



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Does a plant protein substitution strategy endow hybrid plant protein/surimi gels with nutritional quality surpassing pure meat?

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ABSTRACT: Although the inferior protein bioavailability of purely plant-based meat alternatives relative to animal-derived meats is well-established, the digestion and absorption characteristics of hybrid animal-plant protein products formulated through partial substitution remain poorly characterized. To address this, the hybrid surimi/plant protein (HSP) gels crosslinked by transglutaminase (TGase) with uniform gel strength were prepared by modulating TG dosage (wheat: 18 U/100 g surimi; soybean and rice: 9 U/100 g; pea: 6 U/100 g). *In vitro* gastrointestinal digestion revealed no significant differences ($P > 0.05$) in protein or dry matter digestibility (61.50%–69.15%) across groups. However, the legume-based composites (soybean and pea) exhibited significantly higher digestion rate constants ($k = 0.029 \text{ min}^{-1}$) than cereal-based composites ($k = 0.024 \text{ min}^{-1}$), alongside reduced half-digestion times ($t_{0.5} = 163.3 \text{ min}$ vs. 170.9 min), indicating enhanced digestibility. Particle size distribution analysis showed a significant reduction ($P < 0.05$) in pea protein gel particle size after pepsin digestion, whereas wheat protein gels retained larger residual particles ($D_{50} = 27.48 \text{ }\mu\text{m}$) post-pancreatin digestion, suggesting greater hydrolytic resistance. Electrophoresis confirmed digestion products were predominantly $<20 \text{ kDa}$ fragments. Notably, pea protein digests exhibited potent antioxidant activity, strongly correlated with six high-abundance antioxidant peptides identified by mass spectrometry: TLEDQLSEIK, VLNAGVIEGQF, DAGDGVTHNPVYEG, VGLLHSQNTSL, LASLYPAVVEDTTK, and LLVVPQNF. Collectively, partial plant protein substitution conferred favorable protein digestibility and nutrient acquisition potential to hybrid surimi gels, with pea protein emerging as particularly valuable for enhancing nutritional functionality in meat-reduced formulations.

Keywords: hybrid protein products; digestion kinetics; antioxidant peptides; meat reduction; plant protein

1. Introduction

Surimi products are valued for their distinctive elastic texture, fine microstructure, and high-protein/low-fat nutritional profile. As a significant segment of the global aquatic processing industry, they are widely utilized in quick-frozen and ready-to-eat foods (Z. Wang, Chen, Hu, Liu, Li, Guo, et al.,

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Received 22 July 2025

Received in revised form 21 August 2025

Accepted 9 October 2025

2025). However, population growth and resource constraints have intensified concerns regarding protein supply security. Transitioning the surimi industry toward sustainable production is therefore recognized as a critical strategy to address this challenge (Gordon, Choi, Amanipour, Hu, Nikkhah, Koysuren, et al., 2025). Recent efforts focus on partially replacing myofibrillar protein with plant proteins to develop meat-reduced surimi gels as a viable and sustainable approach (St. Pierre, Darwin, Adil, Aviles, Date, Dunne, et al., 2024). Previous work by our group demonstrated that while plant protein substitution maintains amino acid balance, it often induces texture deterioration (T. Ye, Chen, Lu, Zhao, Chen, Lin, et al., 2024). Preliminary studies indicated that transglutaminase (TGase)-catalyzed cross-linking effectively mitigates these structural defects, enabling final products to achieve commercially viable textural and sensory properties (unpublished data).

Concurrently, plant-based meat alternatives (PBMA) have become a prominent research focus in food science (Zhan, Guo, Podgorski, Zeng, Xu, Peng, et al., 2025). Successful development requires not only replication of meat's sensory attributes (appearance, texture, flavor) but also alignment with key nutritional characteristics, including amino acid composition, digestibility, and bioavailability, to meet consumer health demands (Du, Tu, Liu, Ding, Zeng, & Pan, 2023). The nutritional value of food proteins fundamentally depends on their *in vivo* digestibility and absorption efficiency, beyond amino acid composition alone (Sridhar, Saïd, Thomas, Denis, & Lechevalier, 2023; Zhou, Amer, Sultani, Nasr, Wang, Corradini, et al., 2023). Thus, thorough characterization of amino acid profiles, digestive dynamics, and protein bioavailability is essential for accurate nutritional evaluation of PBMA (Ishaq, Irfan, Sameen, & Khalid, 2022).

Protein digestibility is governed by multiple factors, including its structural conformation, aggregation morphology, matrix binding, and anti-nutritional factors (McClements, 2023). Processing-induced denaturation and cross-linking significantly alter digestibility and bioactive peptide release (Lee, Jo, Choi, & Jung, 2024). Jang and Lee (2024) reported that PBMA typically employ techniques like high-moisture extrusion, shear-cell technology, or 3D printing to mimic meat texture; combining plant proteins with process optimization can moderately improve protein bioavailability. Yang, Zheng, Ma, Zhang, Sun, and Fang (2023) compared the commercial soy/pea-based PBMA with animal meats (beef, chicken, pork), revealing significantly higher protein digestibility in animal meats (64.7%-75.3%) versus PBMA (58.6%-63.2%), with enhanced release of bioactive peptides post-digestion. Ishaq, Irfan, Sameen, and Khalid (2022) noted that plant proteins digest faster in the stomach but break down slower in the small intestine. A conclusion further validated by Xie, Cai, Zhao, Liu, Xu, Zhou, et al. (2022) using SDS-PAGE and LC-MS/MS analysis, which showed more thorough degradation of animal proteins and greater release of peptides/functional peptides post-digestion. Additionally, Sánchez-Terrón, Martínez, Freire, Molina-Infante, and Estévez (2024) found in animal studies that rats fed a beef-based diet exhibited significantly higher protein digestibility (98.12%) than those fed gluten (92.03%) or tofu (92.72%) ($P < 0.01$), further evidencing the relatively lower digestibility of plant-derived proteins and potential impacts on

nutrient absorption. Collectively, these studies indicate reduced nutritional availability of PBMA proteins relative to animal-derived counterparts. For hybrid gels prepared by partial plant protein substitution (surimi/plant protein blends), key questions remain: What are their digestive properties (e.g., *in vitro* gastrointestinal degradation behavior, digestion product composition, and bioactivity)? Can they achieve levels comparable to pure surimi? Research on digestion/absorption of hybrid animal-plant protein gels remains scarce; systematic studies on *in vitro* digestibility characteristics, including degradation kinetics and product profiles, of plant protein/surimi hybrid gels prepared via enzymatic cross-linking modulation, are notably lacking, with comparative data insufficient.

Therefore, this study prepared hybrid surimi/plant protein (HSP) gels with uniform gel strength via TGase-mediated cross-linking, using partial substitution of surimi with soy, pea, wheat, or rice proteins at optimized TGase levels. An *in vitro* gastrointestinal digestion model systematically compared pure surimi gel with HSP gels regarding: protein and dry matter digestibility, protein degradation kinetics (digestion rate constant, half-digestion time), particle size evolution during digestion, and the bioactivity of digestion products. Electrophoresis and mass spectrometry analyses further elucidated polypeptide composition and identified bioactive peptides. Ultimately, this work screens advantageous plant proteins for hybrid gels based on nutritional digestibility and provides a reference for developing sustainable processing technologies for meat gel products.

2. Materials and methods

2.1 Materials

Silver carp surimi (production date: November 2023) was sourced from Honghu Jingli Aquatic Food Co., Ltd. (Hubei, China). Upon arrival, the frozen surimi was transported via cold-chain logistics, portioned into uniform units, vacuum-sealed, and stored at -18 °C until further processing. Microbial TGase (1850 U/g activity; Anhui Bomei Biotechnology Co., Ltd.) was stored in dry, airtight containers at -80 °C to maintain activity prior to application. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,4,6-Tris(1,3,5-triazin-2-yl)-1,3,5-triazine (TPTZ) were purchased from Solarbio Science & Technology Co., Ltd. Ferric chloride, copper sulfate, and sodium potassium tartrate tetrahydrate were obtained from Hongxi Biotechnology Co., Ltd. (analytical grade). N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycylglycine (FAPGG), sodium hydroxide, Tris(hydroxymethyl)aminomethane, and bovine serum albumin (BSA) were acquired from the same supplier (analytical grade). Glycine, glacial acetic acid, angiotensin-converting enzyme (ACE), absolute ethanol, ninhydrin hydrate, and hydrochloric acid were sourced from Sangon Biotech (Shanghai) Co., Ltd. (all analytical grade).

2.2 Preparation of the plant protein

Four plant proteins were prepared according to the previous report (T. Ye, Chen, Li, Chen, Lin, Zheng, et al., 2024). Specifically, wheat flour was mixed with water at a flour-to-water ratio of 2:1 (w/w) to form

dough. The dough was then washed with five times its weight of water to remove starch. The resulting wet gluten was dried at 75 °C for 6 h and subsequently milled into a powder, then wheat protein with protein content of 80.01% was obtained. Soy protein (91.90% purity) was extracted from defatted soy flour. Defatted soy flour was dispersed in a sodium hydroxide solution (pH 7.1) at a solid-to-liquid ratio of 1:10 (w/w) and stirred at 45 °C for 50 min. The mixture was centrifuged to collect the supernatant, whose pH was adjusted to 4.6 using acid to precipitate the protein. The precipitated protein was recovered, neutralized to pH 7.0, sterilized, and finally spray-dried. Soaked broken rice kernels were ground into a slurry, which was then treated with amylase (0.1% w/w, 10,000 U/g) for 2 h under continuous stirring. The mixture was filtered, and the residue was resuspended in water and subjected to enzymatic hydrolysis using cellulase (0.2% w/w, 20,000 U/g) and α -amylase (0.1% w/w, 10,000 U/g). After filtration, the solid residue was dried and milled to obtain rice protein. The process yielded rice protein that had a protein content of 83.30%. Pea protein was obtained based on the following protocol. Peas were soaked in deionized water for 24 h, then wet-ground to produce a starch-protein slurry. The pH of the slurry was adjusted to 4.5 to precipitate the protein, which was separated by centrifugation. The recovered protein was neutralized to pH 7.0 and spray-dried. The final material obtained was pea protein and its protein content was measured at 90.41%.

2.3 Preparation of HSP-TG gels

Frozen surimi blocks were thawed at 4 °C for 16 h and cut into small pieces. The thawed surimi (150 g) was homogenized and mixed with one of four plant proteins, wheat protein (12.46 g), soy protein (10.84 g), rice protein (11.96 g), or pea protein (11.02 g), to achieve a surimi protein substitution level of 30%. The moisture content of the mixture was adjusted to $(77.36 \pm 0.89)\%$ with ice water, and the total protein content was maintained at 15.5%.

