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Unveiling anti-inflammatory peptides from Lion's Mane mushroom (*Hericium erinaceus*): Preparation, bioactivity assessment, and peptides identification

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ABSTRACT

Lion's Mane mushroom (*Hericium erinaceus*) is acknowledged worldwide for its substantial contribution of medicinal compounds and nutrients, including protein. The efficient extraction and hydrolysis of proteins are essential for revealing their bioactive properties. This study demonstrates that the optimal pulsed electric field (PEF)-assisted extraction achieved a 42.44 % increase in protein extraction efficiency relative to traditional alkaline extraction ($p < 0.05$). *H. erinaceus* protein contained 44.59 % essential amino acids and exhibited 71.33 % *in vitro* digestibility. Pepsin-trypsin hydrolysis produced the most significant anti-inflammatory activity, resulting in a 36.2 % reduction in nitric oxide and a 31.8 % decrease in interleukin-6 levels ($p < 0.05$). Subsequent fractionations employing membrane ultrafiltration and size exclusion chromatography effectively purified the peptides, resulting in enhanced anti-inflammatory activity ($p < 0.05$). This research discovered nine important peptide sequences containing 50–100 % hydrophobic amino acids in Lion's Mane mushroom proteins, which could aid in the synthesis of natural anti-inflammatory peptides.

1. Introduction

Inflammation plays a crucial role in the progression of various chronic diseases, including cancer. Prolonged instances of untreated acute inflammation can lead to the development of chronic conditions, which may manifest as various chronic inflammatory disorders. Common treatments for these conditions include anti-inflammatory synthetic drugs; however, many of these medications prove ineffective in managing chronic reactions and are associated with adverse side effects. This has led to a variety of initiatives underway aimed at developing alternative anti-inflammatory treatments derived from natural products (Rivera-Jiménez et al., 2022).

Mushrooms are becoming essential components of functional foods and nutraceuticals due to their variety of health and nutritional advantages. The presence of remarkable bioactive compounds, such as polysaccharides, phenolic compounds, terpenoids, and proteins, accounts for their recognized bioactivities, particularly anti-inflammatory effects by lowering the synthesis of nitric oxides and interleukin-6 (IL-6), which have been utilized as indicators of inflammatory responses (Aursuwanna et al., 2022; Ayimbila & Keawsompong, 2023; Contago &

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Conte-Junior, 2025; Wang et al., 2014). The Lion's Mane mushroom (*H. erinaceus*) has attracted considerable interest in the field of mushroom research due to its long-standing recognition of its medicinal properties. Hericenone A, Hericenone C, Hericenone H, Erinacine D, and Erinacine P are identified as principal chemicals that facilitate anti-inflammatory activity in *H. erinaceus* (Contago & Conte-Junior, 2025). Nevertheless, *H. erinaceus* possesses a substantial protein content (20.8 %), and the current literature regarding the anti-inflammatory properties of *H. erinaceus* proteins is scarce.

Proteins are inherently present in a variety of combinations with other molecules, including secondary metabolites, carbohydrates, and lipids, which complicate their unrestricted utilization. Consequently, protein extraction is essential for the utilization of a variety of characteristics, properties, and functions (Tang et al., 2024). Various conventional and sophisticated extraction strategies are now being used to extract mushroom proteins (Kumar et al., 2021). Pulsed electric field (PEF) is an emerging technology that can be used to enhance protein extraction yield by changing cell membrane permeability, resulting in fast permeation of cellular components due to the high potential voltage (Zhang et al., 2021). Moreover, PEF markedly increases *in vitro* protein digestibility by exposing protein structures, hence facilitating enzymatic attack (Thongkong et al., 2023). Therefore, PEF presents a promising approach that may assist in both protein extraction and structural modification for the production of enzymatically bioactive peptides.

Bioactive peptides are frequently concealed within the structure of their parent proteins, making the bioactive properties of proteins inaccessible in their native state. As a result, the most common and straightforward method of producing bioactive peptides is protein hydrolysis using commercial enzymes (Akbarian et al., 2022). Various enzymes, *e.g.* neutrase, alcalase, and papain, have been employed to generate bioactive peptides from various mushrooms, including angiotensin I- converting enzyme (ACE) inhibitory peptide from *Agaricusbisporus*, tyrosinase inhibitory peptide from split gill mushrooms, as well as antioxidant and anti-inflammatory peptides from *Ganoderma lucidum* mushroom (Arnamwong et al., 2024; Aursuwanna et al., 2022; Landi et al., 2022). Nevertheless, the stability of peptides *in vivo* is generally unfavorable as they are susceptible to degradation by gastrointestinal digestive enzymes, which leads to their loss of activity. In the meantime, the peptides that are produced through the simulated digestion of the gastrointestinal tract demonstrate exceptional stability (Molina-Valero et al., 2024; Yang et al., 2019). Thus, this approach provides the possibility to generate novel bioactive peptides from *H. erinaceus* with several health advantages including anti-inflammatory properties (Molina-Valero et al., 2024; Yang et al., 2019).

The objective of this study was to gain insight into the nutritional potential and anti-inflammatory properties of *H. erinaceus* proteins that were obtained through PEF-assisted extraction and subsequent *in vitro* gastrointestinal digestion. Additionally, the efficacy of purification processes was investigated in order to identify potential anti-inflammatory active peptide sequences.

2. Materials and methods

2.1. Chemicals and reagents

The following chemical reagents were purchased from Sigma-Aldrich Company (St. Louis, MO, USA): bovine serum albumin, pepsin (E.C.3.4.23.1, ≥250 units per milligram solid) from porcine gastric mucosa, trypsin (E.C.3.4.21.4, ≥10,000 BAEE units/mg protein) from the bovine pancreas, and trichloroacetic acid (TCA). Additionally, streptomycin (Gibco, United States), fetal bovine serum (Merck KGaA, Germany), and Dulbecco's Modified Eagle's Medium (Gibco, USA) were acquired.

2.2. Sample collection and preparation

Lion's Mane mushroom (21.24 ± 0.07 % protein, 4.37 ± 0.17 % ash, 39.98 ± 0.82 % total dietary fiber, 4.72 ± 0.82 % fat, and 29.69 ± 1.37 % available carbohydrates) were obtained from a Siam Organic Mushroom Farm in Chiang Mai, Thailand. The samples were washed, cut into 1–2 mm pieces, and freeze-dried. Then, the samples were ground into fine particles using a grinder, sieved by using a sieve (250 μm), and the powder was used for further experiments.

2.3. Protein extraction from H. erinaceus

Extraction of protein from *H. erinaceus* was carried out according to the method described by Thongkong et al. (2023) with slight modifications. After adding distilled water (1:20 w/v) to a powdered mushroom sample, NaOH was used to adjust the pH to 10. After 10 min of stirring with a magnetic stirrer, a pulsed electric field (PEF) was applied with 10,000, 15,000, 20,000, and 25,000 pulse numbers at two different electric field strengths (5 kV/cm and 10 kV/cm). Following that, a magnetic stirrer was used to mix each of the eight treatments for 1.5 h at room temperature. The control treatment was also administered without the use of PEF. The mixture of each treatment was then centrifuged (Himac CR 22N, GMI, USA) at 6000 rpm and 4 °C for 20 min. The supernatant was collected and, the total protein content was quantified using the Biuret method. The most effective treatment, providing the highest extracted protein, was then chosen to prepare the protein concentrate for further studies.

In order to determine the impact of PEF on mushroom cell walls following protein extraction, the micrographs in PEF-treated and untreated mushrooms coated with gold were examined using a field emission scanning electron microscope (TESCAN, Brno, Czech Republic) at 15 keV at 2000x.

2.4. Preparation of mushroom protein concentrate (MPC)

Mushroom protein concentrate (MPC) was prepared by using the most effective PEF-assisted extraction from 2.3, followed by isoelectric precipitation at pH 4.5, and then centrifuged at 6000 rpm, 4 °C for 20 min. After that, the precipitate was washed and centrifuged. Then the precipitate was adjusted to pH 7.0 and finally freeze-dried (MPC) as reported in Phongthai et al. (2018) with slight modifications. Then, protein yield was calculated using the following equation and compared with the conventional extraction (control) method.

$$\textit{Protein yield}\ (\%) = \frac{\textit{Amount of extracted protein in g}}{\textit{Amount of total protein in original mushroom powder}} \times 100$$

2.5. Characterization of MPC

2.5.1. Analysis of amino acid profile

Amino acid content in the extracted protein was analyzed by the AOAC Official Method using gas chromatography (6890N; Agilent Technologies, Santa Clara, CA, USA) coupled with a transmission quadrupole mass spectrometer (5973 Inert; Agilent Technologies). Initially, *H. erinaceus* proteins were dissolved in 6 M HCl, followed by incubation at the temperature of 110 °C for a day. Then, the pH level of this mixture was adjusted to 2.2 by dilution using a sodium citrate buffer. The quantity of individual amino acids was determined by comparing those of 17 standard amino acids using a Zebron ZB-AAA gas chromatography column (10 m × 0.25 mm × 0.25 μm) as mg/100 g of protein.

