± 1.07), together with the smallest mean cell diameter (1.91 ± 0.14 mm) and high cell elongation (1.49 ± 0.01), indicating a fine and highly aerated crumb. All faba-based cakes had significantly lower cell numbers than CEP, with TG-treated samples (TGB, TGL, TGH) showing particularly low hole counts (0.43 – 0.67) and larger cell diameters (up to 2.72 ± 0.30 mm), consistent with a coarser, more closed crumb. AC-treated cakes spanned a wider range: ACM and ACH exhibited higher hole counts (1.70 ± 0.94 and 2.14 ± 1.13 , respectively) and elongation values comparable to CEP (1.49 – 1.50), whereas ACL remained closer to FPI in both cell number and hole count. Overall, the FPI, TG- and AC-treated cakes showed intermediate cell densities and elongation values, combined with larger cell diameters than CEP, reflecting crumb structures that were less fine and open than the egg-based reference.

3.3.5. Crust and crumb colour

To compare the crust and crumb colour of the different cakes, the ΔE values were calculated, considering the L^* , a^* and b^* values. The calculated values are displayed in Table 4. The higher the ΔE value, the

higher the difference compared to the control (CEP) cake. For crust colour, ACM (3.98 ± 0.61) and ACH (3.89 ± 0.84) the highest ΔE values analysed, indicating the most pronounced deviation from CEP. Other samples showed moderate crust colour differences ($\Delta E=1.37$ (TGH)– 3.29 (TGB)), with no significant differences between them. Crumb colour differences were more substantial. ACM (11.65 ± 1.52) and ACH (11.72 ± 0.04) showed again the highest ΔE values, significantly exceeding all other samples. The remaining samples exhibited lower but notable crumb colour differences, with FPI (6.42 ± 2.67), ACL (6.42 ± 2.67) and TGH (6.78 ± 0.46) being the lowest and ACB (8.44 ± 0.19) showing the greatest deviation in this group.

4. Discussion

While faba bean protein demonstrates potential as an innovative egg replacement in baking (Halm et al., 2025), its utilisation requires further optimisation. This study investigates whether enzymatic processing can enhance FPI functionality for baking applications. Transglutaminase (TG) was selected to promote protein crosslinking for improved texture, and Alcalase (AC), an endo-protease, to hydrolyse proteins for enhanced solubility. Both enzymes were applied at three treatment levels to assess their impact on FPI. The resulting enzyme-treated FPI were characterised, their techno-functional properties analysed, and their performance evaluated in a pound cake model system.

The impact of processing on FPI is illustrated in the SEM micrographs. Both enzyme treatments involved heat application in aqueous systems: 37°C for TG and 50°C for AC. In contrast to the smooth, rounded morphology typical of spray-dried protein powders (Vogelsang-O'Dwyer et al., 2020), the heat-treated blanks exhibit a gel-like, fused aggregate structure driven by increased protein-protein interactions, as observed in heated pea protein isolate (Asen and Aluko, 2022). TG treatment progressively intensified this aggregation in a concentration-dependent manner, consistent with the formation of covalent ϵ -(γ -glutamyl)lysine isopeptide bonds, as reported for mung bean and soy proteins (Cui et al., 2020; Vijayan et al., 2024). In contrast, AC treatment produced increasingly fragmented, smaller, and rougher particles with increasing concentration, reflecting the progressive proteolytic breakdown by this serine endopeptidase, which preferentially cleaves adjacent to hydrophobic residues (Doucet et al., 2003; Tacias-Pascacio et al., 2020).