Based on preliminary experiments indicating differential TGase responsiveness among plant protein/surimi composites, where legume proteins showed higher sensitivity than cereal proteins and pea protein induced the strongest activation, TGase was added at doses of 18, 9, 9, and 6 U per 100 g surimi to the wheat protein, soybean protein, rice protein, and pea protein groups, respectively, to catalyze the cross-linking reaction with the aim of preparing mixed gel products with equivalent gel strength. Subsequently, 2.5% (w/w) NaCl was incorporated, and the mixture was chopped in a mincer (Model LZ223A, Midea Life Electric Manufacturing Co., Ltd., Foshan, Guangdong, China) for 0.5 min to form a homogeneous paste.

The paste was stuffed into 32 mm polyethylene casings and sealed tightly with a manual clip machine. Gelation was carried out using a two-step water bath heating process: 40 °C for 1 h followed by 90 °C for 30 min. After heating, the gels were cooled under running tap water for 10 min and stored at 4 °C until analysis. The resulting gels prepared with wheat, soybean, rice, and pea proteins were labeled as W, S, R, and P, respectively. A control gel (CG) without plant protein was also prepared.

2.4 Verification of HSP-TG gel strength

The gel strength was performed in accordance with the international standard ISO 23855:2021 (Standardization, 2021). The HSP-TG gels were equilibrated at room temperature for 0.5 h before being cut into cylindrical specimens measuring 20 mm in height and 3.15 cm in diameter. Textural properties were then analyzed using a texture analyzer (Model TA-XT Plus, Stable Micro System Co., London, U.K.) equipped with a P/0.5S spherical probe. The test parameters were as follows: pre-test speed 1 mm/s, test speed 1 mm/s, post-test speed 5 mm/s, compression distance 15 mm, and trigger force 5 g. The breaking force of the HSP-TG gel was defined as the force corresponding to the first peak in the puncture curve, and the breaking distance was the corresponding deformation distance. Gel strength (g·cm) was calculated as the product of breaking force (g) and breaking distance (cm). Each sample was tested in at least five replicates.

2.5 Simulated gastrointestinal digestion of HSP-TG gels

The simulated digestion procedure was adapted from the method described by Xie, et al. (2022), with minor modifications. The HSP-TG gels were sliced and homogenized using a disperser (Model: MQ5035, Braun Electric Co., Ltd., Shanghai, China) at 3000 r/min for 3 min to simulate oral processing, yielding a surimi gel bolus with a particle size of approximately 1 mm. For simulated gastric digestion, 10.0 g of the chewed-like HSP-TG gel sample was accurately weighed and mixed with 10 mL of simulated saliva (Feijing Biotechnology Co., Ltd.). The mixture was incubated in a shaking incubator at 37°C and 30 r/min for 2 min. Then, 20 mL of simulated gastric fluid (artificial gastric juice, pepsin activity 30 U/mL, Feijing Biotechnology Co., Ltd.) was added, and the pH was adjusted to 3.0 using 1 mol/L HCl. The sample was then incubated at 37°C and 30 r/min for 2 h to simulate gastric digestion, resulting in the gastric digestate. Simulated intestinal digestion: Following gastric digestion, the pH of the digesta was adjusted to 7.0 using 1 mol/L NaOH. Then, 40 mL of simulated intestinal fluid (artificial intestinal juice, trypsin activity 30 U/mL, Feijing Biotechnology Co., Ltd.) was added. The mixture was incubated at 37°C and 30 r/min for 2 h to simulate intestinal digestion, resulting in the final gastrointestinal digestate.

2.6 Protein and dry matter digestibility of HSP-TG gels

An accurate amount of HSP-TG gel was weighed and recorded as m_0 . After *in vitro* digestion, the resulting chyme was centrifuged at 7830 r/min for 10 min at 5°C. The supernatant and pellet were collected for subsequent analysis. The pellet was dried to a constant weight at 50°C in a drying oven (DHG-9245A, Shanghai Aozhen Instrument Manufacturing Co., Ltd.), and the final dry weight was recorded as m_1 . The protein contents of the original gel and the dried pellet were determined using the Kjeldahl method. Samples were digested with concentrated sulfuric acid in the presence of a catalyst (6 g K₂SO₄ and 0.12 g CuSO₄ per 100 mL H₂SO₄) at 420 °C for 60 min using a fully automated Kjeldahl analyzer (Kjeltec™ 9 fully automatic Kjeldahl analyzer, FOSS, Denmark). The digested sample was then distilled and titrated automatically with 0.1 M hydrochloric acid to determine the nitrogen content, with a nitrogen-to-protein conversion factor of 6.25. The protein digestibility of the surimi gel was calculated using Equation (1):

$$\text{Protein digestibility} = \frac{(m_0 p_0 - m_1 p_1)}{m_0 p_0} \times 100 \quad (1)$$

where m_0 is the mass of HSP-TG gel before digestion (g), m_1 is the mass of dried residue after digestion (g), p_0 is the protein content of the surimi gel before digestion (%), and p_1 is the protein content of the dried residue after digestion (%).

Meanwhile, the precipitates obtained after simulated gastric and gastrointestinal digestion of HSP-TG gels were transferred to petri dishes and dried to a constant weight in a 50°C oven. Additionally, an equal portion of undigested mixed gel samples was dried under the same conditions, and the final dry weight was accurately recorded. The dry matter digestibility was calculated using the following formula:

$$\text{Dry matter digestibility} = \left(1 - \frac{M_1}{M_0}\right) \times 100 \quad (2)$$

where M_1 is the dry matter weight of the HSP-TG gel after digestion (g), and M_0 is the dry matter weight of the undigested HSP-TG gel (g).

2.7 Free amino acid release kinetics of HSP-TG gels

The mixed surimi gel samples underwent simulated gastrointestinal digestion following the procedure outlined in Section 2.5. At predetermined time points (0, 24, 48, 72, 96, 120, 160, 200, and 240 min), 200 μ L of the digestion fluid was collected, centrifuged, and the supernatant was stored for analysis. The concentration of free amino acids was determined using a microplate reader (SpectraMax® iD5, Molecular Devices) based on the ninhydrin colorimetric method. A standard calibration curve was prepared using glycine: 0.1 g of glycine was dissolved and diluted to 100 mL, then further diluted to various concentrations. The absorbance was measured at 570 nm, and the standard curve was established as $y = 0.0417x$, $R^2 = 0.9929$. Acetate buffer solution was prepared by mixing 20 g sodium acetate, 60 mL distilled water, and 1 mL glacial acetic acid, and diluting to 100 mL. The ninhydrin color reagent was prepared by dissolving 1.5 g of hydrated ninhydrin in 25 mL ethylene glycol and diluting to 100 mL.

For each sample, distilled water, buffer solution, ninhydrin solution, and the sample supernatant were sequentially added. The mixtures were heated in a boiling water bath for 30 min, cooled, and transferred to a 96-well plate. Absorbance was measured at 570 nm. The concentration of free amino acids in the digestion fluid at each time point was used to analyze the digestion kinetics of the mixed surimi gel, thereby characterizing the dynamic release profile of amino acids during digestion. The kinetic model used to fit the simulated digestion process was the Logistic model:

$$C(t) = \frac{C_{\max} \cdot C_0}{C_0 + (C_{\max} - C_0) \exp(-kt)} \quad (3)$$

$$t_{0.5} = \frac{1}{k} \ln\left(\frac{C_{\max} - C_0}{C_0}\right) \quad (4)$$

$$r_{\max} \frac{dC(t)}{dt} = \frac{kC_{\max}}{4} \quad (5)$$

Where $C(t)$ is the concentration of free amino acids at digestion time t (g/100 g); C_0 is the initial concentration of free amino acids (at $t = 0$); $C_{\max}$ is the maximum (saturated) concentration of amino acids at the digestion endpoint; k is the digestion rate constant (min^{-1}); t is the digestion time (min); $t_{0.5}$ is the

half-saturation time (min); $r_{\max}$ is the maximum digestion rate at the inflection point, expressed as g/(100 g·min).

2.8 Particle size distribution of HSP-TG gel digestates

The particle size distribution of the precipitates obtained after gastric and gastrointestinal digestion of HSP-TG gels was measured using a laser particle size analyzer (DT-9300Z, Bettersize Instruments Ltd., Dandong, China). Water was used as the dispersion medium with a refractive index of 1.333. Under ultrasonic dispersion, an appropriate amount of sample (with a refractive index within the range of 10%–15%) was added to the sample cell. The material refractive index was set to 1.52. After thorough dispersion of the sample, the instrument was activated for automatic measurement, and the particle size distribution data were recorded.

2.9 Digestate protein profiling by SDS-PAGE

The HSP-TG gel samples (3.0 g) after gastric and gastrointestinal digestion were mixed with 27 mL of 5% SDS solution and homogenized at 4800 r/min for 2 min using a handheld homogenizer. The homogenate was then heated in an 85 °C water bath for 1 h. After extraction, the samples were centrifuged at $6850 \times g$ and 5°C for 20 min, and the supernatant was collected into centrifuge tubes. Protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) assay using a UV-1600 UV-Vis spectrophotometer (Shanghai Meipuda Instrument Co., Ltd.), and protein concentrations were adjusted to be uniform across samples. Next, 40 µL of each sample was mixed with 10 µL of reducing loading buffer (Cao, Pan, Zheng, Chen, Yin, Liu, et al., 2024) loaded onto the gel. Electrophoresis was performed using a DYY-6C electrophoresis system (Beijing Liuyi Biotechnology Co., Ltd.) with initial settings of 200 mA current and 80 V voltage. After sample equilibrium, the voltage was adjusted to 180 V. Electrophoresis was stopped when the sample front migrated to approximately 8 mm from the bottom of the gel.

2.10 Microscopic observation of HSP-TG gel digestates

A 0.2 g portion of the mixed gel digestion precipitate was stained in the dark for 10 min with 20 µL of fluorescein isothiocyanate (FITC, 0.1% in ethanol). After staining, 5.0 mL of deionized water was added to thoroughly mix the sample. Then, 500 µL of the mixture was transferred onto a microscope slide and dried/fixed in a 50°C oven for 20 min. The samples were observed using a laser scanning confocal microscope (FV3000, Olympus Corporation).

2.11 Determination of antioxidant activity and ACE inhibitory activity of HSP-TG gel digestates

2.11.1 FRAP assay

The FRAP working reagent was prepared fresh daily by combining 10 mmol/L TPTZ (in 40 mmol/L HCl), 20 mmol/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, and 0.1 mol/L acetate buffer (pH 3.6) in a 1:1:10 (v/v/v) ratio followed by thorough vortexing; subsequently, 0.1 mL of digested sample supernatant was mixed with 2.45 mL of this reagent, incubated in the dark at 37°C for 60 min, and absorbance at 593 nm was measured against a

deionized water blank, with results expressed as mg Trolox equivalents per gram sample (mg Trolox/g) using daily-prepared Trolox standard curves.