2.5.2. Determination of amino acid score (AAS)

Amino acid score (AAS) was calculated using the following equation.

$$\text{AAS} = \frac{\text{Essential amino acid content in MPC (mg per g protein)}}{\text{Recommened essential amino acid (mg per g protein)}}$$

The recommended essential amino acid, given by the Joint WHO/FAO/UNU Expert Consultation (2007), is provided in Table S-1 (Supplementary data).

2.5.3. Determination of in vitro protein digestibility

After preparing a protein solution (3 % w/v) and adjusting the pH to 1.5, it was incubated for five minutes at 37 °C. Pepsin was added to this reaction mixture in a 1:100 ratio of pepsin to protein and stirred continuously in a digestion chamber at 37 °C for 2 h. Then the pH was adjusted to 7.0, and trypsin was added in a 1:100 ratio of trypsin to protein and kept on digestion for 2 h.

The enzyme process was then terminated using a water bath that was kept at 95 °C. After that, 10 % TCA (100 mL) was added and centrifuged at 4500 rpm for 20 min by using a temperature-control centrifuge (OHAUS, USA). Precipitate was taken, freeze-dried, and analyzed for the remaining protein content. The protein digestibility was calculated as reported in Phongthai et al. (2016).

$$\textit{Protein digestibility}\ (\%) = \left\{\frac{(W_a \times P_a) - (W_u \times P_u)}{(W_a \times P_a)}\right\} \times\ 100$$

where, W_a and P_a are the weight and protein content of the sample taken for hydrolysis. W_u and P_u are weight and protein content of remaining undigested sample.

2.5.4. Determination of protein digestibility-corrected amino acid score (PDCAAS in vitro)

Protein digestibility corrected amino acid score (PDCAAS) was calculated using the following equation:

$$\text{PDCAAS value} = \text{AAS of the limiting amino acids} \times \textit{in vitro}\ \text{Potein digestibility}$$

2.6. Preparation of mushroom protein hydrolysates (MPH)

In vitro gastro-small intestinal hydrolysis was conducted according to the procedure conducted by Phongthai and Rawdkuen (2020) with slight modifications. After preparing the protein solution (3 % w/v), the pH of the *H. erinaceus* protein solution was adjusted to 1.5 and kept at 37 °C for 5 min. Pepsin was added to this reaction mixture in a 1:100 ratio of pepsin to protein (6.5 units of enzymes) and stirred continuously in a digestion chamber at 37 °C for 2 h. After that, the pH of the solution was increased to 7.0, and trypsin was added in a 1:100 ratio of trypsin to protein (260 units of enzymes) and kept on digestion for 2 h. The enzyme reaction was terminated by boiling at 95 °C for 10 min using a boiling water bath. Then the mixture of pepsin-trypsin hydrolyzed fraction was filtered using Whatman No. 4 filter paper, the filtrate was lyophilized, and the MPH was kept at −18 °C. The same procedure was carried out to prepare the pepsin hydrolyzed fraction without adding the trypsin. The final pH of both pepsin and pepsin-trypsin hydrolyzed fractions were maintained at 7.0 and these fractions were prepared in triplicate to conduct bioactivity evaluations.

2.7. Determination of secondary structure by Fourier-transform infrared spectroscopy (FTIR)

FTIR spectrometer (Jasco-4X, Japan) was used to determine the transmission infrared spectra of non-hydrolyzed and enzyme (pepsin and pepsin-trypsin) hydrolyzed mushroom protein samples by following the method described in Thongkong et al. (2023). After being grounded with KBr, the samples (~2 mg) were pressed into a thin pellet. At a resolution of 4 cm^{-1}, the measurement was taken from the 400 to 4000 cm^{-1} range. The amide I region's spectra (1600 to 1700 cm^{-1}), were divided into multi-component peaks based on particular wavelengths at 1657 cm^{-1} for α-helices, at 1611 and 1626 cm^{-1} for β-sheets, at 1673 and 1688 cm^{-1} for β-turns, and at 1642 cm^{-1} for random coils using the software OriginPro 2024 (OriginLab Corporation, Northampton, Massachusetts, USA).

2.8. Determination of anti-inflammatory activity of mushroom proteins

2.8.1. Cell culture

RAW 264.7, a mouse macrophage cells, were purchased from ATCC (ATCC, Manassas, VA, United States). The cells were grown in Dulbecco's Modified Eagle's Medium with 10 % fetal bovine serum (Merck KGaA, Germany), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco, United States) and incubated in a 37 °C humidified incubator with 5 % CO_2. All cellular experiments were performed in laboratory room (Registered No. 2–0040–0008–2), which has received peer evaluation approval at biosafety level 2 (BSL2) from the National Research Council of Thailand (Certification No. 02–2021/00,005).

2.8.2. Determination of cell viability

The potential cytotoxicity of *H. erinaceus* proteins and peptide fractions to RAW 264.7 macrophage cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test according to a previously published protocol in Hankittichai et al. (2020). Briefly, cells were seeded in 96-well-plate at 5×10^4 cells per well and incubated in complete medium for twenty-four hours. Cells were treated with *H. erinaceus* protein concentrate and hydrolysates at different concentrations from 0 to 800 μg/mL for an additional 24 h. After adding the MTT solution (0.5 mg/mL in PBS) to each well, the plates were incubated at 37 °C with 5 % CO_2 for 30 min. After aspirating the culture supernatants, DMSO (200 μL) was added to each well to dissolve a purple-colored formazan crystal. After that, a microplate reader (Micro Tek Instruments, USA) was used to measure the absorbance at a wavelength of 590 nm.

2.8.3. Determination of nitric oxide production

The nitric oxide test was carried out by measuring the total nitrate and nitrite concentration as an indicator of nitric oxide production using a commercial Griess reagent (Sigma, United States), as mentioned in Hankittichai et al. (2020). In summary, RAW 264.7 cells at 3×10^5 cells were seeded in 24-well plate and cultured for 24 h. The *H. erinaceus* protein concentrate (25, 50, 100 and 200 μg/mL) and the HE mushroom peptide fractions (3.75, 7.5, and 15 μg/mL) were added 4 h before stimulated with 1 μg/mL of lipopolysaccharide (Sigma, USA). Cells were further incubated for 20 h. The 100 μL of culture supernatant from every well was taken and combined with an equivalent amount of Griess reagent in a new 96-well plate. The mixture was incubated 15 min at room temperature. A pink-colored of nitrite ions was measured using a microplate reader (BioTek Instruments, United States) at 540 nm.

2.8.4. Determination of IL-6 production

RAW 264.7 cells at 3×10^5 cells/well were seeded in 24-well-plated for overnight. The protein hydrolysate and lipopolysaccharide (LPS) were solubilized in deionized water to treat RAW 264.7 cells. Consequently, DI water served as the vehicle control for LPS-treated RAW 264.7 cells. The *H. erinaceus* proteins (25, 50, 100 and 200 μg/mL) and peptide fractions (3.75, 7.5, and 15 μg/mL) were added 4 h before stimulated with LPS at 1 μg/mL. Cells were further incubated for 20 h. The enzyme-linked immunosorbent (ELISA) assay was performed using ELISA MAXTM Deluxe Set (BioLegend, San Diego, CA, USA) to detect the IL-6 production in culture supernatant. The method was followed according to manufactured manual and in Hankittichai et al. (2020). Briefly, the capture antibody in coating buffer (100 μL of 1x capture antibody) was applied to the ELISA 96-well plate and incubated at 4 °C overnight. After four rounds of cleaning with wash buffer (0.05 % Tween-20 in PBS) the plate was blocked for an hour at room temperature using 200 μL of blocking buffer. After cleaning the plate four times with

wash buffer, the standard mouse IL-6 at range 0–500 pg/mL and sample supernatants were added into each well and incubated at room temperature for 2 h. After washing with wash buffer 4 times, 100 μL of a specific detection antibody was added into each well at room temperature for 1 h. After washing with wash buffer 4 times, the 100 μL of horseradish peroxidase (HRP)-conjugated avidin (at ratio of 1:1000) was added and incubated for 30 min. After washing, the 100 μL of freshly prepared TMB substrate solution was added and incubated in the dark for 15–30 min to develop the signal. Then, 100 μL of stop solution (2 N of H_2SO_4) was used to stop the reaction. The absorbance at 450 nm was measured using a microplate reader (BioTek Instruments, United States). Out of 2.9.3 and 2.9.4, the best anti-inflammatory fraction was chosen for fractionation by ultrafiltration.