The SDS-PAGE results confirm structural changes in the FPI. In the untreated FPI, four major bands are visible at approximately 21, 37, 50, and 70 kDa, corresponding to the α - and β -subunits of legumin-like 11S globulins and vicilin, respectively (Wright and Boulter, 1974, 1972). A similar band pattern is observed in TGB. In contrast, ACB displays smudged bands, likely due to partial denaturation and aggregation at 50°C , with the SDS concentration insufficient to fully solubilise the proteins (Wallace et al., 2015). TGL exhibits a blurred accumulation around 250 kDa, which may indicate protein crosslinking (Nivala et al., 2021). Increasing the concentration of TG also results in decreased intensity of the α -legumin and vicilin bands, suggesting these proteins preferentially crosslink to form higher oligomers, whereas the β -legumin band remains largely unchanged. This observation aligns with the proposed folding of legumin proteins, where hydrophilic α -legumins are surface-exposed and hydrophobic β -legumins are buried, making the former more susceptible to crosslinking. Additionally, vicilins are noted for their higher solubility (Nivala et al., 2017). The introduction of AC visibly reduced all the typical bands observed in FPI. In ACL, faint bands around 15 kDa are noticeable, whereas ACM and ACH exhibit further hydrolysis, showing only blurred bands below 10 kDa. Liu et al. (2019a) reported comparable results, indicating that the enzyme effectively hydrolysed FPI. The progressive disappearance of high molecular weight bands in TG-treated samples, reflecting increased crosslinking and polymerisation, is consistent with the observed reduction in protein solubility and the impaired emulsification stability of these samples, as larger aggregated protein species have reduced surface activity at oil-water

Table 4
Appearance and colour of the pound cakes.

	Cake Appearance				Colour	
	Number of cells	Number of holes	Cell diameter [mm]	Cell elongation	ΔE (crust)	ΔE (crumb)
CEP	3170 ± 224^a	2.55 ± 1.07^a	1.91 ± 0.14^e	1.49 ± 0.01^a	0.00 ± 0.00^b	0.00 ± 0.00^c
FPI	$2047 \pm 190^{b,c,d,e}$	$0.91 \pm 0.67^{b,c}$	$2.47 \pm 0.23^{c,d}$	$1.45 \pm 0.01^{b,c,d}$	$2.63 \pm 1.39^{a,b}$	6.24 ± 0.54^b
TGB	1919 ± 113^e	0.54 ± 0.66^c	$2.68 \pm 0.20^{a,b}$	$1.44 \pm 0.02^{c,d}$	3.29 ± 1.09^a	8.17 ± 0.69^b
TGL	1918 ± 113^e	0.43 ± 0.62^c	2.72 ± 0.30^a	1.44 ± 0.02^d	$2.81 \pm 1.71^{a,b}$	8.14 ± 0.30^b
TGM	$2139 \pm 181^{b,c}$	$0.93 \pm 0.69^{b,c}$	2.36 ± 0.21^d	1.45 ± 0.02^b	$1.96 \pm 1.22^{a,b}$	7.20 ± 0.37^b
TGH	2163 ± 197^b	0.67 ± 0.85^c	$2.41 \pm 0.29^{c,d}$	$1.45 \pm 0.02^{b,c}$	$1.37 \pm 0.49^{a,b}$	6.78 ± 0.46^b
ACB	$1951 \pm 136^{d,e}$	$0.31 \pm 0.45^{c,d}$	$2.60 \pm 0.22^{a,b,c}$	1.44 ± 0.02^d	$1.45 \pm 1.23^{a,b}$	8.44 ± 0.19^b
ACL	$1999 \pm 152^{c,d,e}$	$0.86 \pm 0.76^{b,c}$	$2.51 \pm 0.23^{b,c,d}$	1.46 ± 0.02^b	$2.27 \pm 0.60^{a,b}$	6.42 ± 2.67^b
ACM	$2062 \pm 135^{b,c,d,e}$	$1.70 \pm 0.94^{a,b}$	1.94 ± 0.18^e	1.50 ± 0.02^a	3.89 ± 0.84^a	11.65 ± 1.52^a
ACH	$2089 \pm 119^{b,c,d}$	$2.14 \pm 1.13^{a,b}$	2.04 ± 0.13^e	1.49 ± 0.01^a	3.98 ± 0.61^a	11.72 ± 0.04^a

CEP = whole egg powder; FPI = faba bean protein isolate; TG = Transglutaminase treatment, AC = Alcalase treatment, B = Blank, L = Low concentration, M = Medium concentration, H = High concentration. Values are presented as the average $\pm$ standard deviation. No significant difference occurred between values in the same column with the same lowercase letter ($p < 0.05$).

interfaces. Conversely, the shift towards low molecular weight peptides below 10 kDa in ACM and ACH aligns with the marked improvements in solubility observed for these samples, while the near-complete loss of intact protein structure explains the disruption of gelation capacity.