2.11.2 ABTS radical scavenging activity assay

The ABTS radical cation solution was prepared by mixing 6 mL of 7 mmol/L 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) solution with 176 μ L of 140 mmol/L potassium persulfate, followed by 15 h dark incubation. The resulting ABTS solution was diluted with anhydrous ethanol to achieve an absorbance of 0.700 at 734 nm. For the assay, 30 μ L of digest supernatant was combined with 270 μ L of deionized water, followed by addition of 3.7 mL ABTS working solution. The reaction mixture was incubated at room temperature in the dark for 6 min, and absorbance was measured at 734 nm with anhydrous ethanol as blank. Radical scavenging activity (%) was calculated using Equation (6):

$$\text{ABTS radical scavenging rate} = \frac{A_0 - A}{A_0} \times 100 \quad (6)$$

Where A_0 is the absorbance of the blank, and A is the absorbance of the sample.

2.11.3 DPPH radical scavenging activity assay

A 0.5 mmol/L DPPH stock solution was prepared by accurate weighing of 9.75 mg DPPH dissolved in anhydrous ethanol and brought to 50 mL final volume, and 0.2 mmol/L working solution was obtained by mixing 10 mL stock solution with 15 mL anhydrous ethanol (yielding final 0.2 mmol/L concentration). For assay, 0.6 mL digest supernatant was mixed with 1.4 mL deionized water, followed by addition of 2 mL DPPH working solution. The reaction system was incubated at 25°C in dark for 30 min with absorbance measured at 517 nm using anhydrous ethanol as blank. Radical scavenging activity (%) was calculated using Equation (7):

$$\text{DPPH radical scavenging rate} = \frac{A_0 - A}{A_0} \times 100 \quad (7)$$

Where A_0 is the absorbance of the blank, and A is the absorbance of the sample.

2.11.4 Determination of ACE inhibitory activity

ACE inhibitory activity was measured using a microplate reader with N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as the substrate. A 0.2 mol/L Tris-HCl buffer solution (pH 8.3) and a 0.4 mg/mL FAPGG substrate solution were prepared. In the assay, 10 μ L of ACE enzyme solution (0.1 U/mL) was added to each well, followed by 10 μ L of HSP-TG gel gastric or intestinal digestion sample. For the blank control wells, 10 μ L of buffer was added in place of the sample. Subsequently, 150 μ L of FAPGG substrate solution was added to each well. The initial absorbance was measured immediately at 340 nm (0 min, recorded as A_1 and A_{01}). The absorbance was measured again after 30 min (recorded as A_2 and A_{02}). The ACE inhibitory activity was evaluated by comparing the change in absorbance of the gastric and gastrointestinal digestion samples before and after 30 min.

2.12 Determination of total polyphenol and flavonoid contents in HSP-TG gel digestates

Total polyphenol content was determined as follows: 1.0 mL of the surimi gel digestion supernatant was transferred to a test tube, and mixed sequentially with 5.0 mL of deionized water, 1.0 mL of Folin-Ciocalteu reagent (0.2 mol/L), and 3.0 mL of Na₂CO₃ solution (7.5%, w/v). The resulting mixture was vortexed thoroughly and incubated in the dark at 25°C for 2 h. Absorbance was then measured at 765 nm using a spectrophotometer. A blank solution was used as the zero reference, and a gallic acid standard curve was employed for quantification. Results were expressed as milligrams of gallic acid equivalents per milliliter of sample (mg GAE/mL). Total flavonoid content: 6 mL of digestion supernatant was added to a test tube, followed by 0.4 mL of sodium nitrite solution (5%). The solution was mixed and allowed to stand for 6 min. Then, 1.2 mL of sodium hydroxide solution (4%) was added, mixed, and allowed to stand for 15 min. Absorbance was measured at 510 nm. A blank was used as the zero reference, and rutin was used as the standard to construct a calibration curve. Results were expressed as milligrams of rutin equivalents per gram of sample (mg RE/g).

2.13 LC-MS/MS analysis of HSP-TG gel digestates

The method was adapted with slight modifications from Yang, Zheng, Ma, Zhang, Sun, and Fang (2023). After simulated gastrointestinal digestion, the HSP-TG gel digestate was centrifuged, and the resulting supernatant was diluted to a final protein concentration of 2 mg/mL. Then, 1 mL of the diluted sample was mixed with 1 mL of 50 mmol/L ammonium bicarbonate (NH₄HCO₃) solution to dissolve the sample. Subsequently, 0.1 mL of dithiothreitol (DTT) solution (100 mmol/L) was added, and the mixture was incubated in a 60 °C water bath for 1 h to reduce disulfide bonds. Finally, 0.55 mL of iodoacetamide (IAM) solution (100 mmol/L) was added, and the mixture was incubated in the dark for 40 min to alkylate sulfhydryl groups. The treated samples were desalted using a desalting column, and solvents were removed by vacuum centrifugation at 45°C to complete the sample preparation.

High-performance liquid chromatography (HPLC) separation conditions: Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B was 80% acetonitrile in water containing 0.1% formic acid. The chromatographic column used was an Acclaim PepMap™ RSLC (150 µm × 150 mm, Thermo Scientific). The flow rate was set at 600 nL/min with an analysis time of 60 min. The injection volume was 1 µL. The gradient program for mobile phase B during the separation was as follows: 0 to 5 min, linear increase from 6% to 9%; 6 to 20 min, linear increase from 9% to 14%; 21 to 50 min, linear increase from 14% to 30%; 51 to 58 min, linear increase from 30% to 40%; 59 to 60 min, linear increase from 40% to 95%. The separated peptides were analyzed by an electrospray ionization (ESI) hybrid ion trap-Orbitrap mass spectrometer (Thermo Fisher Scientific) over a duration of 120 min, operating in positive ion mode. The mass spectrometry data acquisition strategy was as follows: after each full scan (MS¹), sequential MS² fragmentation scans were acquired. The scan range was set from 300 to 1400 m/z, with a resolution of 70,000 for MS¹ and a maximum ion injection time of 60 ms, and a resolution of 17,500 for MS² with a maximum ion injection time of 80 ms.

2.14 Data analysis

Data were processed using SPSS 20.0 software and expressed as mean $\pm$ standard deviation (Mean $\pm$ SD). Statistical significance among groups was determined using one-way analysis of variance (ANOVA) followed by a post hoc test for multiple comparisons. The goodness-of-fit of the Logistic model to the digestion kinetics data was evaluated by the coefficient of determination (R^2), which was calculated via nonlinear regression analysis using Origin 2018.

For the LC-MS/MS analysis, the raw mass spectrometry data files were analyzed for peptide sequencing using PEAKS Studio 10.6 software. The peptide mass tolerance was set at 20 $\mu\text{g/mL}$ (parts per million, relative mass error), and the fragment mass tolerance was set at 0.02 Da. The raw mass spectrometry data were searched against specified target protein databases using professional software. The search was conducted with a non-specific enzymatic digestion setting, where carbamidomethylation of cysteine was set as a fixed modification, while oxidation of methionine and acetylation at the peptide N-terminus were designated as variable modifications. The databases utilized included the proteome of silver carp (*Hypophthalmichthys molitrix*), combined separately with the proteomes of wheat (*Triticum aestivum*), soybean (*Glycine max*), rice (*Oryza sativa* subsp. *japonica*), and pea (*Pisum sativum*). The mass tolerance parameters for the spectrometry analysis were set as follows: a mass deviation of twenty parts per million was allowed for MS1 (precursor ions), and a mass deviation of 0.02 Da was permitted for MS2 (fragment ions).

3. Results and discussion

3.1 Verification of gel strength

Our previous study showed that the legume/surimi protein systems enhanced the gel strength more effectively than the cereal/surimi systems when mediated by TGase at the same substitution level. To enable systematic comparison of digestive properties in plant protein/surimi composite gels (HSP-TG gels), TGase dosage was optimized per formulation: 18 U/100 g surimi for the wheat group; 9 U/100 g for both the soybean and rice groups; and 6 U/100 g for the pea group. While no significant differences in breaking force occurred among the four plant protein gel groups ($P > 0.05$), their values were consistently slightly lower than the surimi control. For breaking distance, only the pea protein group (1.12 cm) differed significantly from the rice protein group (1.00 cm) ($P < 0.05$), with no statistical differences observed among other groups ($P > 0.05$). Gel strength values were measured as 965.06 g $\cdot$ cm for the control group, and 803.86, 780.90, 768.39, and 947.86 g $\cdot$ cm for the wheat, soybean, rice, and pea groups, respectively. No significant intergroup differences occurred ($P > 0.05$; Fig. 1). These results confirm that the adjusted TGase dosages yielded uniform gel strength across all composite formulations, providing a valid basis for comparative analysis of digestive properties.

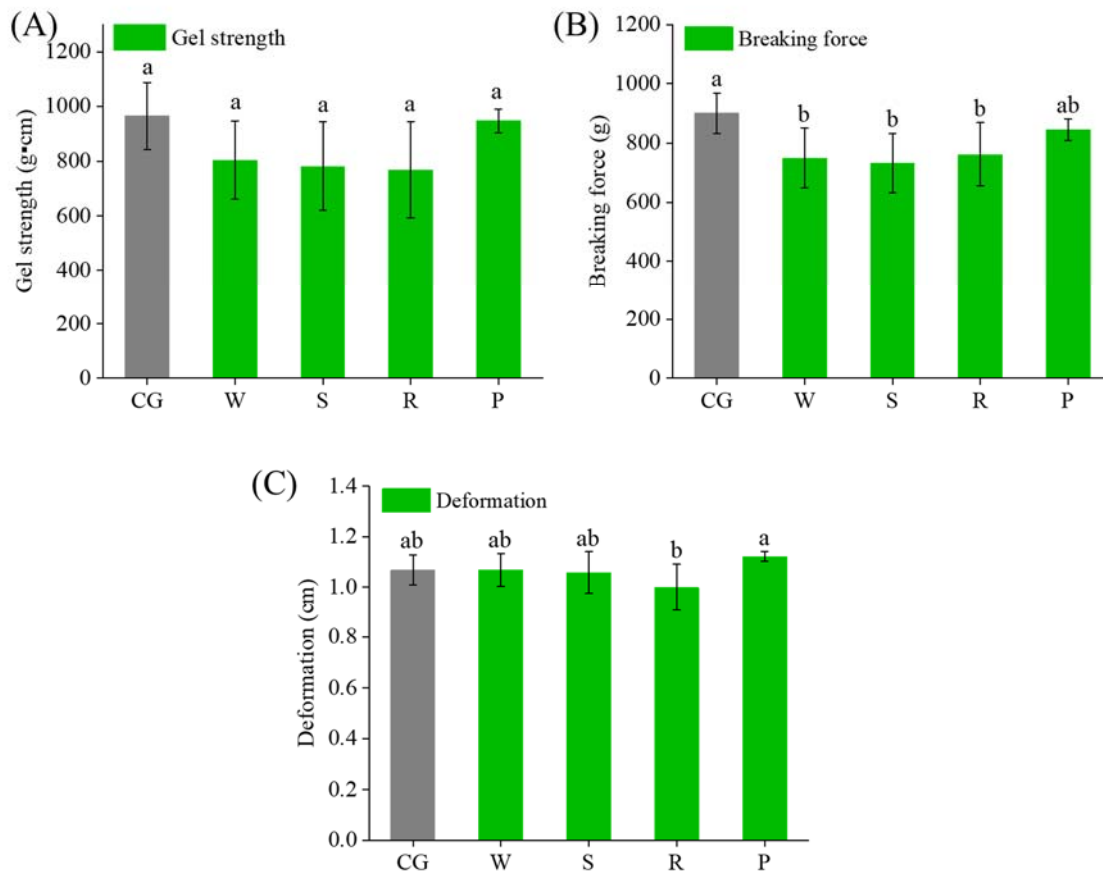


Fig. 1 Gel strength (A), breaking force (B), and deformation (C) of the hybrid surimi/plant protein (HSP) gels catalyzed by transglutaminase (TGase). CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by TGase at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly. Different lowercase letters (a~b) in A-C indicate significant differences across different types of HSP gels ($P < 0.05$).