2.9. Fractionation and purification of anti-inflammatory peptides

2.9.1. Fractionation of mushroom protein hydrolysate (MPH) by ultrafiltration

The most potent anti-inflammatory active peptide fraction was isolated utilizing a membrane ultrafiltration technique (Amicon® stirred cells, MERCK, Darmstadt, Germany), as described by Fashakin et al. (2023), with a few modifications. The sample (0.5 % w/v) solution was prepared, homogenized, and then separated using molecular weight (MW) cut-offs of 10, 5, and 3 kDa membranes to collect the fractions with MW >10 kDa, 5–10 kDa, 3–5 kDa, and <3 kDa. All fractions were lyophilized, and the anti-inflammatory activity was assessed to identify the most potent anti-inflammatory fraction for subsequent purification via size exclusion chromatography.

2.9.2. Fractionation of selected hydrolysate fraction by size exclusion chromatography

A minor alteration of the methods previously reported by Fashakin et al. (2023) was implemented in this study. The homogenized solution obtained by dissolving the lyophilized sample obtained from membrane fractionation (1.0 g in 400 mL of deionized water) was injected through a versatile preparative liquid chromatography system (LC-Forte/R-II, YMC Co., LTD., Japan), equipped with a preparative column (Jaigel-W255, Japan Analytical Industry Co., Ltd., Japan). A UV detector at 280 nm and 220 nm wavelengths, in suction mode, was used for the elution of the fraction at a flow rate of 10 mL/min. Fractions were collected separately using the fraction collector, and then each fraction was lyophilized to identify the potent anti-inflammatory active sequences.

2.10. Identification of peptide sequence by LC-MS/MS

The methodology outlined by Fashakin et al. (2023) was adapted to identify the amino acid sequences of potential anti-inflammatory peptides through LC-MS/MS analysis. The lyophilized peptide fractions obtained from preparative HPLC were dissolved in water with 0.1 % formic acid with a concentration of 0.2 μg/mL. Orbitrap HF mass spectrometry with an ESI ion source working at 3.2 kV was employed to study the peptidomics properties. A C18 column was loaded with peptides (1 μg/ 5 μL) with 2 technical replications. Throughout the separation, the temperature of the column was maintained at 60 °C. Water containing 0.1 % formic acid (A) and acetonitrile containing 0.1 % formic acid (B) made up the mobile phase with 0 min at 95 % A, 15 min at 60 % A, 20 min at 20 % A, 25 min at 5 % A, and 35 min at 95 % A as the gradient elution program. A solution of 0.1 % formic acid/water was administered following each injection to avoid overloading of samples in between runs. To generate MS spectrum results employing the Top 3 approach, the most common precursor ions with charge states (+1 to +5) were dynamically chosen over a wide array of survey scans (150–2000 m/z). The dynamic exclusion lasted 20 s. Precursor isolation was performed at a mass of 1.4 m/z, and MS/MS scans were acquired at an initial mass of 120 m/z. Survey scans with a resolution of 120 k were collected at 400 m/z. The fragmentation spectra were resolved to 30,000 at m/z 200, and 29 was the HCD-normalized collision energy. Quality control of the sample preparations and LC-MS/MS parameters were performed in order to confirm the repeatability data. Both chromatographic analysis and MS-acquisition analysis were performed on two LC-MS runs.

2.11. Statistical analysis

Every assay was performed in triplicate, and the mean ± SD was used to express the results. The analysis of the variance in the test results was determined using the IBM-SPSS software (Version 17). Tukey's HSD was used to compare the mean values of each parameter, and tests for significance were conducted below the 0.05 level.

3. Results and discussion

3.1. Protein extraction using pulsed electric field (PEF)-assisted extraction

The total soluble protein obtained from PEF-assisted extraction is shown in Table 1. The pulse number significantly improved protein extraction efficiency ($p < 0.05$). However, using the maximum number of pulses and electric field strengths can reduce the amount of protein retrieved. Meanwhile, the total soluble protein in the treatments with 10,000 pulses at both electric field strengths and 15,000 pulses at 10 kV/cm did not differ substantially from the control (stirring at room temperature for 1.5 h, $p > 0.05$). The maximum soluble protein (2266.44 ± 40.23 mg) and protein yield (34.71 ± 1.32 %) were achieved at 5 kV/cm with 25,000 pulses ($p < 0.05$), representing a 42.44 % increase compared to the control treatment.

At both electric field strengths, an increase in pulse number resulted in a better protein extraction efficiency. However, the extracted protein was significantly diminished at 10 kV/cm with an increased pulse number in contrast to 5 kV/cm. Similar research was conducted by Liu et al. (2013) and Calleja-Gómez et al. (2022), and the tendencies identified in these studies correspond with those in our examination. Calleja-Gómez et al. (2022) investigated the effective ness of PEF-assisted process at different electric field strengths (2–3 kV/cm) for protein extraction from *Agaricus bisporus*. As the electric field strengths intensified, protein recovery increased until reaching 2.5 kV/cm, after which it declined with additional increases in electric field strength. Liu et al. (2013) explained the reasons for decreasing the protein recovery at such high electric field strengths (50 kV/cm) in soybean protein extraction that when increasing the electric field strengths gradually to 30 kV/cm, unfolding of protein molecules was observed. Moreover, when increasing the electric field strengths further up to 50 kV/cm, reassembly in unfolding proteins was detected. According to Taha et al. (2023), PEF treatment caused surface hydrophobicity to increase at electric field strengths of 10 kV/cm, while decreasing at higher electric

Table 1
Total soluble protein obtained from PEF-assisted extraction.

Electric field strength (kV/cm)	Number of pulses	Total soluble protein (mg)
5 kV/cm	10,000	1654.89 ± 15.31^{dc}
	15,000	1675.10 ± 17.58^{c}
	20,000	1820.52 ± 10.09^{b}
	25,000	2266.44 ± 40.23^{a}
10 kV/cm	10,000	1614.16 ± 11.75^{dc}
	15,000	1615.45 ± 22.03^{dc}
	20,000	1670.14 ± 27.87^{c}
	25,000	1821.88 ± 19.41^{b}
Conventional extraction (pH 10, 1.5 h, Room temperature)		1591.13 ± 45.99^{d}

Note: Values were expressed as means ($n = 3$) ± SD. Tukey HSD was used to compare the results. Different superscripts in each column represent significantly different mean values ($p < 0.05$).

field strengths. PEF facilitated hydrophobic interactions between unfolded proteins, leading to the formation of soluble protein aggregates (Taha et al., 2023). In conclusion, one of the main causes of extracted protein decrease may be the aggregation of unfolded protein structures.

The electroporation process occurred when the mushroom was exposed to an electric field that produced an electric potential. If the potential exceeds a specific threshold, pores develop in the membrane's weak areas. The strength of the electric field and the duration of the treatment can be increased to increase the size of the openings, while a low-intensity PEF treatment may result in smaller pores. The electroporation effects due to PEF on cellular structure can be observed under the scanning electron microscopy as shown in Fig. 1. A scanning electron microscopy (SEM) microstructure image showed that the mushroom cell wall had an integral block structure (A); in contrast, the PEF-treated sample (B) had a clear appearance of exposed pores surrounding the cell wall, as shown by white arrows. This confirms that the elevated protein levels in the PEF-treated mushroom samples are linked to the presence of numerous micropores or cavities. Previous studies indicated that enhancing cell permeability at the critical electrical potential resulted in structural alterations in the cells and the electrical disintegration of rapeseed cell membranes, thereby releasing intracellular proteins into the solution (Yu et al., 2015).