Another parameter used to describe the influence of enzymes on FPI is the degree of hydrolysis (DH). As expected, untreated blank samples and those treated with transglutaminase showed no detectable DH, since TG catalyses protein crosslinking rather than peptide bond cleavage (Zhang et al., 2009). Liu et al. (2019a) used alcalase (0.01 U/g protein) on FPI to enhance the physical and oxidative stability of oil-in-water emulsions. AC was added to an aqueous dispersion of 5% (w/v) FPI, which was treated at 50 °C; 0.2 M NaOH was specifically added to achieve DH values of 4%, 9%, and 15%, respectively. The results correspond to ACL ACM, and ACH in this study, as the hydrolysis was conducted under comparable conditions, with only the AC concentration varied and without pH monitoring. Another study used pepsin, trypsin, flavourzyme, and neutrase on faba bean protein to achieve DH values ranging from 2% to 16% (Eckert et al., 2019). Comparable DH-dependant behaviour has been reported for other legume proteins, for soy (9%) and chickpea (13%) isolates (Dent et al., 2023). AC typically generates low to moderate DH values that increase solubility and interfacial activity, whereas higher DH or prolonged hydrolysis can reduce thermal stability, promote aggregation and impair gelation, and similar trends have been observed for limited AC hydrolysis of pea protein in gel systems (Sawant et al., 2024). This indicated that the DH range for AC-treated FPI in this study (3–9%) falls within the broader window where legume protein hydrolysates often show improved solubility and interfacial properties, while compromising gelation. Lower DH observed for AC in FPI compared to other proteases may be attributed to substrate specificity. AC preferentially cleaves peptide bonds on the carboxyl side of hydrophobic amino acids, which may be restricted by the compact globular structure of legumin and vicilin in faba bean protein (Liu et al., 2019a; Samaei et al., 2020).

Furthermore, the reductions in free amino acids observed in the TG-treated samples highlight the pronounced crosslinking effect, consistent with findings in black bean protein isolate and soy protein (Hu et al., 2015; San et al., 2025). While AC treatment typically leads to a general increase in free amino acids due to extensive hydrolysis, as supported by SEM, SDS-PAGE, and DH results, the release of individual amino acids depends on their position within the protein structure and the substrate specificity of the enzyme (Vogelsang-O'Dwyer et al., 2023). Accordingly, the exceptions observed for histidine, methionine, serine, threonine, and tryptophan in the AC-treated samples are likely due to a combination of enzyme specificity, the structural context of these residues, and their resistance to further enzymatic degradation. The apparent reduction or disappearance of certain free amino acids (e. g., leucine, valine, tyrosine, isoleucine) in ACM and ACH samples is counterintuitive given the progressive hydrolysis detected by the OPA method. However, it is important to note that the AccQ-Tag HPLC method used here specifically quantifies free, unconjugated amino acids, and does not detect amino acids incorporated into peptide fragments. Alcalase preferentially cleaves on the carboxyl side of hydrophobic residues, and at higher degrees of hydrolysis, extended breakdown of hydrophobic domains results in these residues being predominantly present as internal residues of short peptides rather than as free amino acids (Liu et al., 2019a; Tacias-Pascacio et al., 2020). Consequently, the OPA-based DH, which measures all newly generated free amino groups including peptide N-termini, continues to increase, while the HPLC-detected pool of specific free amino acids declines.