3.2 Protein and dry matter digestibility of HSP-TG gels

As shown in Fig. 2, the HSP-TG gels exhibited obvious structural degradation characteristics during the simulated digestion process. Following oral mastication, the gels fragmented into irregular granules. Upon entering the gastric digestion phase, the small gel particles were further decomposed into a chyme-like substance. Finally, after intestinal digestion, even finer chyme particles were formed, which demonstrated reduced adhesion to the vessel walls. HSP-TG gels from all plant protein sources (wheat, soybean, rice, and pea) underwent comparable morphological changes at each stage, indicating largely consistent digestive behavior.

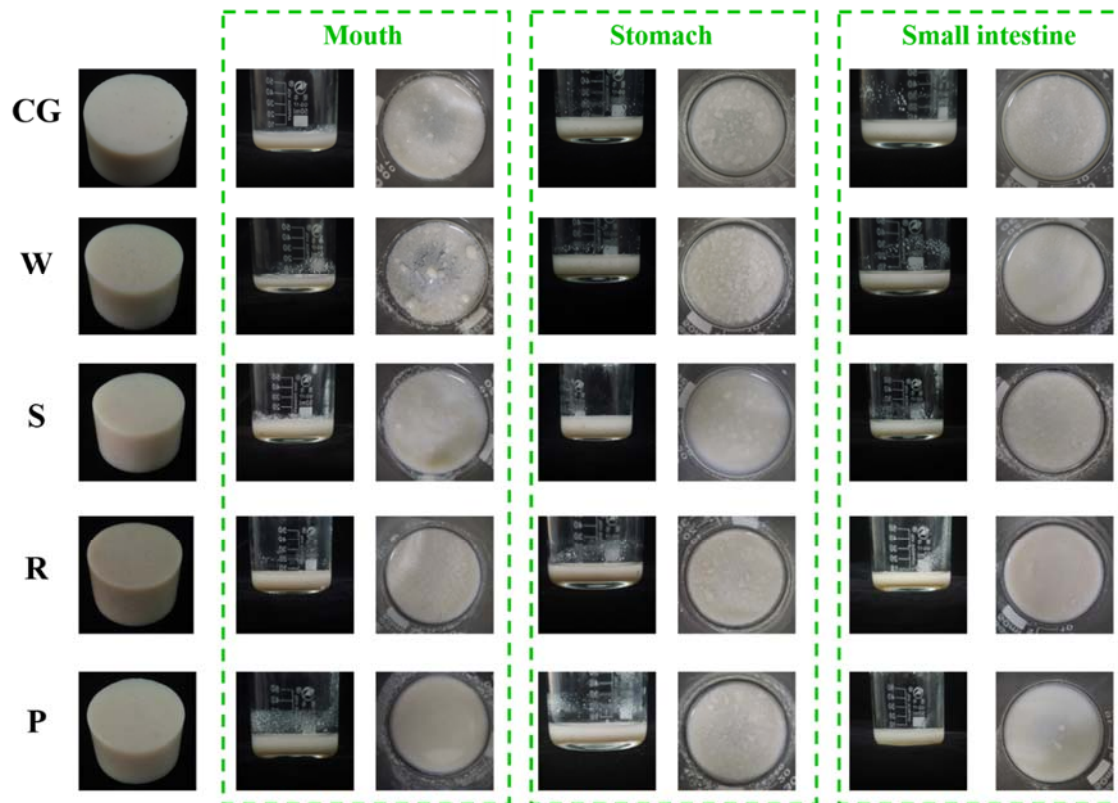


Fig. 2 Morphological changes of transglutaminase (TG)-catalyzed hybrid surimi/plant protein (HSP) gels following *in vitro* simulated digestion phases: oral mastication, gastric (G), and gastrointestinal (GI) digestion. CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by TGase at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly.

©After gastric digestion, the protein digestibility of the pure surimi gel (control group) was 14.79%, while those of the wheat, soybean, rice, and pea protein HSP-TG gels were 17.29%, 10.19%, 16.30%, and 25.55%, respectively, with no significant differences observed among the groups ($P > 0.05$). During intestinal digestion, protein digestibility rose significantly to 59.86% (control), with wheat (50.40%), soybean (50.14%), and rice (52.23%) composites showing moderately lower values. Notably, pea HSP-TG gel achieved 64.96% digestibility, though intergroup differences remained non-significant ($P > 0.05$; Fig. 3A). Fig. 3B displays dry matter digestibility of HSP-TG gels throughout simulated digestion. The trend of dry matter digestibility changes was similar to that of protein digestibility: after gastric digestion, the dry matter digestibility of the control group was 42.50%, while those of the wheat, soybean, rice, and pea composite gels were 41.50%, 37.50%, 35.23%, and 38.06%, respectively. Following simulated intestinal digestion, the dry matter digestibility increased to 69.15%, 68.03%, 62.28%, 61.50%, and 61.78% for the control, wheat, soybean, rice, and pea groups, respectively, with no significant differences observed among all groups ($P > 0.05$). Collectively, *in vitro* digestion revealed minor and statistically insignificant differences in the protein or dry matter digestibility among the HSP-TG gels.

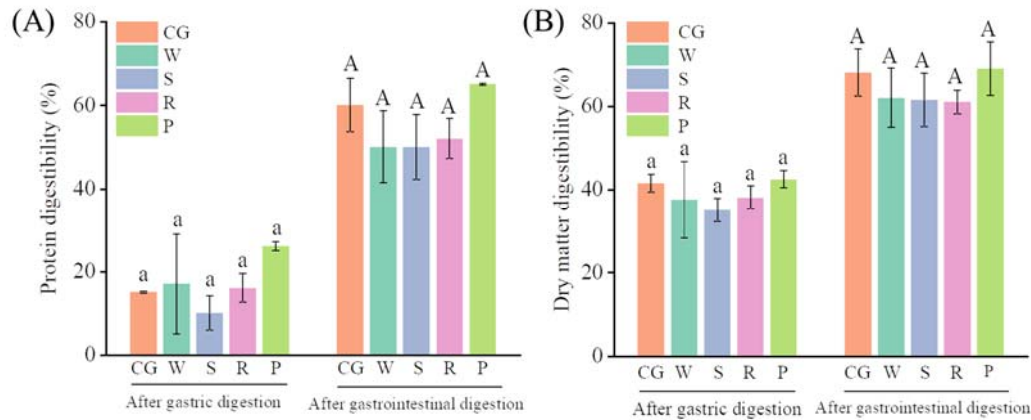


Fig. 3 Protein (A) and dry matter (B) digestibility of the hybrid surimi/plant protein (HSP) gels catalyzed by transglutaminase (TG). CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by TGase at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly. Different lowercase letters in (A) and (B) indicate significant differences after gastric digestion ($P < 0.05$). Different capital letters in (A) and (B) indicate significant differences after gastrointestinal digestion ($P < 0.05$).

The composition and structure of dietary proteins, especially the differences between plant and animal sources, fundamentally influence their digestive behavior and nutrient release (Hu, Ye, Cheng, Lee, & Yang). The native structure of protein molecules is governed by the type, number, and sequence of amino acids within the polypeptide chains (Zhou, Luo, Jin, Wu, Liu, & Chen, 2025). One fundamental difference between plant and animal proteins lies in the higher proportion of storage proteins found in plant sources, which account for approximately 50% of the total protein content. These storage proteins are typically characterized by large, compact three-dimensional conformations (Day, Cakebread, & Loveday, 2022). Based on their solubility, plant storage proteins are traditionally classified into four major categories: water-soluble albumins, salt-soluble globulins, 60-80% ethanol-soluble prolamins, and dilute acid-soluble or alkali-soluble glutelins (Tan, Nawaz, & Buckow, 2023). In cereal grains, prolamins and glutelins represent the predominant protein fractions, whereas in legumes, globulins constitute the dominant storage proteins, comprising more than 70% of the total protein content (Zhang, Boateng, Xu, & Zhang, 2024).

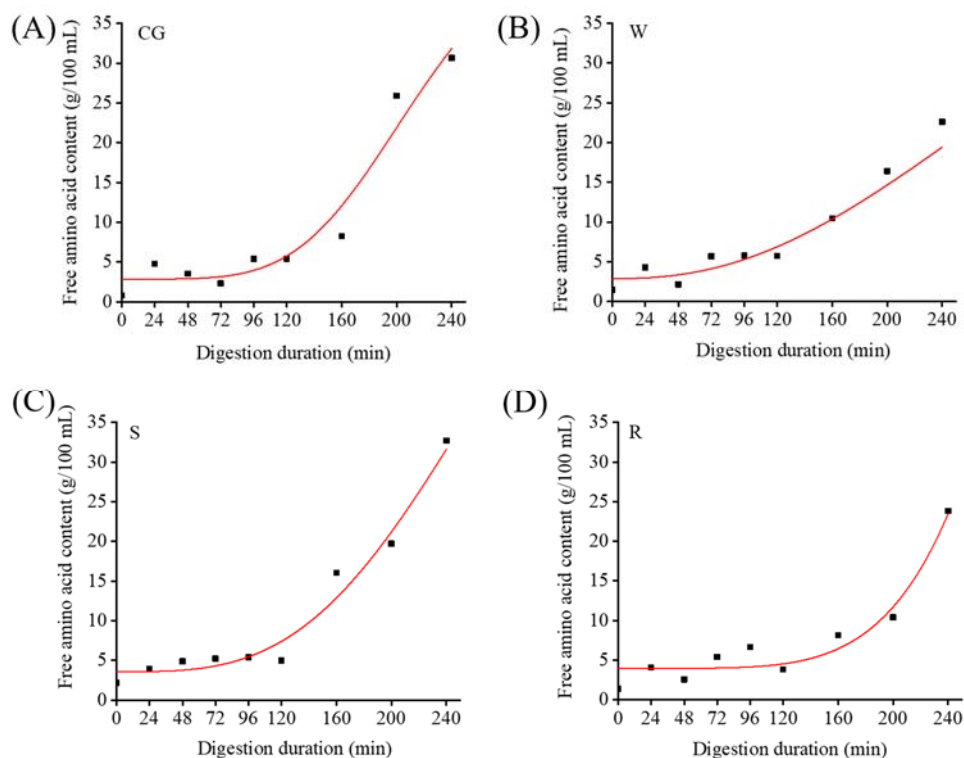
Taking glutelins (as representatives of cereal proteins) and globulins (as representatives of legume proteins) as examples, systematic structural differences have been observed between the two. At the secondary structure level, glutelins are typically enriched in glutamine and proline residues, and are primarily composed of β -sheets and random coils, with a relatively low content of α -helices. In contrast, globulins exhibit a significantly higher proportion of α -helical structures (Zhang, Boateng, Xu, & Zhang, 2024). In terms of tertiary structure, globulins adopt compact, well-defined globular conformations, characterized by a hydrophobic core shielded by a hydrophilic outer surface. Although glutelin monomers exhibit some degree of folding, their structural tendency favors more extended or fibrous morphologies (Tan, Nawaz, & Buckow, 2023). The most pronounced difference lies in their quaternary structure: globulins (e.g., 7S and 11S fractions) are assembled into discrete oligomers, such as trimers or hexamers, mainly stabilized by non-covalent interactions, including hydrophobic forces, hydrogen bonding, van der