Our research and other studies indicate that the application of PEF markedly enhances protein extraction efficiency. Nonetheless, establishing a continuous process that is suitable for industrial applications may pose challenges for manufacturers. However, PEF may also serve as a batch pre-treatment method prior to implementing a continuous extraction process. Moreover, the uneven source materials may lead to suboptimal PEF application, thus adversely affecting protein extraction efficiency. Therefore, it is essential for manufacturers to undertake thorough optimization processes.

3.2. Characterization of HE proteins

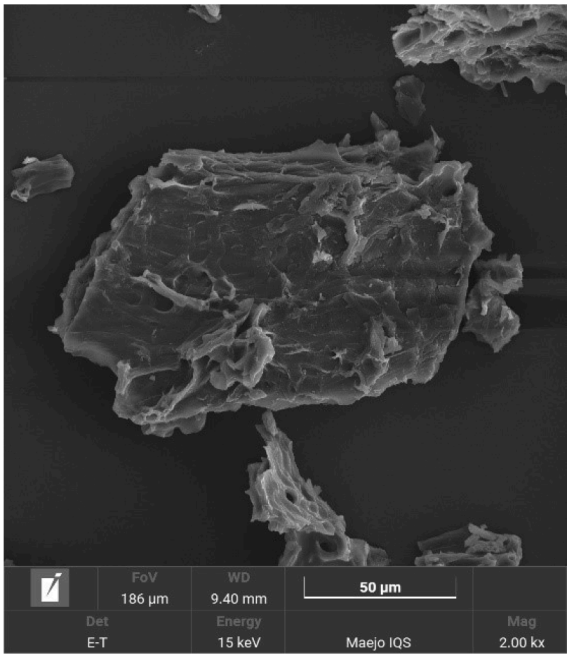
3.2.1. Amino acid profile

Table 2 indicates that all investigated essential amino acids were present in the HE proteins, constituting 44.59 ± 0.31 % of the total amino acids. The most abundant amino acids were aromatic amino acids (59.68 ± 0.38 mg/g protein), leucine (58.12 ± 0.30 mg/g protein), valine (47.06 ± 0.46 mg/g protein), and lysine (35.45 ± 0.94 mg/g protein). Further, all the values obtained in the current study are higher than those obtained by Rodríguez-Martín et al. (2024) in chickpea flour, which contained histidine (6.32 ± 0.07 mg/g protein), isoleucine (9.89 ± 0.19 mg/g protein), leucine (17.69 ± 0.21 mg/g protein), lysine (16.26 ± 0.22 mg/g protein), sulfur-containing amino acids (SAA) (3.92 ± 0.19 mg/g protein), aromatic amino acids (20.62 ± 0.54 mg/g protein), threonine (8.9 ± 0.14 mg/g protein), and valine (10.39 ± 0.20 mg/g protein). However, studies conducted by Yi-Shen et al. (2018) and Sá et al. (2021) indicated that the sesame seed meal and mung bean protein isolate contained higher SAA and lysine with values of 47.5 mg/g of SAA and 62.4 mg/g of lysine, respectively.

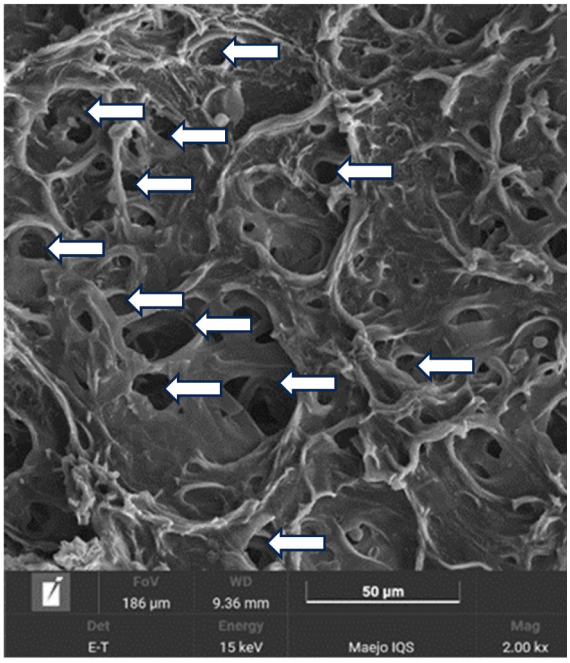
Saisavoey et al. (2021) and Feng et al. (2021) determined that hydrophobic amino acids demonstrate superior bioactive characteristics in peptides generated from plants. The *H. erinaceus* protein comprised 45.70 ± 0.69 % hydrophobic amino acids as the predominant group. Consequently, it possesses considerable potential for use as a raw material in the production of bioactive nutraceutical compounds.

3.2.2. Amino acid score (AAS)

Essential amino acid content obtained from our study was compared with the reference pattern given by WHO/FAO/UNU Expert Consultation (2007). The amino acid score (AAS) of *H. erinaceus* protein is shown in Table 3. Sulfur-containing amino acids (SAA) and lysine were found to be the limiting amino acids in *H. erinaceus* proteins in relation to all age categories. The AAS for lysine is 0.62, 0.68, 0.74, 0.74, 0.75, and 0.79, whereas the AAS for SAA is 0.40, 0.43, 0.47, 0.49, 0.49, and 0.51



(A)



(B)

Fig. 1. Microstructures appeared in SEM images; (A) Control sample with magnification x 2000, and (B) PEF treated sample with magnification x 2000.

for age categories of 6–11 months, 1–2 years, 3–10 years, 11–14 years, 15–18 years, and 18+ years, respectively. These results are consistent with the findings of the previous study, which found that, in relation to the amino acid requirement for children, lysine was the limiting amino acid in *Terfezia claveryi* and *Tricholoma terrum*, with AAS of 0.71 and 0.67, respectively, while SAA was the limiting amino acid of *Pleurotus ostreatus* and *Agaricus macrosporus* proteins, with AAS of 0.61 and 0.50,

Table 2
Amino acid content (mg/g protein) in *H. erinaceus* mushroom proteins.

Essential amino acids (EAA)									
Histidine	Isoleucine	Leucine	Lysine	SAA*	AAA**	Threonine	Valine	SUM	%EAA
16.41 ±0.60	29.75 ±0.13	58.12 ±0.30	35.45 ±0.94	11.21 ±0.10	59.68 ±0.38	33.02 ±0.90	47.06 ±0.46	264.24 ±8.92	44.59 ±0.31

Non-essential amino acid (non-EAA)							
Aspartic acid	Serine	Glutamic acid	Proline	Glycine	Alanine	Arginine	SUM
65.01 ±1.11	29.43 ±0.71	74.05 ±1.39	25.39 ±1.44	30.46 ±0.35	38.15 ±0.76	39.32 ±0.65	328.30 ±6.40

SAA*- sulphur containing amino acids (methionine and cysteine); AAA**-aromatic amino acids (phenyl alanine and tyrosine).

Table 3
Amino acid score of *H. erinaceus* proteins.

Essential amino acids (EAA)	Amino Acid Score						Limiting amino acids
	6–11 month	1–2 years	3–10 years	11–14 years	15–18 years	18+ years	
Histidine	0.82	0.91	1.03	1.03	1.03	1.09	
Isoleucine	0.93	0.96	0.96	0.99	0.99	1.00	
Leucine	0.88	0.92	0.95	0.97	0.97	1.00	
SAA*	0.40	0.43	0.47	0.49	0.49	0.51	First
Lysine	0.62	0.68	0.74	0.74	0.75	0.79	Second
AAA**	1.15	1.30	1.46	1.46	1.49	1.57	
Threonine	1.07	1.22	1.32	1.32	1.38	1.44	
Valine	1.09	1.12	1.18	1.18	1.18	1.21	

SAA*-sulfur-containing amino acids, AAA**-aromatic amino acids

respectively (Dabbour & Takruri, 2002).

As a result, *H. erinaceus* proteins are incomplete proteins containing two limiting amino acids; hence, they may be blended with high-lysine and SAA proteins to yield a complete protein. Sá et al. (2021) indicated that the amino acid score (AAS) of sulfur-containing amino acids (SAA) in sesame seed meal protein was 2.04, while Kowalczewski et al. (2019) determined that the AAS of lysine in potato juice protein concentrate was 1.23. Therefore, a mixture of sesame seed meal and potato juice protein concentrates with *H. erinaceus* proteins in a 1:1:1 ratio can offer an extensive selection of essential amino acids comparable to the nutritional value of animal-based proteins, yielding 1.28 for SAA and 1.01 for lysine, respectively. Consequently, *H. erinaceus*-based protein blends can be utilized in food formulation to address malnutrition by providing an extensive selection of amino acids.