The structural modifications of FPI may enhance its techno-functional properties and increase its potential to mimic the ideal characteristics of egg proteins (Halm et al., 2025, 2024). Protein solubility is a critical parameter influencing the functionality of protein ingredients (Vogelsang-O'Dwyer et al., 2020). Statistical analysis revealed a positive correlation between the degree of hydrolysis (DH) and protein solubility at pH 7 (R-value: 0.87; $p < 0.05$). Notably, the introduction of

AC significantly improved protein solubility, particularly in the substrate treated with 1.0 U/g protein AC, approaching the solubility observed for CEP. This observation aligns with previous studies, which attribute increased solubility to the formation of smaller peptides with reduced molecular size, thereby enhancing the capacity to form hydrogen bonds with water (Liu et al., 2019a, 2010). Conversely, the application of TG led to an overall reduction in solubility. For example, Nivala et al. (2017) reported a decrease in FPI solubility from 83% to 60% at a TG concentration of 60 U/g. While TG can promote deamidation under optimal conditions, resulting in a lower isoelectric point and increased solubility (Renzetti et al., 2008), the balance between deamidation and transamidation depends on the availability of lysine side chains and reaction conditions. In our system, the strong decrease in solubility across TGL-TGH, together with the SEM and rheological evidence for extensive network formation, indicates that ϵ -(γ -glutamyl)-lysine crosslinking and aggregation dominated over any deamidation-driven increase in net charge (Schmid et al., 2011; Zhang et al., 2009). Additionally, in the presence of salts, decreased electrostatic repulsion and increased hydrophobicity may favour aggregation and insolubility (Gaspar and de Góes-Favoni, 2015).

The results indicate that water-holding capacity (WHC) is negatively correlated with protein solubility (R-value: -0.91 ; $p < 0.05$). The higher WHC observed for TGB and ACB can be attributed to heat and moisture treatment followed by freeze-drying. During processing, protein denaturation and aggregation occur, exposing polar sites and hydrophilic groups that enhance water interaction. Additionally, freeze-drying may preserve an open, porous microstructure that facilitates water absorption, though this latter explanation is based on qualitative SEM observations and should be regarded as indicative rather than conclusive (Álvarez-Castillo et al., 2021; Bühler et al., 2020). The addition of TG reduces the WHC of TGB as its concentration increases. Although the enzyme can enhance water-holding at optimal doses, higher levels result in excessive crosslinking, creating a dense and less porous protein network, as seen in SEM micrographs, which cannot retain as much water (Min et al., 2025). In contrast, Vijayan et al. (2024) reported a threefold increase in WHC of mung bean protein isolate following TG treatment at 2 IU/g protein, attributed to a stiffer, denser gel network capable of trapping water more efficiently. A significant decrease in WHC is observed with the application of AC, yielding values lower than native FPI and replicating the benchmark results of CEP for ACM and ACH. After hydrolysis with AC, the apparent reduction in polar side chain availability likely reflects several mechanisms. Firstly, Alcalase preferentially cleaves adjacent to hydrophobic amino acids, which progressively exposes and then removes hydrophilic residues from accessible positions as peptide fragments. Secondly, the short peptides generated at high DH may adopt compact conformations or self-associate, burying polar side chains. Thirdly, the free amino group and carboxyl terminus introduced by each cleavage event form a zwitterionic pair that partially neutralises the contribution to net polarity. Collectively, these effects reduce the density of freely accessible polar groups available to interact with water, lowering WHC (Xu et al., 2021). This is consistent with Islam et al. (2022), who reported that Alcalase hydrolysis of soy protein resulted in smaller molecular weight peptides that negatively affected WHC, attributing the decline to reduced capacity of short peptides to retain water. Oil-holding capacity (OHC) is positively correlated with water-holding capacity (WHC), exhibiting similar trends across the tested samples (R-value: 0.70 ; $p < 0.05$). Notably, freeze-drying exerts a pronounced effect on OHC, as processed samples, including the blanks and enzyme-treated samples display higher OHC values than the native FPI, reaching levels comparable to CEP. This observation aligns with findings by Keivaninahr et al. (2021), who reported that faba bean protein isolate and egg white protein have comparable OHC values. Multiple studies confirm that freeze-dried proteins consistently exhibit significantly higher OHC than their spray-dried or native counterparts (Allameh et al., 2024; Dong et al., 2024; Wang et al., 2022). Although enzymatic hydrolysis with AC