Waals interactions, and electrostatic attractions (Sha & Xiong, 2020). In contrast, glutelins form large, covalently cross-linked fibrous networks through extensive intermolecular disulfide bonding, resulting in supramolecular assemblies with high molecular weight, enhanced stability, and elastic properties (Y. Wang, Cai, Li, Gao, & Lai, 2023).

The degree of protein denaturation and aggregation during subsequent processing (Nie, Xiong, & Jiang, 2025), as well as the “food matrix effect” formed by other coexisting components, such as dietary fiber, lipids, and antinutritional factors, upon entry into the digestive system, have all been shown to influence the digestion and absorption of proteins in the human gastrointestinal tract (Xie, Cai, Zhou, & Li, 2024).

3.3 Kinetics of free amino acid release

Fig. 4 illustrates the release kinetics of free amino acids from HSP-TG gels during simulated digestion, along with the fitted curves based on the kinetic model. As shown in the figure, during the gastric digestion phase (0–120 min), the release of free amino acids proceeded relatively slowly across all experimental groups, with cumulative release maintained within the range of 5–7 g/100 g. Upon transition to the intestinal digestion phase (120–160 min), a significant ($P < 0.05$) acceleration in amino acid release was observed in all groups, with the release rates increasing by approximately 2.1–3.8 times compared to the gastric phase. A nonlinear regression analysis was performed using the Logistic model (Equation 3) to fit the *in vitro* release kinetics of free amino acids, and the fitting parameters are presented in Table 1. All groups exhibited a coefficient of determination (R^2) above 0.94 (ranging from 0.941 to 0.985), indicating that the Logistic model provided a good fit for describing the digestion behavior of HSP-TG gels (Htwe, Yin, Duan, Liu, Wei, Xiao, et al., 2025). Analysis of the *in vitro* digestion kinetic parameters (e.g., digestion rate constant, lag time) revealed significant differences in digestive behavior among the gel samples.



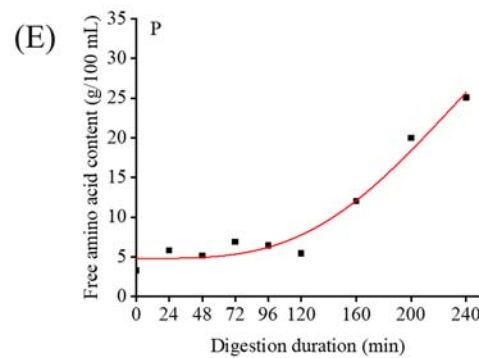


Fig. 4 Release kinetics and model-fitting curves of free amino acids of the hybrid surimi/plant protein (HSP) gels catalyzed by transglutaminase (TGase). CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by TGase at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly.

In terms of digestion efficiency, the HSP-TG gel prepared with soy protein exhibited the highest digestion rate constant ($k = 0.035 \text{ min}^{-1}$), which was significantly higher than that of the other groups and represented a 25% increase compared to the pure surimi control group ($k = 0.028 \text{ min}^{-1}$), indicating superior digestive kinetic performance. This was followed by the rice protein HSP-TG gel ($k = 0.027 \text{ min}^{-1}$) and the pea protein HSP-TG gel ($k = 0.025 \text{ min}^{-1}$), while the wheat protein group showed the lowest digestion rate constant ($k = 0.023 \text{ min}^{-1}$). Notable differences were also observed in the total amount of free amino acids released. The soy protein HSP-TG gel achieved the highest cumulative release ($C_{\max} = 32.69 \text{ g/100 g}$), followed by the pure surimi control (30.65 g/100 g). Intermediate values were recorded for the pea protein group (25.08 g/100 g) and rice protein group (23.84 g/100 g), while the wheat protein group released the least amount (22.61 g/100 g). Regarding the digestion lag time, the rice protein group exhibited the longest half-digestion time ($t_{0.5} = 173.5 \text{ min}$), representing an 8.1% increase compared to the control, whereas the soy protein group showed the shortest lag time (158.2 min), suggesting greater susceptibility to enzymatic hydrolysis.

Table 1 Digestion kinetic parameters of the hybrid surimi/plant protein (HSP) gels catalyzed by transglutaminase (TG) in simulated gastrointestinal digestion based on logistic model

Sample	Logistic model fitting parameters					
	C_0 (g/100g)	$C_{\max}$ (g/100g)	k (min^{-1})	$t_{0.5}$ (min)	$r_{\max}$ (g/(100g·min))	R^2
CG	0.82	30.65	0.028	160.5	0.214	0.968
W	1.44	22.61	0.021	168.2	0.119	0.985
S	2.16	32.69	0.035	158.2	0.286	0.974
R	1.39	23.84	0.027	173.5	0.161	0.941
P	3.26	25.08	0.023	168.3	0.144	0.963

CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by TGase at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly. C_0 : Initial free amino acid concentration; $C_{\max}$: Maximum free amino acid concentration at digestion endpoint; k : Digestion rate constant (higher values indicate faster digestion kinetics); $t_{0.5}$: Time required to reach half-maximal concentration (digestion lag time); $r_{\max}$: Maximum release rate during digestion process; R^2 : Goodness-of-fit index (values closer to 1 indicate better model fitting).

The results demonstrated that the legume protein group (soy and pea proteins) exhibited overall superior digestibility compared to the cereal protein group. Specifically, its average digestion rate constant (0.029 min^{-1}) was 20.8% higher than that of the cereal protein group (0.024 min^{-1}), accompanied by a shorter half-digestion time (163.3 min vs. 170.9 min) and a greater cumulative protein release (28.89 g/100 g

vs. 23.23 g/100 g). Among all samples, the soy protein composite gel showed the best digestive performance, with a digestion rate constant of 0.035 min^{-1} , which was 66.7% higher than that of the best-performing cereal protein, wheat protein (0.021 min^{-1}), and a cumulative release of 32.69 g/100 g, 37.1% higher than that of the top cereal protein, rice protein (23.84 g/100 g). In contrast, the cereal protein group (wheat and rice proteins) displayed typical sustained-release characteristics, including slower digestion rates ($0.021\text{--}0.027 \text{ min}^{-1}$) and longer half-digestion times (168.2–173.5 min), indicating a more gradual and controlled release process.

The process and efficiency of protein digestion are regulated by a variety of physicochemical and structural factors (Fan, He, Cao, Woldemariam, Cai, Wang, et al., 2025). Protein digestion primarily begins in the stomach due to the presence of pepsin in gastric juice; however, the majority of digestion occurs in the small intestine, where various proteases such as trypsin and chymotrypsin are active (Y. Wang, Cai, Li, Gao, & Lai, 2023). The rate and extent of protein digestion depend on how easily proteases can access the peptide bonds within the polypeptide chains. Differences in protein type, processing and cooking methods, as well as the composition of the surrounding food matrix, result in variations in molecular size, surface hydrophobicity, surface charge, and dispersibility of proteins. These factors affect the three-dimensional conformation of proteins in the digestive fluid system, determining the accessibility of peptide bonds between specific amino acids to digestive enzymes, ultimately leading to differences in digestive behavior (Lee, Jo, Choi, & Jung, 2024).

Chen, Fan, Wang, Zhang, Wang, Hou, et al. (2024) found that during the digestion process, the secondary structure of β -casein was significantly altered, as reflected by a reduction in β -sheet and α -helix content, while the proportions of β -turn and random coil structures were increased. As a result, the protein molecules were rendered more loosened and disordered, which facilitated the exposure of cleavage sites and allowed greater access for digestive enzymes, thereby enhancing protein digestibility. Physical processing techniques have also been proven effective in inducing structural alterations that promote digestive efficiency. Ultrasound treatment (20 kHz, pulse mode: 10 s on / 5 s off, amplitude 60%, duration 10 min) was applied to buckwheat protein isolates, and structural transitions were induced via the cavitation effect of microbubbles. β -turn and β -sheet structures were transformed into anti-parallel β -sheet, random coil, and α -helix conformations. Meanwhile, buried hydrophobic groups were exposed, intramolecular cross-links were disrupted, large protein aggregates were fragmented into smaller pieces, and surface properties were modified. Thus, protein digestibility was improved as a result of the structural modifications and the disintegration of protein aggregates (Jin, Okagu, Yagoub, & Udenigwe, 2021). Protein digestion and absorption were also reported to be slowed down by dietary fiber through several mechanisms: the viscosity of gastrointestinal contents was increased; hydrogel structures were formed to physically entrap proteins; and complexes were formed between proteins or digestive enzymes and dietary fiber, resulting in steric hindrance or altered enzymatic activity. These effects collectively reduced the effective interaction between

proteases and substrate proteins in both the stomach and small intestine (H. Ye & Yu, 2024; Yu, Wang, Zou, Cai, Pan, & Chen, 2023).

3.4 Particle size distribution of HSP-TG gel digests

Fig. 5 illustrates the particle size distribution profiles of HSP-TG gels after simulated gastrointestinal digestion. After simulated gastric digestion, all samples exhibited a unimodal distribution. The peak particle size for the control, wheat protein, soy protein, and rice protein gel groups was observed at approximately 227.2 μm . In contrast, the peak for the pea protein group shifted leftward to 202.8 μm , and the peak width significantly broadened, indicating that pea protein was more readily degraded by pepsin, but the resulting protein particles showed greater variability in size. Following intestinal digestion, the peak particle sizes of all groups markedly decreased to a range of 10.56–32.91 μm , suggesting extensive breakdown of gel particles under trypsin action.