3.2.3. *In vitro protein digestibility*

Basically, proteins are regularly altered upon entering the gastrointestinal tract due to a specific enzymatic hydrolysis, which degrades them into small fragments *e.g.* tripeptides, dipeptides, and amino acids. Under the gastrointestinal tract, the endopeptidase, pepsin, is more selective in its ability to degrade peptide bonds in which the carboxyl group is supplied by aromatic amino acids such as tyrosine, phenylalanine, tryptophan, and leucine. In the meanwhile, trypsin, the most specialized pancreatic protease, degrades the carboxyl-terminal peptide bonds of arginine or lysine in polypeptide chains (Goodman, 2010b). As a result of this specific enzyme function, the digestibility of proteins is dependent upon the substrate present in the protein structure, resulting in a range of digestibility. The *H. erinaceus* protein has an *in vitro* digestibility of 71.33 ± 0.30 %. Meanwhile, the protein digestibility values for *Tricholoma terrum, Terfezia claveryi, Pleurotus ostreatus,* and *Agaricus macrosporus* were 52.6 %, 61.4 %, 73.4 %, and 80.5 %, respectively, as reported by Dabbour et al. (2002). Conversely, the protein isolates from sesame (87.57 ± 4.41 %), flaxseed (85.41 ± 2.04 %), and canola (82.13 ± 2.86 %) had somewhat higher digestibility values (Sibt-E-Abbas et al., 2022). According to the research reported by Wang et al. (2023), the protein digestibility for mushrooms was in the range of 72–83 %, whereas that was 82 % for rice, 74 % for soybeans, 98 % for eggs, 97–98 % for dairy, and 92–94 % for meat. Moreover, the structural characteristics of proteins are intimately linked to their digestibility. Low protein digestibility may cause by many factors including large molecular weight, complicated structures, high degree of folding, and aggregations. Additionally, trypsin inhibitor, the residual cellulose, pectin, glucan, and mannan found in plant, fungal, and algae proteins might hinder the interaction of digestive enzymes with proteins, hence reducing digestibility (Zeng et al., 2022).

In general, protein exhibits even greater bioactivities after being hydrolyzed by several proteases, as reported by Akbarian et al. (2022) and Aursuwanna et al. (2022). Nevertheless, the peptide fragments generated by non-gastrointestinal proteases are highly susceptible to degradation by gastrointestinal digestive enzymes, which leads to their loss of activity. Thus, the *in vitro* gastrointestinal tract has the potential to both stimulate bioactivity and maintain the stability of peptides, providing a viable method for conserving the bioactivity of peptides upon their entry into the gastrointestinal tract.

3.2.4. *Protein digestibility–corrected amino acid score (PDCAAS)*

PDCAAS is a method of evaluating protein quality that considers both human amino acid requirements and digestibility. It is calculated as the ratio of the first limiting amino acid in a test protein to the corresponding amino acid in a reference amino acid pattern, then corrected for digestibility (FAO/WHO/UNU, 2007). High value of PDCASS indicates high protein quality. As mentioned earlier, the physical and chemical properties of the proteins or related molecules existing in protein sources determine their bioviability. Even if protein has a perfect amino acid composition in line with the scoring pattern, its capacity to provide essential amino acids for human function will be impaired if it is not entirely digested and absorbed.

In this study, PDCAAS for limiting amino acids (lysine and sulfur-containing amino acids) of *H. erinaceus* protein depending on the different age groups is shown in Table S-2 (Supplementary data). PDCAAS for sulfur-containing amino acids (SAA), the first limiting amino acid, was only 28, 31, 34, 35, 35, and 36, whereas PDCAAS for lysine, the second limiting amino acid, was 44, 48, 53, 53, 54, and 56, for the age categories of 6–11 months, 1–2 years, 3–10 years, 11–14

years, 15–18 years, and 18+ years, respectively. However, the PDCAAS values in our study are greater for lysine than the result obtained in the research done by Dabbour et al. (2002), for *Terfezia claveryi* (43 %) and *Tricholoma terrum* (35 %), while for SAA, the PDCAAS value was lower than in *Pleurotus ostreatus* (45 %) and *Agaricus macrosporus* (40 %) proteins. Nonetheless, enhancing the PDCAAS may involve combining *H. erinaceus* protein with other protein sources that are abundant in specific limiting amino acids. Alterations in amino acid composition and amino acid score may result in improved protein digestion, facilitating a higher PDCAAS.

3.3. Protein secondary structure changes

FTIR spectroscopy has been frequently used to detect the secondary structure of proteins. The amide I band region (1600–1700 cm^{-1}) was found to be the most sensitive for alteration of the secondary structure of proteins, so this region was selected for the deconvolution purpose. Fig. 2 shows the peak-fitting spectra of unhydrolyzed, pepsin hydrolyzed, and pepsin-trypsin hydrolyzed fractions after the deconvolution of the amide I region. Ratios of secondary structure components of α-helices, β-sheets, β-turns, and random coils are shown in Table 4. All secondary structure components were altered remarkably by the digestion process. The deconvolution process revealed that the fundamental components of unhydrolyzed *H. erinaceus* protein consisted of β-sheets (32.17 ± 0.24 %), α-helices (24.89 ± 0.32 %), random coils (22.33 ± 0.20 %), and β-turns (20.63 ± 0.71 %). Following *in vitro* gastrointestinal digestion, β-sheets exhibited a significant decrease from 32.17 ± 0.24 % to 24.54 ± 0.18 %, representing an approximate reduction of 23.74 %. During this period, β-turns increased from 20.63 ± 0.71 % to 26.57 ± 0.34 %. The increments and losses observed were significant in the pepsin and pepsin-trypsin digestions ($p < 0.05$).

However, the content of α-helices significantly increased only in pepsin digestion. These results are consistent with the results obtained by Zhang et al. (2022) by observing the loss of β-sheets while gaining more α-helices during *in vitro* digestion of green wheat proteins. It was found that the loss of β-sheets is due to the unfolding of β-sheet structure during digestion (Zhang et al., 2022). Further, the random coil content of green wheat proteins also increased in the same study. However, in our study, a significantly high number of random coils was observed only in the pepsin digested fraction. According to the report of Yang et al. (2016), proteins with a larger random coil and a correspondingly lower β-sheet component have a less compact and more unordered molecular structure that is easier to be hydrolyzed. Therefore, in our study, the pepsin hydrolyzed fraction may have the desirable secondary structure composition for further hydrolysis by pepsin-trypsin hydrolysis.

Table 4
The secondary structure content of *H. erinaceus* mushroom proteins before and after digestion.

Treatment	α-helices	β-sheets	β-turns	Random coils
Unhydrolyzed proteins	24.89 ± 0.32^{b}	32.17 ± 0.24^{a}	20.63 ± 0.71^{c}	22.33 ± 0.20^{b}
Pepsin hydrolyzed proteins	26.28 ± 0.39^{a}	26.49 ± 0.33^{b}	23.53 ± 0.47^{b}	23.70 ± 0.20^{a}
Pepsin-trypsin hydrolyzed proteins	26.13 ± 0.22^{a}	24.54 ± 0.18^{c}	26.57 ± 0.34^{a}	22.80 ± 0.18^{b}

Note: Values were expressed as means ($n = 3$) ± SD. Tukey HSD was used to compare the results. Different superscripts in each column represent significantly different mean values ($p < 0.05$).

3.4. Determination of anti-inflammatory activity

3.4.1. The effect of protein fraction on RAW 264.7 cells viability

Inflammation is a biological response of the body to microorganism or tissue injury. Even though this process is important for defending and healing process of many diseases, over production of an inflammatory mediators can cause severe tissue damage. Therefore, inhibiting inflammatory mediators may prevent the damage cause by inflammatory diseases. Macrophages play a role in innate immune response especially upon bacterial-LPS activation. RAW 264.7 mouse macrophage cell was used to determine anti-inflammatory activity of protein and peptides. Before testing anti-inflammatory activities, finding a non-toxic concentration of proteins or peptides was performed using cell viability assay (MTT assay). The results showed that all three fractions (undigested protein, pepsin-digested fraction, and pepsin-trypsin digested fraction) at 0–200 μg/mL were not toxic to macrophage cells. Therefore, all three fractions at 25, 50, 100 and 200 μg/mL were used in subsequent experiments to investigate anti-inflammatory properties. Interestingly, undigested protein at 25 μg/mL to 100 μg/mL could promote cell proliferation approximately 1.5 times (Fig. 3). Moreover, pepsin digested fractions and pepsin-trypsin digested fractions at 25–400 μg/mL significantly increased cell viability to 2–2.6 times and 2.3–3 times, respectively. However, proteins at 400–800 μg/mL and pepsin-trypsin digested fractions at 800 μg/mL were significantly toxic to those cells. Increasing cell viability is normally related to increase cell number which reflex proliferative activity. How *H. erinaceus* peptide fractions enhance cell proliferation should be confirmed with cell counting and further investigated.