typically reduces OHC by disrupting the protein network, the structural modifications induced by freeze-drying can partially offset this reduction, resulting in processed samples with superior OHC compared to native proteins.

In addition to WHC, gelling properties determine the functionality of protein ingredients. Samples treated with TG exhibited similar improvements in gelling properties as observed for their WHC. Applying TG to pea protein resulted in increased gel strength at higher enzyme concentrations. This increase in gel strength was associated with improved water retention, as a strong gel matrix more effectively encapsulates water (Li et al., 2024). However, excessive crosslinking can hinder gel formation, indicating that there is an optimal level of crosslinking for achieving desirable gel strength and water retention. This optimal level is potentially achieved in TGL. In line with this, G' profiles mirrored the behaviour of G'' and confirmed that TG-treated samples remained predominantly elastic. In contrast, AC hydrolysis almost completely disrupted gelation, with ACL, ACM and ACH exhibiting very low G' values. When considered together with the moderate DH values achieved here (2.97–9.00%), these findings are consistent with the DH-dependent loss of gelation reported for other AC-hydrolysed legume proteins (Dent et al., 2023; Islam et al., 2022; Sawant et al., 2024).

The techno-functional properties closely related to WHC are foam capacity (R-value: -0.68 ; $p < 0.05$) and foam stability (R-value: 0.68 ; $p < 0.05$). The effect of TG on foaming is minimal, as FPI already exhibits values comparable to CEP. Notably, an intermediate enzyme concentration of TG (TGM) is beneficial for foam stabilisation, matching the foam stability observed in CEP. Thus, an optimal TG dosage may strengthen the protein network and improve its ability to trap and retain air, similar to the function of egg proteins in traditional foams (Kim and Chin, 2024; Liu et al., 2019b; Nivala et al., 2021, 2017). Enhanced protein solubility significantly increased the foam capacity of samples treated with AC. Eckert et al. (2019) reported increased foam capacity following pepsin treatment, attributing this to peptide-peptide interactions that facilitate rapid migration to the air-water interface and formation of an extensive intermolecular network. Conversely, higher AC concentrations reduced foam capacity, indicating that an optimised degree of hydrolysis is necessary to prevent excessive peptide fragmentation and loss of foaming ability (Di Filippo et al., 2024). The higher foam stability in ACM and ACH compared to ACL may be due to the dense packing of small peptides, which form films resistant to collapse and coalescence (Horax et al., 2017).

A low separation rate indicates good emulsifying behaviour, which is crucial for producing high-quality food products such as pound cake (Halm et al., 2025). A recent study comparing fourteen plant-based egg replacers in a pound cake model reported separation rates in the range of approximately 0.5–2.1%/min for oat- and legume-based ingredients, indicating that plant proteins generally form less stable emulsions than whole egg (Halm et al., 2026). None of the analysed samples in the present study were able to replicate the superior emulsification qualities of CEP. Among the FPI-derived samples, ACL showed the numerically lowest separation rate ($1.75 \pm 0.21\%/min$); however, this did not differ significantly from untreated FPI ($1.96 \pm 0.04\%/min$), indicating that low-concentration Alcalase treatment did not meaningfully improve emulsification stability under the conditions tested. To the best of our knowledge, directly comparable LUMiSizer data for commercial egg replacers are not yet available, so a quantitative benchmark against market products was beyond the scope of this work. Additionally, previous studies have shown that a low degree of hydrolysis (DH, $\sim 4\%$) produces flexible peptides with increased hydrophobicity, which anchor to the oil-water interface and improve emulsifying properties. In contrast, extensive hydrolysis has the opposite effect, negatively impacting emulsification (Can Karaca et al., 2015; Liu et al., 2019a, 2010; Schröder et al., 2017). The reduced emulsification stability observed in TG-treated samples contrasts partially with findings by Liu et al. (2019b), who reported that TG treatment for 60 min maintained the emulsifying activity of FPI. However, their study also demonstrated