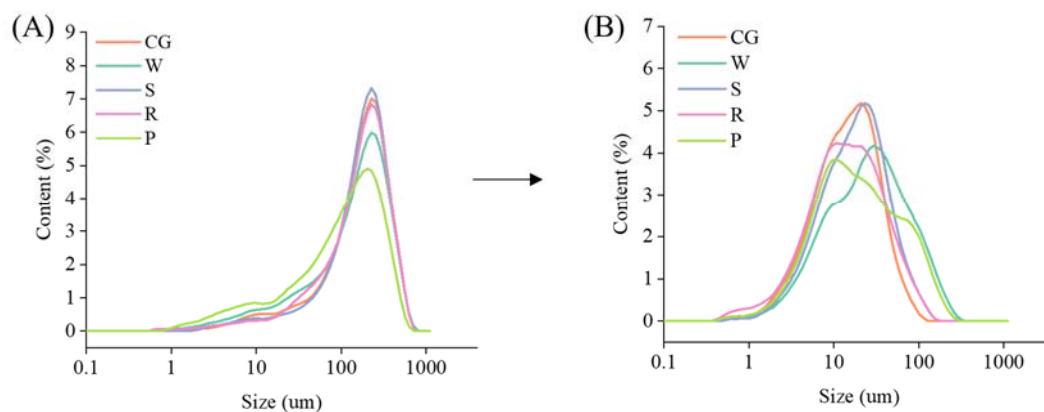


Fig. 5 Particle size distribution of the hybrid surimi/plant protein (HSP) gels catalyzed by transglutaminase (TGase) following *in vitro* gastro-intestinal digestion. CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by TGase at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly.

Table 2 lists the volume-weighted mean diameter ($D_{(4,3)}$) and percentile particle sizes (D_{10} , D_{50} , D_{90}) of HSP-TG gels after digestion. After pepsin digestion, the percentile particle sizes of the pea protein gel were smaller than those of other groups, with $D_{(4,3)}$ and D_{50} showing statistically significant differences ($P < 0.05$). This is attributed to the greater accessibility of pepsin cleavage sites in the pea protein system (Jang & Lee, 2024). After trypsin digestion, the wheat protein HSP-TG gel exhibited significantly higher $D_{(4,3)}$ (42.88 μm) and percentile particle sizes (D_{10} : 5.73 μm , D_{50} : 27.48 μm , D_{90} : 101.84 μm) compared to other groups, suggesting the presence of components resistant to trypsin digestion in this system (Leng, Yang, Zhu, & Qi, 2025). This difference is likely related to particle size effects: smaller particles enhance enzyme accessibility and promote protein hydrolysis, whereas larger particles limit protease access to the protein interior, thereby reducing digestibility (Yang, Zheng, Ma, Zhang, Sun, & Fang, 2023).

Table 2 Mean particle diameters including $D_{(4,3)}$, D_{10} , D_{50} , and D_{90} of the hybrid surimi/plant protein (HSP) gels catalyzed by transglutaminase (TG) following *in vitro* gastric (G), and gastrointestinal (GI) digestion.

Sample	$D_{(4,3)}$	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)
After G				

CG	195.70 ± 21.43a	89.73 ± 75.91a	179.65 ± 20.86a	542.05 ± 192.97a
W	198.75 ± 6.86a	23.62 ± 5.73a	181.60 ± 12.59a	395.20 ± 7.92a
S	195.10 ± 26.1a	30.9 ± 18.37a	179.35 ± 25.67a	378.90 ± 33.52a
R	191.60 ± 2.34a	26.65 ± 14.08a	175.40 ± 20.93a	380.75 ± 31.89a
P	151.25 ± 1.77b	9.63 ± 1.27a	117.00 ± 8.63b	346.75 ± 8.27a
After GI				
CG	21.32 ± 1.73d	4.54 ± 0.10d	16.73 ± 1.67c	44.31 ± 3.88c
W	42.88 ± 2.15a	5.73 ± 0.13a	27.48 ± 0.14a	101.84 ± 10.05a
S	25.28 ± 0.39c	5.30 ± 0.04b	19.32 ± 0.25b	53.23 ± 1.26c
R	22.30 ± 0.24d	3.85 ± 0.14e	14.90 ± 0.22d	50.72 ± 0.85c
P	33.81 ± 1.95b	4.78 ± 0.07c	18.24 ± 0.44b	87.90 ± 6.62b

Values are presented as mean ± SD (n = 3); Different lowercase letters (a~d) in the same column indicate significant differences ($P < 0.05$). CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by TGase at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly.

3.5 Microstructural features of HSP-TG gel digests

CLSM was utilized to observe the morphology of gel particles in the precipitates of HSP-TG gels after simulated oral, gastric, and intestinal digestion. The results are shown in Fig. 6. After simulated oral digestion, distinct microstructural features were observed among the different HSP-TG gel groups. The control group exhibited dense three-dimensional protein networks, whereas the wheat protein mixed gels formed loose aggregates composed of large particles. The soy protein group displayed a typical lamellar network structure, the rice protein group showed a fragmented network structure, and the pea protein group also presented lamellar structures with evident three-dimensional stacking characteristics.

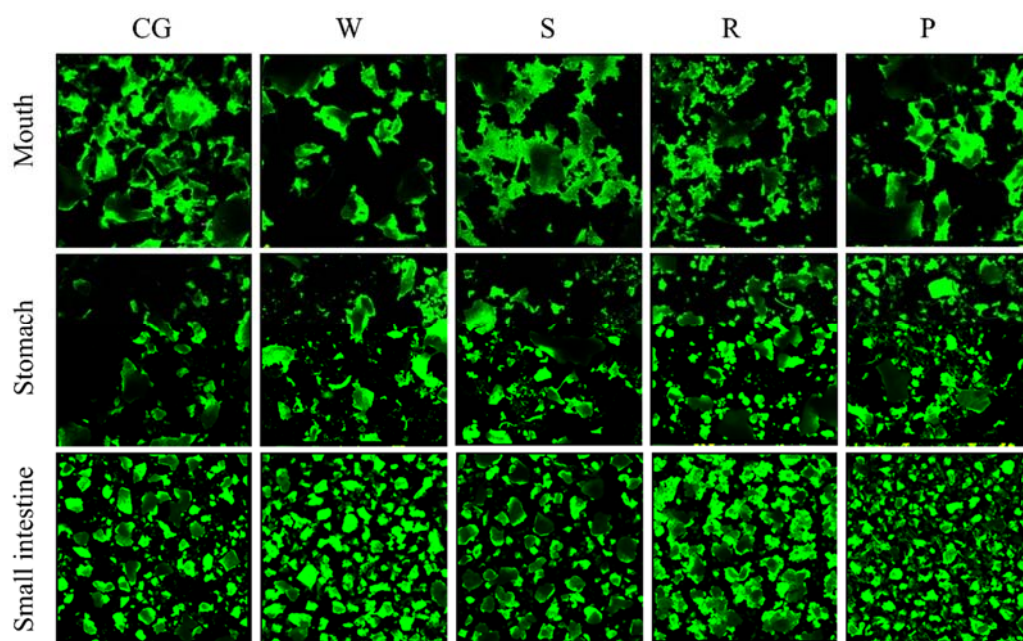


Fig. 6 Confocal fluorescence microscopy images of the transglutaminase (TG)-catalyzed hybrid surimi/plant protein (HSP) gels following in vitro simulated digestion phases: oral mastication, gastric (G), and gastrointestinal (GI) digestion. CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by transglutaminase (TGase) at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly.

After gastric digestion, all protein gel particles underwent significant structural degradation. The HSP-TG gels in the rice protein and pea protein groups formed more small particle aggregates, with the pea protein group exhibiting an uneven particle size distribution and retaining some lamellar structure fragments.

Following intestinal digestion, the gel particles were further reduced in size. The control group and wheat protein HSP-TG gels retained residual three-dimensional structures, while the rice protein group showed relatively larger particle sizes. The pea protein HSP-TG gel particles were significantly reduced in size but still contained undigested flocculent protein aggregates. The wheat protein group had the highest proportion of undigested large particles. These results indicate that the pea protein mixed gels exhibit the highest digestion sensitivity but retain some flocculent aggregates, whereas the wheat protein HSP-TG gels demonstrate certain resistance to digestion.

3.6 SDS-PAGE of HSP-TG gel digests

SDS-PAGE was employed to analyze changes in the protein composition of HSP-TG gels following simulated gastrointestinal digestion. The results are presented in Fig. 7. After simulated gastric digestion, almost no obvious bands above 75 kDa were observed in any of the gel groups, whereas a distinct band appeared at approximately 48 kDa (Fig. 7A), which was identified as actin (Dong, Wang, Xu, & Wang, 2025). Previous experiments indicated that the myosin heavy chain (MHC, ~245 kDa), located near 245 kDa, was the main band in HSP-TG gels and played a crucial role in gel network formation. These findings suggest that during the gastric digestion phase, pepsin preferentially hydrolyzed myosin, resulting in the collapse of the gel's three-dimensional network structure. Additionally, weak bands around 75 kDa were observed in the soy protein and pea protein HSP-TG gels, attributed to β -conglycinin (7S, α') and pea vicilin, respectively, which are major components of their respective proteins (He, Wang, Li, Guo, Dong, Zhu, et al., 2025). The staining intensity of the 7S globulin band after gastric digestion was slightly higher than that of pea vicilin, indicating that pea vicilin is more susceptible to pepsin digestion. Following trypsin digestion, protein bands above 20 kDa almost completely disappeared, with only small amounts of low-molecular-weight protein fragments between 11 and 17 kDa being detected (Fig. 7B). At this stage, a substantial amount of low-molecular-weight proteins and peptides were released from the HSP-TG gels after intestinal digestion.

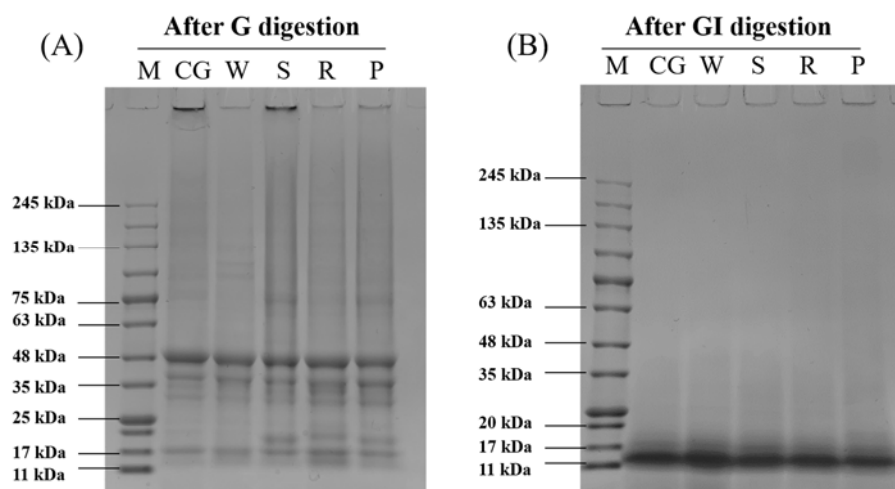


Fig. 7 Protein profile of transglutaminase (TG)-catalyzed hybrid surimi/plant protein (HSP) gels following in vitro simulated digestion phases: gastric (G), and gastrointestinal (GI) digestion. CG: Control group (pure surimi). M: protein marker. W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by transglutaminase (TGase) at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly.