3.4.2. The effect of protein fraction on the production of nitric oxide (NO)

Nitric oxide (NO) is overproduced under abnormal circumstances, and NO is considered to be a pro-inflammatory mediator that causes inflammation (Yuan et al., 2017). The results showed that LPS increased NO production in RAW 264.7 macrophage cells compared to non-treated condition. The current study indicates that undigested proteins did not show a statistically significant difference in results compared to the LPS control group ($p < 0.05$); however, a trend suggests that NO production decreases with increasing protein concentration. The lowest level of NO production was observed at the highest protein concentration of 200 μg/mL, as illustrated in Fig. 4. Interestingly, pepsin digested fraction significantly reduced nitric oxide formation at a concentration of 200

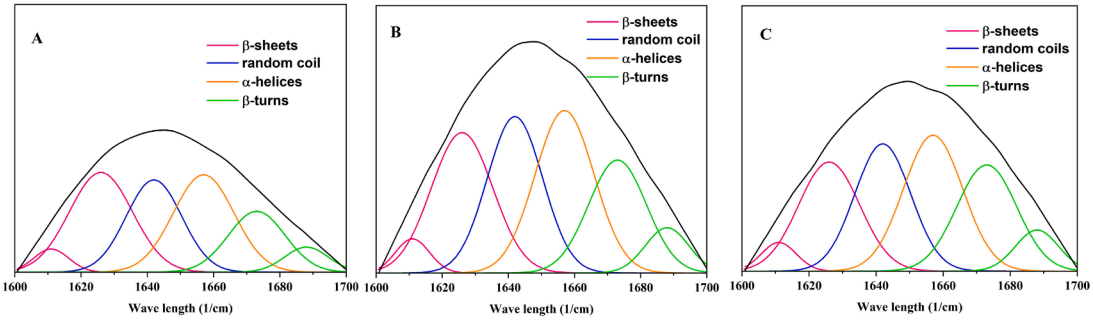


Fig. 2. The fitted peaks of unhydrolyzed (A), pepsin hydrolysed (B) and pepsin-trypsin hydrolysed (C) *H.erinaceus* mushroom proteins.

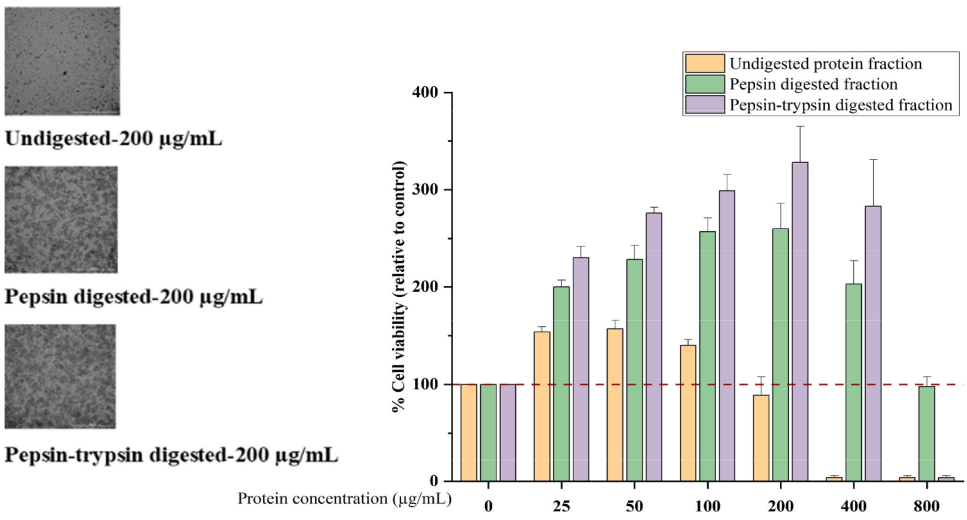


Fig. 3. Effects of unhydrolyzed, pepsin hydrolyzed and pepsin-trypsin hydrolyzed fractions on the survival of RAW 264.7 macrophages at 48 h. Bars indicate the standard deviation of the mean.

µg/ml, while pepsin-trypsin digested fraction significantly reduced NO formation at a concentration of 100–200 µg/ml ($p < 0.05$). The reduction percentages in NO formation were 7.9 % and 14.5 % for undigested proteins, 14.2 % and 27.5 % for pepsin-digested fractions, and 26.9 % and 36.2 % for pepsin-trypsin digested fractions at concentrations of 100 µg/mL and 200 µg/mL, respectively. This can be speculated that digested peptides or smaller fraction peptides somehow enhanced anti-inflammatory properties. Akbarian et al. (2022) pointed out that most of the bioactive peptides are generally buried in the structure of mature proteins, and these peptides may function when released by the hydrolysis processes. Sandoval-Sicairos et al. (2021) conducted similar research on germinated amaranth *in vitro* gastrointestinal digested fractions, demonstrating a 42.0 % and 53 % reduction in NO formation compared to the LPS-treated control at concentrations of 1 and 3 mg/mL, respectively.

The findings of our study indicated that all three protein fractions exhibited anti-inflammatory effects through the reduction of nitric oxide production. *In vitro* pepsin-trypsin digestion significantly reduces inflammatory effects by decreasing NO production at a concentration of 100–200 µg/mL ($p < 0.05$). This fraction demonstrates the highest potential for inhibiting inflammation when compared to undigested and pepsin-digested proteins. However, employing *in vitro* anti-inflammatory investigations may not reliably anticipate the physiological impact of anti-inflammatory peptides in the human immune system due to the complexity of intracellular defenses and cell signaling pathways.

3.4.3. *The effect of protein fractions on production of Interleukin-6 (IL-6)*

Elevated levels of IL-6 have been associated with rheumatoid arthritis and other chronic inflammatory diseases, and autoimmune diseases induced through experimental methods are notably affected by IL-6 (Yuan et al., 2017). In this study, the results indicated that both hydrolyzed and unhydrolyzed protein fractions effectively suppressed the pro-inflammatory cytokine IL-6, which was induced by LPS, in a dose-dependent manner (Fig. 4).

Fig. 4 indicates that IL-6 production was significantly lower in the presence of pepsin and pepsin-trypsin hydrolyzed fractions at a concentration of 100 µg/mL, when compared to the LPS-treated control ($p < 0.05$). The formation of IL-6 was reduced by 13.8 % and 15.1 % for undigested fractions, 23.0 % and 26.8 % for pepsin-digested fractions, and 28.6 % and 31.8 % for pepsin-trypsin digested fractions at concentrations of 100 and 200 µg/mL, respectively. Garcia-Mora et al. (2015) conducted a comparable study examining the anti-inflammatory properties of pinto bean proteins. The hydrolysate produced by Alcalase exhibited the most significant inhibition of IL-6 secretion, achieving a 28 % reduction compared to the control treatment.

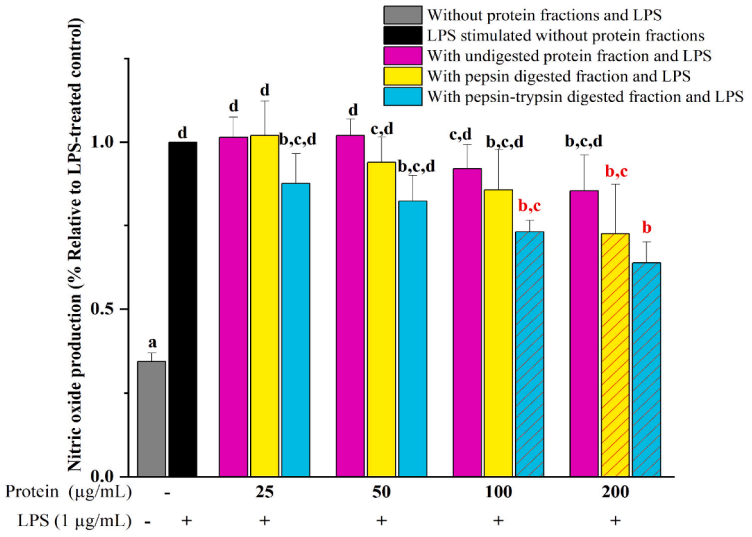
Macrophages are significant immune cells that secrete pro-inflammatory cytokines (such as IL-1β and IL-6), chemokines, nitric oxide, and reactive oxygen species (ROS) that regulate inflammation and host defence. Excessive macrophage activation, however, might have negative consequences as well, like septic shock, which can result in multiple organ dysfunction syndrome and even death. Chronic inflammation, including inflammatory bowel disorders and rheumatoid arthritis, can arise from persistent pro-inflammatory activity (Yuan et al., 2017). Thus, a viable way to stop unfavorable inflammatory effects is to modulate macrophage activation appropriately.