that prolonged TG treatment (120 and 240 min) decreased emulsifying activity due to excessive surface hydrophobicity, consistent with the pattern observed here. The higher protein concentration used in the present study (13%w/v vs 3% w/v) likely promoted more extensive crosslinking and aggregation, shifting the balance towards impaired interfacial activity even at lower TG doses.

A thorough understanding of how techno-functional properties influence the final product is essential for selecting effective egg substitutes. While enzyme treatment can enhance certain properties, the overall quality of the pound cake depends on the combined interactions of all techno-functional attributes. Accordingly, bake loss determined for the samples is potentially related to the analysed gelling properties (R-value: -0.85 ; $p < 0.05$). The complex protein structures present in FPI, TGB, ACB, and TGL provide stronger gelation, which improves water retention and restricts water evaporation during baking, as supported by previous findings (Salehi, 2020). In contrast, dense protein structures (TGM, TGH) or excessively hydrolysed proteins (ACL, ACM, ACH) are unable to gel effectively and thus fail to retain water. The specific volume is significantly higher in the CEP cake, which is expected, as the enzymatic modifications were unable to replicate the unique characteristics of CEP (Halm et al., 2025). Alp and Bilgiçli (2008) reported an improvement in the volume index of cakes with increasing TG concentration, which is consistent with the results of the cakes in the current study. Additionally, correlation analysis provided insights into a key parameter: emulsifying properties appear to have a significant effect on the specific volume of the pound cakes (R-value: -0.87 ; $p < 0.05$). Lower emulsion separation rates in TGH and ACL, compared to other concentrations, could have contributed to increased volumes. Other studies have also reported the positive influence of enhanced emulsifying properties on cake volume (Halm et al., 2025; Lin et al., 2017; Paraskevopoulou et al., 2015). From a mechanistic perspective, improved emulsifying stability promotes the formation of finely dispersed oil droplets and facilitates the incorporation of air during batter mixing, consistent with the observed correlation between emulsifying properties and specific volume. Proteins with enhanced interfacial activity can rapidly adsorb at oil-water and air-water interfaces, forming viscoelastic films that stabilise both fat droplets and entrapped air bubbles. This stabilisation reduces coalescence and disproportionation, allowing air cells to persist and expand during baking, ultimately contributing to increased cake volume and a finer crumb structure. In contrast, poor emulsifying properties can lead to phase separation and unstable air cells, which collapse during baking, thereby limiting volume development (Khalilian Movahhed et al., 2016; Rahmati and Mazaheri Tehrani, 2014). It should be acknowledged that batter rheology was not measured in this study. Batter viscosity and air incorporation during mixing are key determinants of cake quality, and their assessment would provide valuable mechanistic insight into the observed differences in specific volume and crumb structure between samples. This represents a limitation of the current study and should be addressed in future work.

The texture of cakes is a key determinant of consumer acceptance. In this study, exceptionally high values were observed for all texture descriptors, except for adhesiveness, in the CEP cake. A specialised recipe, optimised for plant proteins and with reduced fat and sugar levels compared to a standard pound cake recipe, was employed to facilitate the incorporation of the plant-based egg replacement. In a previous study, CEP was incorporated into a standard pound cake formulation, resulting in lower values for texture parameters that are considered more representative of consumer-preferred texture (Halm et al., 2024). A hardness value of 7.99 ± 0.47 N was determined in the current study, whereas a formulation with reduced CEP inclusion (based on 7.5% protein), as described by Halm et al. (2025), yielded a higher hardness of 9.31 ± 0.79 N. Comparable values were observed in TGH and ACL, suggesting that their distinct structural and techno-functional modifications positively influenced hardness. Chewiness and hardness are interrelated indicators of cake texture (Agrahar-Murugkar et al., 2016;