The digestibility of proteins and the nature of the resulting peptides are critical determinants of their subsequent absorption and physiological effects (Li, Ma, Acevedo-Fani, Lu, Singh, & Ye, 2025). Free amino acids and small peptides, mainly dipeptides and tripeptides, generated during protein digestion are efficiently absorbed in the small intestine. Insufficient digestion of proteins in the small intestine leads to the presence of undigested proteins and larger peptides that cannot be absorbed at this site. This may reduce the availability of absorbable amino acids and peptides from dietary protein and lower overall utilization efficiency (Cao, Pan, Zheng, Chen, Yin, Liu, et al., 2024). Moreover, undigested proteins and peptides reaching the colon serve as substrates for gut microbiota fermentation, significantly influencing microbial composition and metabolic products. However, these effects are complex. On one hand, excessive protein fermentation may promote the proliferation of certain bacteria such as proteolytic bacteria. This results in the production of potentially harmful metabolites including ammonia, hydrogen sulfide, amines, phenols, and indoles, which can disrupt microbial balance (dysbiosis) and adversely affect intestinal health (Yu, Nie, Chen, Zou, Xu, & Chen, 2024). On the other hand, under specific conditions where undigested proteins coexist with certain dietary fibers, fermentation can enhance the production of beneficial metabolites such as butyrate. Butyrate serves as a key energy source for colon epithelial cells and plays a positive role in maintaining intestinal barrier function and overall gut health (Jackson, Yao, Bulut, Cantu-Jungles, & Hamaker, 2024).

3.7 Antioxidant and ACE inhibitory activities of HSP-TG gel digests

To investigate bioactivity differences among the digests of HSP-TG gels, *in vitro* antioxidant and ACE inhibitory activities were assessed. Results are presented in Fig. 8. After gastric digestion, the digest from the pea protein HSP-TG gel exhibited a significantly higher ferric reducing antioxidant power (FRAP) value ($7.03 \mu\text{g Trolox/mL}$) compared to digests from other groups (3.33 to $4.29 \mu\text{g Trolox/mL}$) ($P < 0.05$). Following intestinal digestion, FRAP values increased further in digests from all gel groups (9.30 to $10.76 \mu\text{g Trolox/mL}$), although no significant differences were observed among them ($P > 0.05$) (Fig. 8A). Regarding ABTS radical scavenging activity, the digest obtained from the pea protein gel after simulated intestinal digestion (60.57%) demonstrated significantly higher activity than those from other groups (25.30% to 40.63%) ($P < 0.05$) (Fig. 8B). After simulated gastric digestion, digests from the rice and pea protein gels showed significantly enhanced DPPH radical scavenging activity, with rates of 60.90% and 64.93% , respectively. These values were markedly higher than those of digests from other treatment groups (20.34% to 39.20%) ($P < 0.05$) (Fig. 8C). However, following intestinal digestion, DPPH radical scavenging rates became comparable across all digests (45.12% to 52.34%), with no statistically significant differences ($P > 0.05$).

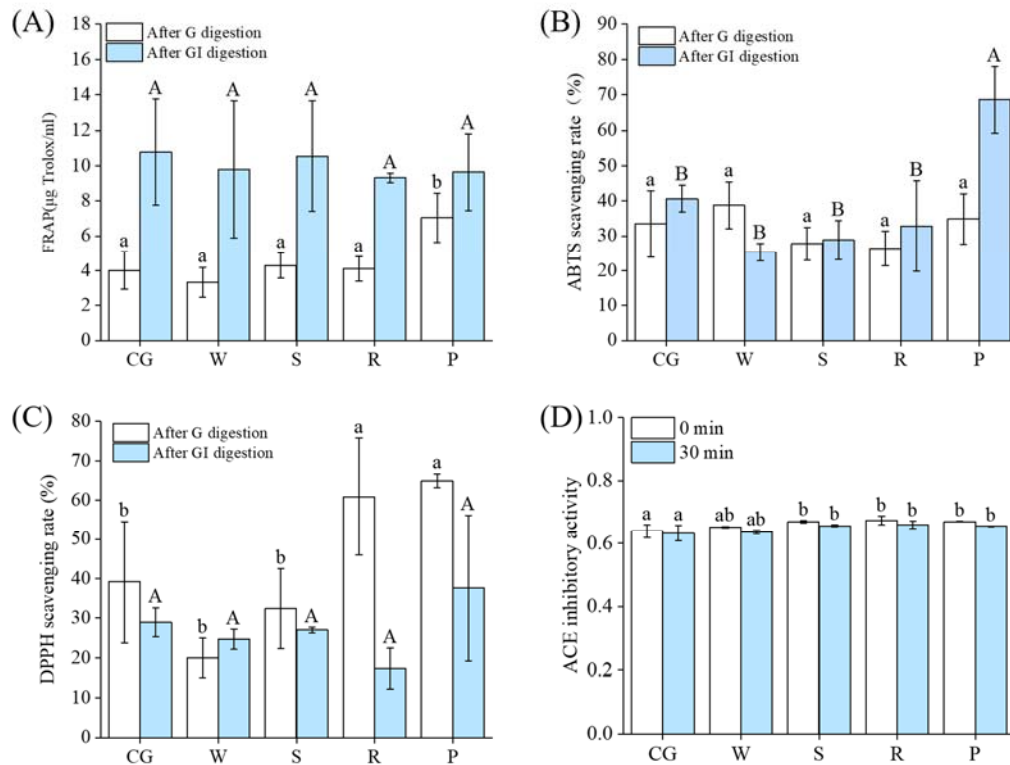


Fig. 8 Antioxidant activities (A, B, and C) and angiotensin-converting enzyme (ACE) inhibitory activity (D) of the hybrid surimi/plant protein (HSP) gels catalyzed by transglutaminase (TGase) following *in vitro* simulated digestion phases: gastric (G) and gastrointestinal (GI) digestion. Different letters (a-b or A-B) indicate significant differences ($P < 0.05$). CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by TGase at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly.

Auclair, Eustachio Colombo, Milner, and Burgos (2024) reported that replacing red meat, processed meats, and dairy products with plant-based protein foods may reduce the risk of chronic diseases and thereby increase life expectancy. Similarly, Sridhar, Bouhallab, Croguennec, Renard, and Lechevalier (2023) claimed that plant-based meat alternatives exhibit high nutritional similarity to animal-based meats and may contribute to reduced risks of cardiovascular diseases and obesity. Since hypertension is a major risk factor for cardiovascular diseases and ACE inhibitory peptides are known to play a significant role in blood pressure regulation, we evaluated the ACE inhibitory activity of digests from four plant-based proteins. This investigation aimed to provide a preliminary assessment of their potential antihypertensive effects and broader health benefits. However, no significant ACE inhibitory activity was detected in any of the HSP-TG gel digests (Fig. 8D). These results suggest that the gastric digests of rice and pea protein gels contained specific antioxidant components. Furthermore, the pea protein gel digest demonstrated stronger ABTS radical scavenging ability following gastrointestinal digestion.

3.8 Total polyphenol and flavonoid contents in HSP-TG gel digests

The total polyphenol and flavonoid contents in the digests of HSP-TG gels were quantified, and the results are summarized in Table 3. Total polyphenol content across all gel groups ranged from 149.74 to 158.43 mg gallic acid equivalents/mL, while total flavonoid content ranged from 51.19 to 72.38 mg rutin

equivalents/mL. Statistical analysis revealed no significant differences ($P > 0.05$) in either total polyphenol or total flavonoid content among the treatment groups. These results indicate that peptides exhibiting antioxidant activity were released during the gastrointestinal digestion of the rice and pea protein HSP-TG gels.

Table 3 Total polyphenol and flavonoid contents in the digesta of the hybrid surimi/plant protein (HSP) gels catalyzed by transglutaminase (TG).

Sample	Total phenolic content (mg gallic acid equivalents/mL)	Flavonoid content (mg rutin equivalents/mL)
CG	147.27 $\pm$ 1.71 ^b	44.41 $\pm$ 4.52 ^a
W	157.16 $\pm$ 7.61 ^a	51.19 $\pm$ 21.39 ^a
S	149.74 $\pm$ 2.24 ^{ab}	72.38 $\pm$ 19.14 ^a
R	152.85 $\pm$ 6.09 ^{ab}	58.45 $\pm$ 5.95 ^a
P	158.43 $\pm$ 3.70 ^a	66.43 $\pm$ 13.65 ^a

Values are presented as mean $\pm$ SD ($n = 3$); Different lowercase letters (a~b) in the same column indicate significant differences ($P < 0.05$). CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by transglutaminase (TGase) at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly.

3.9 Peptide profile analysis of HSP-TG gel digests by LC-MS/MS

Peptide profiling of the digestion fluids derived from HSP-TG gels subjected to simulated gastrointestinal digestion was performed using LC-MS/MS. Comprehensive statistical analyses were conducted on the number of reliably identified peptides, molecular weight distribution, relative amino acid composition, differences in peptide composition, and the abundance of high-intensity peptides. The results are summarized in Fig. 9. A total of 1,863 confidently identified peptides were detected in the digest of the pure surimi gel. In comparison, the HSP-TG gels prepared with wheat, soy, rice, and pea proteins yielded 1,782, 979, 1,039, and 1,080 peptides, respectively (Fig. 9A). As shown in Fig. 9B, the molecular weights of peptides in all digested samples were below 5 kDa. The control group (pure surimi gel) exhibited the highest proportion of peptides with molecular weights below 1 kDa, accounting for 42.67%. In contrast, the HSP-TG gels predominantly contained peptides in the 1.0-1.5 kDa range, with proportions of 45.45%, 42.39%, 46.39%, and 46.20% in the wheat, soy, rice, and pea protein groups, respectively. These findings indicated that a slightly higher proportion of low molecular weight peptides was released from pure surimi gel during digestion compared to the HSP-TG composite gel.