According to our results, the pro-inflammatory cytokines IL-6 and NO-induced by LPS were inhibited in a dose-dependent manner by both hydrolyzed and unhydrolyzed protein fraction. However, the pepsin-trypsin hydrolysate of *H. erinaceus* showed the highest anti-inflammatory benefits, with significant inhibition of pro-inflammatory mediators NO and IL-6 by 26.9 % and 28.6 %, respectively, at 100 µg/mL compared to the LPS-stimulated RAW 264.7 macrophages. It was found that enzymatic digestion could effectively liberate bioactive fragments from natural-origin proteins. Pepsin and trypsin enzymes are capable of preferentially breaking internal peptide bonds of the protein molecules and forming several peptides. Pepsin cleaves the bonds where carboxyl groups are supplied by tyrosine, phenylalanine, tryptophan, and leucine, whereas trypsin cleaves the bonds where the C-terminal side is provided by lysine or arginine (Goodman, 2010). Regarding the pepsin-trypsin hydrolyzed fraction, these two enzymes might cause peptide fractions to have more hydrophobic and positively charged amino acids. Many studies found that hydrophobic and positively charged amino acids in peptides exhibit high bioactive properties (Feng et al., 2021; Han et al., 2018; Ji et al., 2020; Wang et al., 2021). Since the pepsin-trypsin hydrolyzed fraction showed the highest anti-inflammatory activity with the greatest reduction of formation of NO and IL-6, in our study, this fraction was selected for further fractionations.

3.5. *Fractionation of anti-inflammatory peptides*

3.5.1. *Membrane ultrafiltration*

3.5.1.1. Cell viability. This study examined the cytotoxic effects of various molecular weight fractions of pepsin-trypsin hydrolyzed



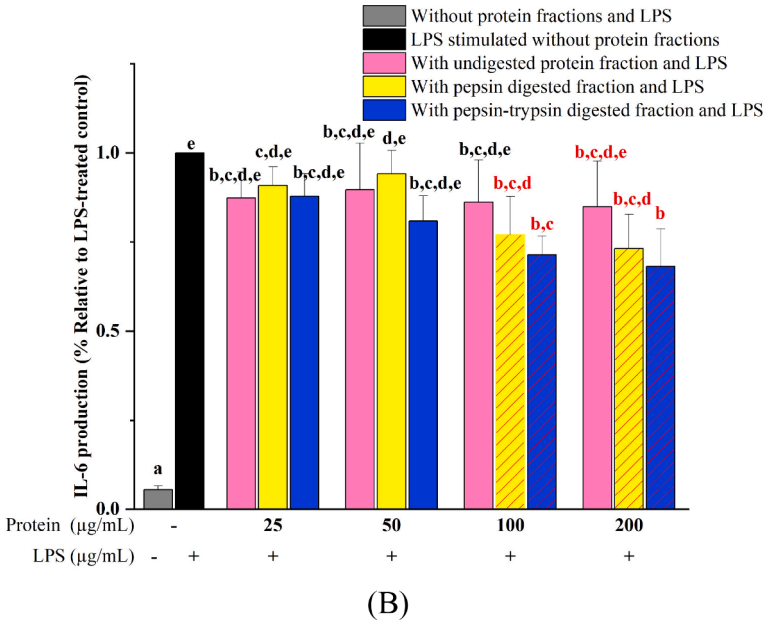
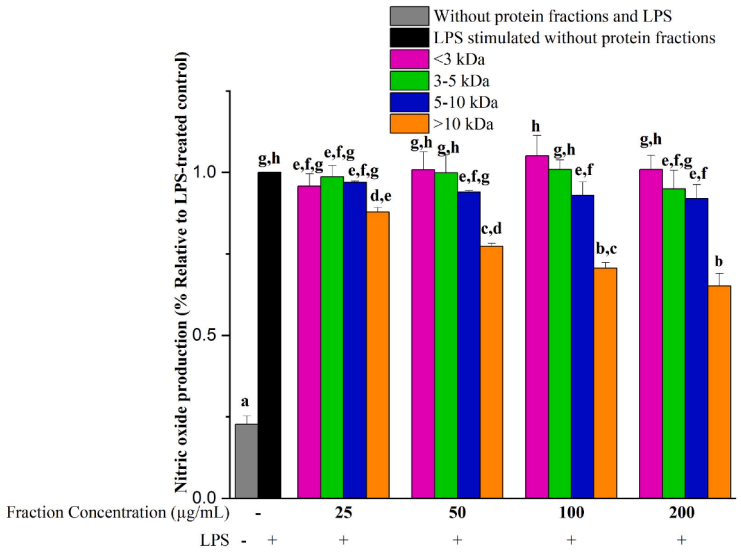


Fig. 4. Effects of unhydrolyzed, pepsin hydrolysed and pepsin-trypsin hydrolysed samples on LPS stimulated nitric oxide (A) and interleukin-6 (B) levels in RAW 264.7 macrophages. Tukey HSD was used to compare the results. Different lower-case letters show significant differences among treatments for each fraction ($p < 0.05$ compared to the LPS control group). Bars indicate that the standard deviation of the mean value.

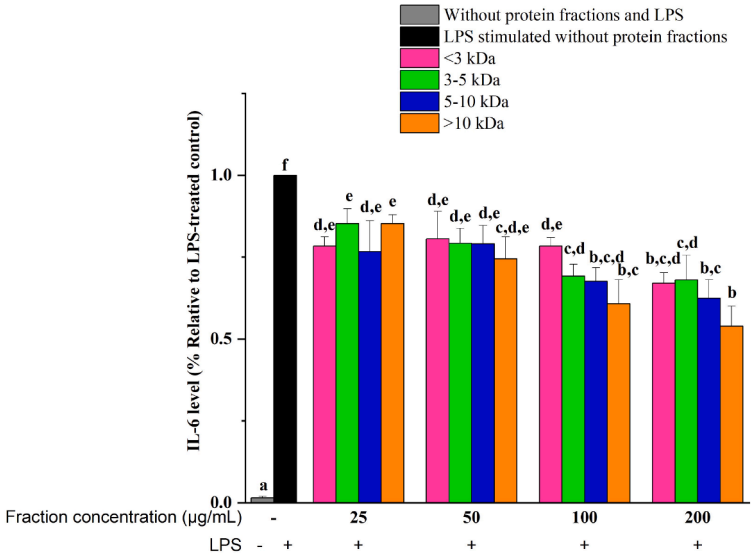
fractions on the survival of murine macrophage RAW 264.7 cells. The optimal concentration that is non-cytotoxic to the cells was identified for potential application in subsequent experimental studies. The fractions with molecular weights (MW) of <3 kDa, 3–5 kDa, 5–10 kDa, and >10 kDa were assessed at concentrations varying from 0 to 1000 µg/mL. Subsequently, cell viability was assessed using the MTT assay. The findings indicated that the <3 kDa fraction exhibited no toxicity at a maximum concentration of 500 µg/mL, while all other fractions demonstrated no toxicity at a maximum concentration of 250 µg/mL (Supplementary data). The non-toxic level was established at 200 µg/mL for subsequent studies.

3.5.1.2. The effect of fractionated peptides on the production of NO and IL-6. The fraction hydrolyzed by pepsin-trypsin were subjected to membrane ultrafiltration to yield fractions of varying MWs: >10 kDa, 5–10 kDa, 3–5 kDa, and <3 kDa. The production of NO and IL-6 was analyzed to assess the anti-inflammatory activity across these four fractions. The fractions with MWs greater than 10 kDa and between 5–10 kDa significantly decreased NO production compared to the LPS-treated control, at concentrations of 25 µg/mL and 100 µg/mL, respectively ($p < 0.05$, Fig. 5). Nonetheless, production was not significantly reduced by the 3–5 kDa and <3 kDa fractions ($p > 0.05$).