Hedayati and Mazaheri Tehrani, 2018). In contrast to the high chewiness observed in the CEP cake (12.20 ± 0.74 J), optimal values reported in previous studies ranged from 4.55 ± 0.30 J to 5.58 ± 0.45 J (Halm et al., 2025, 2024). TGH and ACL demonstrated chewiness values similar to those reported in earlier studies. Correlation analysis revealed a significant relationship between chewiness and the gelling behaviour of the samples (R-value: 0.75; $p < 0.05$), suggesting that the ability of legume proteins to form a stable gel influences the textural properties of cake (Akbin et al., 2025; Zhang et al., 2024). The texture parameters springiness, resilience, cohesiveness, and adhesiveness were not improved by the introduction of enzymes into the cake formulation, although these parameters are linked to the gelling and emulsifying properties of proteins. These characteristics reflect the material's ability to withstand compression and indicate the formation of a stable, interconnected protein matrix (Paraskevopoulou et al., 2015). Regarding these parameters, the applied enzymatic modifications appear limited in their ability to recreate the unique network-forming properties of egg proteins in cake systems (Liu et al., 2019a; Ouraji et al., 2020). Murray-Nalbandian et al. (2026) similarly reported that faba bean protein produced significantly lower resilience (31.81 ± 2.22) and cohesiveness (0.67 ± 0.01) compared to egg white protein (48.28 ± 0.80 and 0.80 ± 0.02 , respectively) in a high-ratio cake system, confirming that these textural parameters are particularly resistant to improvement through protein substitution alone.

The crumb structure and colour of a cake are crucial determinants of a consumer's initial perception. Regarding the appearance of the cakes, increasing the concentration of TG improved the perceived quality of the pound cake, while increasing the AC concentration had the opposite effect. Image analysis, compared to the CEP cake, revealed similarities among ACM, ACH, and CEP in terms of number of holes, cell diameter, and cell elongation. Correlation analysis attributed these similarities to the protein solubility of the ingredients. Although AC cakes may score well on objective crumb structure metrics, their overall appearance is negatively affected by changes in colour, surface texture, and subtle uniformity issues that are not captured by standard image analysis. These factors combine to create a less appealing product, despite a comparable internal cell structure to egg-based cakes. This highlights the importance of considering both quantitative and qualitative assessments when evaluating food quality. Colour measurements provide a more accurate representation of the cakes' actual appearance. Colour analysis revealed significant differences in both crust and crumb colour among the control cakes (FPI, TGB, ACB). However, TGH and ACL treatments were able to reduce these differences relative to CEP, thereby slightly improving cake quality. Crust and crumb colour were found to correlate with pH (Crust: R-value: -0.78 , $p < 0.05$; Crumb: R-value: -0.88 , $p < 0.05$) and total titratable acidity (TTA) (Crust: R-value: 0.83, $p < 0.05$; Crumb: R-value: 0.86, $p < 0.05$) of the samples. No significant differences in pH were observed among the TG samples. Structural changes induced by TG reduced the number of free amino acids, making acidic groups less accessible for titration (Beirami-Serizkani et al., 2021). In a previous study, faba bean concentrate with a higher pH and a lower TTA, resulting from heat and moisture treatment, improved the crumb and crust colour of pound cakes (Halm et al., 2025). For AC treatment, higher concentrations caused a significant decrease in pH and an increase in TTA. Accordingly, the colour should improve at higher concentrations; however, the opposite was observed. AC treatment releases acidic groups, lowering pH and simultaneously increasing TTA (Liu et al., 2019a). The released free amino groups (particularly lysine) are highly reactive in the Maillard reaction, accelerating the formation of melanoidins during baking and resulting in a darker colour (Oh et al., 2013). Melanoidin formation was not quantified in this study (e.g. by measuring absorbance at 420 nm), so this explanation should be regarded as a plausible mechanism rather than direct evidence. Future work should therefore include spectrophotometric assessment of melanoidins to confirm the contribution of Maillard-derived pigments to the darker crust and crumb colour of AC-treated cakes.