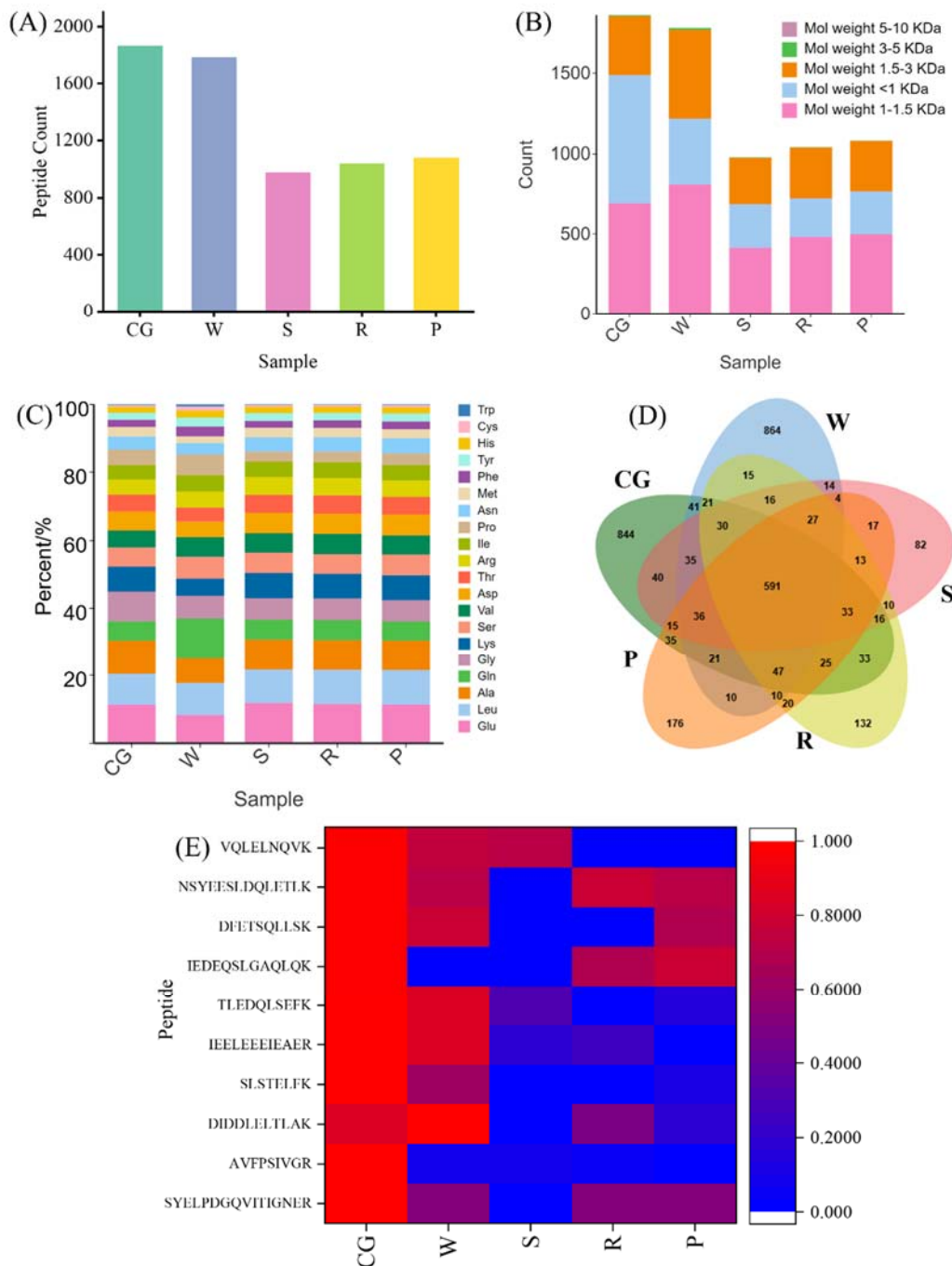


Fig. 9 Number of confidently identified peptides (A), peptide molecular weight profile (B), relative amino acid composition of identified peptides (C), discrepancies in the peptide compositions (D), and heatmap of high-abundance peptides (E) in the digesta of the hybrid surimi/plant protein (HSP) gels catalyzed by transglutaminase (TGase), which were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). CG: Control group (pure surimi). W, S, R, and P: Represent wheat protein/surimi gels, soy protein/surimi gels, rice protein/surimi gels, and pea protein/surimi gels, respectively. These were catalyzed by TGase at dosages of 18, 9, 9, and 6 U/100 g surimi paste, correspondingly.

The structural features of proteins from different sources, including their secondary and tertiary conformations, the aggregation state of protein particles formed during mastication, and the physicochemical characteristics of the digestive environment resulting from the interaction between the food matrix and digestive fluids, including zeta potential, particle size distribution, and apparent viscosity, collectively affect

the accessibility of digestive enzymes in the gastrointestinal tract (Diao, Xue, Mao, Li, Yang, Ke, et al., 2025). Fig. 9C presents the relative amino acid composition of peptides identified from the HSP-TG gels, indicating a relatively balanced amino acid profile across all gel groups. Among the identified peptides, 844, 864, 82, 136, and 176 unique peptides were detected in the control, wheat, soy, rice, and pea protein HSP-TG gels, respectively (Fig. 9D). During digestion, the mixed gel fragments were initially subjected to non-specific cleavage by pepsin in the stomach, which preferentially targets peptide bonds adjacent to hydrophobic amino acids such as phenylalanine, tryptophan, tyrosine, and leucine. The resulting gastric hydrolysates subsequently entered the intestinal phase, where trypsin specifically cleaved at the carboxyl termini of lysine and arginine residues. These hydrolysates were further degraded into small peptides and free amino acids. (Liu, Janssen, Smeets, & Stieger, 2025). Given that plant proteins from different sources possessed distinct amino acid sequences, the resulting gels exhibited structural differences across multiple scales and digestion generated source-specific peptide profiles (Rudge, Nicholson, Cottrell, Collins, Hoffman, Stokes, et al., 2025). Fig. 9E displays the top ten most abundant peptides identified in the digest of the pure surimi gel. It is evident that the soy, rice, and pea protein HSP-TG gels differ markedly from the control group. Although the mixed systems still contain 70% surimi protein, differences in the dynamic digestive behaviors of gels formed with various plant proteins were observed. These variations may influence bioavailability factors such as nutrient absorption and functional activity, which warrant further investigation. Notably, the digestion products of the pea protein HSP-TG gel exhibited superior ABTS radical scavenging activity.

Based on these results, we developed a supervised deep learning framework integrating the ESM-2 protein language model with a convolutional neural network (CNN) to predict ABTS, DPPH, and FRAP radical scavenging activities of peptides. The model was trained and evaluated using distinct datasets for each antioxidant assay: the ABTS dataset contained 540 peptides (training: 432 peptides, 225 active/207 inactive; testing: 108 peptides, 48 active/60 inactive), the DPPH dataset contained 357 peptides (training: 286 peptides, 170 active/116 inactive; testing: 71 peptides, 34 active/37 inactive), and the FRAP dataset contained 571 peptides (training: 457 peptides, 255 active/202 inactive; testing: 114 peptides, 45 active/69 inactive). Each model was rigorously validated through 10-fold cross-validation, achieving mean accuracies of 0.84, 0.92, and 0.87 for ABTS, DPPH, and FRAP, respectively, with peak accuracies reaching 0.98, 0.97, and 0.98. Generalizability was further assessed on independent test sets, where the models attained overall accuracies of 0.84 (ABTS), 0.87 (DPPH), and 0.89 (FRAP), with sensitivities for active peptides of 0.80, 0.88, and 0.92, and specificities for inactive peptides of 0.88, 0.87, and 0.88, respectively. Coupled with relative abundance analysis, six peptides with high relative abundance among the top thirty differential peptides ($\text{Area} \geq 7.10 \times 10^8$) in the pea protein group were identified: TLEDQLSEIK, VLNAGVIEGQF, DAGDGVTHNVPVYEG, VGLLHSQNTSL, LASLYPAVVEDTTK, and LLVVPQNF. These peptides demonstrate not only ABTS antioxidant activity but also FRAP and DPPH antioxidant activities. Further validation and screening are needed to fully explore their bioactivities.

4. Conclusion

By controlling the amount of TGase added (wheat group: 18 U/100 g surimi; soy and rice groups: 9 U/100 g; pea group: 6 U/100 g), HSP-TG gels with uniform gel strength were prepared. Simulated gastrointestinal digestion experiments showed no significant differences in protein digestibility (61.50-69.15%) and dry matter digestibility (61.50-69.15%) among the control, wheat, soy, rice, and pea gel groups. The legume protein groups (soy and pea proteins) exhibited superior digestive performance overall, with an average digestion rate constant (0.029 min^{-1}) significantly higher by 20.8% than that of the cereal protein groups (0.024 min^{-1}), and a shorter half-digestion time (163.3 min vs. 170.9 min). In contrast, the cereal protein groups (wheat and rice proteins) demonstrated typical sustained-release characteristics, showing slower digestion rates ($0.021\text{-}0.027 \text{ min}^{-1}$) and longer half-digestion times (168.2-173.5 min), indicating a more stable release process. After pepsin digestion, the percentile particle sizes of the pea protein gel were smaller than those of other groups, with $D_{(4,3)}$ and D_{50} showing statistically significant differences ($P < 0.05$), which was related to better accessibility of pepsin cleavage sites. Following trypsin digestion, the $D_{(4,3)}$ ($42.88 \mu\text{m}$) and percentile particle sizes (D_{10} : $5.73 \mu\text{m}$, D_{50} : $27.48 \mu\text{m}$, D_{90} : $101.84 \mu\text{m}$) of the wheat protein HSP-TG gel were significantly larger than those of other groups. It was evident that the pea protein mixed gel exhibited the highest digestion sensitivity while retaining flocculent aggregates, whereas the wheat protein HSP-TG gel demonstrated certain resistance to digestion. Protein electrophoresis revealed that after trypsin digestion, HSP-TG gels primarily produced protein fragments below 20 kDa. The digests of rice and pea protein HSP-TG gels after gastric digestion contained certain antioxidant components, and after gastrointestinal digestion, the pea protein HSP-TG gel exhibited relatively strong ABTS radical scavenging activity. Protein profiling of the digests showed that all peptide molecular weights were below 5 kDa, with the control group displaying the highest proportion of peptides below 1 kDa. In the pea protein digest, relatively abundant differential peptides were identified, including six peptides: TLEDQLSEIK, VLNAGVIEGQF, DAGDGVTHNPVYEG, VGLLHSQNTSL, LLVVPQNF, and LASLYPAVVEDTTK. These peptides, with high relative abundance, were considered the material basis for the antioxidant activity observed in the pea protein digest. In summary, these results indicate that plant proteins, such as pea protein, have the potential to enhance the nutritional functionality of surimi products when used as partial substitutes. However, further validation through *in vivo* experiments is still required for its digestion kinetics, antioxidant activity, and bioavailability of bioactive peptides, in order to fully explore the health benefits and application potential of hybrid surimi/plant protein gels.

Conflict of interest

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

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