When considering the production of IL-6, all the fractions significantly decreased the production of IL-6 in all concentration levels from 25 µg/mL (Fig. 5), compared to the LPS-treated control ($p < 0.05$). As



(A)



(B)

Fig. 5. Effects of different peptide fractions obtained by membrane ultra filtration (>10 kDa, 5–10 kDa, 3–5 kDa, and <3 kDa) on LPS stimulated NO (A) and interleukin-6 (B) levels in RAW 264.7 macrophages. Tukey HSD was used to compare the results. Different lowercase letters indicate significant differences among treatments for each fraction ($p < 0.05$, compared to the LPS control group). Bars indicate that the standard deviation of the mean value.

indicated in Fig. 5, the MW >10 kDa fraction showed the most pronounced effect on decreasing NO and IL-6 production by 34.85 % and 46.11 %, respectively, at a concentration of 200 µg/mL. The findings align with those reported by Sandoval-Sicairos et al. (2021), who assessed amaranth peptide fractions. They demonstrated that fractions with molecular weights greater than 10 kDa and between 3–10 kDa resulted in a more substantial decrease in nitric oxide (NO) formation compared to the fraction below 3 kDa. Specifically, the >10 kDa and 3–10 kDa fractions reduced NO production by 65.2 % and 66.6 %, respectively, at a concentration of 1.0 mg/mL. González-Montoya et al. (2018) identified high molecular weight peptides generated from the hydrolysis of germinated soybean protein concentrate by pepsin and pancreatin. These peptides demonstrated a 45 % reduction in nitric oxide production at a concentration of 10 mg/mL, with the 5–10 kDa fraction exhibiting superior activity compared to the <5 kDa fractions. However, findings by Hu et al. (2020) indicated that the low molecular weight (<3 kDa) protein digested fraction of foxtail millet exhibited the greatest inhibition of inflammatory mediator production.

As a result, a critical challenge in membrane filtration processes is the accumulating and buildup of particulates on the membrane surface or within its internal pores, which may result from the use of membrane separation. Fouling that occurs during the filtration process can have a detrimental impact on the efficacy of the filtration system, particularly in terms of the permeate flux. The persistent decrease in flux and the

adverse impact on separation efficiency can result from the progressive accumulation of particles in the pores and on the membrane surface (Saxena et al., 2009). Therefore, in this study, *H. erinaceus* peptide fractions with a molecular weight exceeding 10 kDa were further fractionated using size exclusion chromatography, which improves the precision of peptide size separation. Sequence identification was then performed using LC-MS/MS.

3.6. Identification of anti-inflammatory peptide sequences

Three fractions (F-1, F-2, and F-3) were obtained through size exclusion chromatography, and overall, main nine sequences were identified by LC-MS/MS. The sequences found in F-1 were LLLFRNKN, LLLKKSL, and LLEAWSL, while those found in F-2 were LLLKVKT, VVGPLAKSA, and LLLLLLLL, and F-3 contained LYLLFGV, ELALVVKN, and KNFLFR (Table 5). The mass spectra of nine selected anti-inflammatory sequences are provided in Supplementary data.

Table 5 indicates that the hydrophobic amino acid content of the peptide sequences ranged from 50 % to 100 %, predominantly consisting of leucine, followed by lysine and valine. Each sequence included at least one leucine amino acid. The presence of one or more leucine residues in the anti-inflammatory active sequences aligns with the findings of Feng et al. (2021), Ji et al. (2020), and Wang et al. (2021) regarding the anti-inflammatory properties of peptides derived from selenium-enriched brown rice (Ala-Leu-Leu-Leu-Glu-Ala-Val-Glu-Ser--Glu-Tyr-Glu-Glu-Lys), foxtail millet (Ile-Ala-Leu-Leu-Ile-Pro-Phe), and walnut (Leu-Pro-Phe), respectively. Liu et al. (2022) indicated that highly hydrophobic bioactive peptides possess anti-inflammatory properties due to their ability to bind to cell membranes and induce membrane depolarization, thereby disrupting the inflammatory cascade pathway. Additionally, hydrophobic peptides can form peptide-lipopolysaccharide complexes with LPS, which allows them to scavenge LPS and induce a reversal of cell membrane charge, thus inhibiting LPS-stimulated inflammatory responses (Liu et al., 2022). Zhang et al. (2015) reported that leucine, a hydrophobic amino acid, may significantly influence ERK kinases in the downstream MAPK pathways, as well as PI3K and Akt kinases within the PI3K/Akt signaling pathways. Lysine was identified as the second most accessible amino acid in our sequences. Han et al. (2018) demonstrated that lysine restriction significantly impacted the inflammatory responses in the kidney, liver, and spleen by affecting serum antibody levels, inflammatory cytokines, ERK1/2, and NF-κB signaling in piglets. The majority of anti-inflammatory peptides from various plant sources contained lysine, which specifically influences the nuclear translocation of the transcription factor NF-κB and the activation of the kinase ERK (Han et al., 2018).

In our study, the sequences of leucine followed by lysine and valine in *H. erinaceus* mushroom proteins are identified as the primary amino acids responsible for reducing inflammatory responses in LPS-stimulated macrophage RAW 264.7 cells. This discovery may benefit the nutraceutical industry by enabling the direct synthesis of potent anti-inflammatory peptides with specified sequences for the development of alternative inflammation treatments.

4. Conclusion

The application of pulsed electric field (PEF) effectively improved both the extraction yield and *in vitro* digestibility of protein derived from *H. erinaceus*. The derived protein contained 44.59 % essential amino acids; however, it exhibited deficiencies in sulfur-containing amino acids and lysine. The digestive process utilizing pepsin and trypsin effectively produced protein hydrolysates that exhibited anti-inflammatory properties, resulting in a notable decrease in the levels of nitric oxide (NO) and interleukin-6 (IL-6). Peptides containing 50–100 % hydrophobic amino acids in their sequences were found to demonstrate the strongest anti-inflammatory effect. This study highlighted the anti-inflammatory potential of *H. erinaceus* peptides as promising alternatives for therapeutic applications; however, issues including *in vivo* validation and peptide stability after purification require additional exploration.

Table 5
Peptide sequences derived from *H. erinaceus* protein hydrolysates.

Fraction number	Peptide	Amino acid sequence	Hydrophobic amino acid (%)
F-1 A	LLLFRNKN	Leu-Leu-Leu-Phe-Arg-Asn-Lys-Asn	50.00
F-1 B	LLLKKSL	Leu-Leu-Leu-Lys-Lys-Ser-Leu	57.14
F-1 C	LLEAWSL	Leu-Leu-Glu-Ala-Trp-Ser-Leu	71.43
F-2 A	LLLKVKT	Leu-Leu-Leu-Lys-Val-Lys-Thr	57.14
F-2 B	VVGPLAKSA	Val-Val-Gly-Pro-Leu-Ala-Lys-Ser-Ala	77.78
F-2 C	LLLLLLLL	Leu-Leu-Leu-Leu-Leu-Leu-Leu-Leu	100.00
F-3 A	LYLLFGV	Leu-Tyr-Leu-Leu-Phe-Gly-Val	85.71
F-3 B	ELALVVKN	Glu-Leu-Ala-Leu-Val-Val-Lys-Asn	62.50
F-3 C	KNFLFR	Lys-Asn-Phe-Leu-Phe-Arg	50.00

Ethical statements

No studies in humans and animals.

CRediT authorship contribution statement

Buddhika Silva: Writing – original draft, Methodology, Formal analysis, Data curation. **Korawan Sringarm:** Writing – review & editing, Methodology. **Saranyapin Potikanond:** Writing – original draft, Validation, Methodology. **Pipat Tangjaidee:** Writing – review & editing, Validation, Methodology. **Pensiri Buacheen:** Writing – review & editing, Methodology. **Pornchai Rachtanapun:** Writing – review & editing. **Natthawuddhi Donlao:** Writing – review & editing. **Jaspreet Singh:** Writing – review & editing. **Lovedeep Kaur:** Writing – review & editing. **Utthapon Issara:** Writing – review & editing. **Passakorn Kingwascharapong:** Writing – review & editing. **Suphat Phongthai:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2025.101167.

Data availability

Data will be made available on request.

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