5. Conclusion

This study is the first to systematically investigate the effects of two mechanistically distinct enzymatic strategies, transglutaminase (TG)-induced crosslinking and Alcalase (AC)-mediated hydrolysis, at multiple enzyme concentrations on the techno-functional properties of faba bean protein isolate (FPI), and to directly link these modifications to pound cake quality. By bridging the gap between ingredient characterisation and baking application, this work provides a multi-parameter framework for evaluating enzymatic modification of plant proteins as egg replacers, an approach not previously reported for FPI in a pound cake system.

TG treatment promoted concentration-dependent protein crosslinking, yielding denser protein networks with improved gelation and enhanced water-holding capacity, but at the cost of substantially reduced solubility. At the optimal TG concentration, gelation was maximised, reflecting the formation of an extensive covalent isopeptide bond network. In the cake system, the highest TG concentration delivered the best overall performance, achieving the highest specific volume and improved crust and crumb colour among TG-treated samples, the latter attributed to reduced free amino acid availability and lower titratable acidity. However, springiness, cohesiveness, and resilience remained significantly below the egg-based control across all TG concentrations, confirming that crosslinking alone cannot replicate the unique thermo-setting and network-forming behaviour of egg proteins in a complex baking matrix.

AC treatment generated smaller peptides with markedly improved solubility and foam capacity in a degree-of-hydrolysis-dependent manner, demonstrating that targeted hydrolysis can selectively enhance interfacial properties of FPI. However, gelation was almost entirely disrupted even at the lowest AC concentration, and water- and oil-holding capacities declined progressively with increasing hydrolysis. In the cake system, the lowest AC concentration represented the most favourable balance, producing crumb structure metrics comparable in some respects to the egg-based control, though colour darkening driven by accelerated Maillard reactions and reduced emulsifying stability at higher doses limited the overall product quality.

In both enzymatic strategies, incomplete emulsifying stability and heat-induced gelation emerged as the primary functional limitations, translating into suboptimal batter aeration and gas retention, lower specific volume, and reduced crumb elasticity compared with the egg-based control. These findings highlight a fundamental challenge in plant protein egg replacement: enzymatic modification that improves one functional property frequently compromises another, underscoring the necessity of multi-parameter optimisation rather than single-property targeting.

From a practical standpoint, the highest TG concentration and the lowest AC concentration represent the most promising candidates for further development as egg replacers in pound cake. Future work should prioritise the following directions: firstly, combined TG and AC treatments, applied sequentially or simultaneously, to exploit the complementary nature of crosslinking and hydrolysis in simultaneously improving gelation, solubility, and interfacial activity; secondly, batter rheology measurements to better understand the relationship between ingredient functionality, batter aeration, and gas retention during baking; thirdly, spectrophotometric quantification of Maillard-derived melanoidins to confirm and address the colour limitations observed in AC-treated cakes; and fourthly, sensory evaluation to assess off-flavour and mouthfeel, which are critical determinants of consumer acceptance for plant-based egg alternatives but were beyond the scope of the present study. Enzyme-treated FPI holds significant promise as a sustainable, allergen-friendly egg alternative, but achieving broad functional and sensory equivalence with egg proteins in bakery systems will require holistic, application-driven optimisation strategies.

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Ethical statement - studies in humans and animals

Animals and humans were not involved in this study.

CRedit authorship contribution statement

Juliane Halm: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation. **Laura Nyhan:** Writing – review & editing. **Emanuele Zannini:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Elke K. Arendt:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, 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.

Supplementary materials

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Data availability

Data will be made available on request.

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