Effect of ultrasound-assisted modes on cholesterol esterase inhibition activity and functional properties of whiteleg shrimp (*Litopenaeus vannamei*) head protein hydrolysate

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Abstract

Introduction. This study investigated three ultrasound-assisted modes (ultrasound pretreatment, ultrasound-simultaneous treatment, and ultrasound post-treatment) to enhance cholesterol esterase inhibitory activity and functional properties of protein hydrolysates obtained from whiteleg shrimp (*Litopenaeus vannamei*) heads.

Materials and methods. Whiteleg shrimp heads were hydrolysed using alcalase to produce protein hydrolysates. For each ultrasound-assisted mode, ultrasonic amplitude and processing time were optimized to obtain hydrolysates with the highest cholesterol esterase inhibitory activity. The resulting hydrolysates were subsequently characterized in terms of degree of hydrolysis, half-maximal inhibitory concentration (IC_{50}) for cholesterol esterase inhibition, and techno-functional properties, including solubility, thermal stability, water-holding capacity, and oil-holding capacity.

Results and discussion. The highest cholesterol esterase inhibitory activity of the hydrolysates was obtained at an ultrasonic amplitude of 60% and a treatment time of 25 min for both ultrasound pretreatment and ultrasound-simultaneous treatment modes, while the same conditions (60% amplitude and 25 min) also resulted in the greatest inhibitory activity for the ultrasound post-treatment mode. Under these conditions, the cholesterol esterase inhibition activities of the ultrasound pretreatment, ultrasound-simultaneous treatment, and ultrasound post-treatment hydrolysates were 77.74±1.46%, 67.21±0.60%, and 64.67±0.71%, respectively, representing increases of 1.34-, 1.16-, and 1.12-fold compared with the unsonicated hydrolysate. The IC_{50} values of the ultrasound-treated hydrolysates ranged from 0.68 to 0.78 mg/mL and were significantly higher than that of the reference drug simvastatin (IC_{50} = 0.08324 μg/mL). Across a pH range of 3–8, all ultrasound-treated whiteleg shrimp head protein hydrolysates exhibited high solubility, exceeding 74%, even after thermal treatments at 63 °C for 30 min or 93 °C for 30 s. Regarding fluid-holding capacity, all three ultrasound-assisted modes markedly enhanced oil-holding capacity while reducing water-holding capacity of the hydrolysates.

Conclusions. These findings demonstrate the efficacy of ultrasound treatment in enhancing cholesterol esterase inhibitory activity and other functional properties of whiteleg shrimp head hydrolysate, suggesting its potential use as a natural supportive agent for hypercholesterolemia management and as a green emulsifier to improve food structure.

Introduction

The Vietnam Association of Seafood Exporters and Producers reported that Vietnam's shrimp exports reached approximately USD 4 billion in 2024, with whiteleg shrimp accounting for nearly 70% of this total. During processing, shrimp heads, which constitute 35–45% of the total shrimp weight (Majura et al., 2023), and contain substantial protein content (55.9–57.9% on a dry matter basis) are generated and typically considered waste. Transforming agricultural and industrial wastes into useful products contributes significantly to economic growth while helping to reduce environmental pollution (Stabnikova et al., 2023a, b). Whiteleg shrimp head waste can be valorized to produce protein hydrolysates, which exhibit diverse biological activities, including antioxidant and antimicrobial effects (Rashidian et al., 2021; Vo et al., 2019), and show potential as a source of antidiabetic peptides (Xiang et al., 2021). In addition, these hydrolysates possess functional properties, such as antifreezing and copper-binding capacity (Majura et al., 2023; Vo et al., 2020a).

To date, no studies have been published on cholesterol esterase inhibitory protein hydrolysates derived from whiteleg shrimp heads. Cholesterol esterase liberates free cholesterol from dietary cholesterol esters, accelerating cholesterol absorption. Excessive cholesterol intake may induce excessive cholesterol accumulation in the plasma, increasing the risk of heart disease (Alnuaimi et al., 2023; Jafar et al., 2018). Therefore, inhibiting cholesterol esterase offers a prospective approach to manage hypercholesterolemia. Zhang et al. (2024) reported that compared to simvastatin (a synthetic drug), protein hydrolysates present the advantages of minimal side effects and cost-effectiveness in hypercholesterolemia management.

Bioactive peptides are generally inactive within their native protein sequences and can be released through protease-catalysed hydrolysis, which may be enhanced by ultrasound post-treatment (Mudgil et al., 2023). However, some intact proteins are resistant to enzymatic hydrolysis because their tertiary and quaternary structures limit enzyme access to cleavage sites (Rao et al., 2024). In such cases, ultrasound, a green, non-thermal, and safe physical processing technology, has been shown to effectively assist enzymatic protein hydrolysis (Hong et al., 2022).

The application of ultrasound can generate mechanical effects (e.g., turbulence, shear forces, shock waves, and micro-jets), thermal effects, and chemical effects (such as sonochemical reactions and sonolysis of water), leading to protein disruption and partial denaturation, as well as enhanced mass transfer, thereby improving enzyme accessibility to substrates (Santos et al., 2024; Wali et al., 2017). This technology has been successfully applied to increase the cholesterol esterase inhibitory activity of protein hydrolysates derived from soybean (Chen et al., 2024), rice bran (Fathi et al., 2021), and millet (Rao et al., 2024).

This study investigated the effects of three ultrasound-assisted modes, ultrasound pretreatment (UPr), ultrasound-simultaneous treatment (US), and ultrasound post-treatment (UPo), on the cholesterol esterase inhibitory activity and functional properties of alcalase-derived protein hydrolysates from whiteleg shrimp heads. For each mode, the ultrasonic amplitude and processing time were optimized to obtain hydrolysates with maximal cholesterol esterase inhibition. The resulting hydrolysates were further evaluated for their degree of hydrolysis and functional properties, including solubility, thermal stability, water-holding capacity, and oil-holding capacity.

Materials and methods

Materials

Whiteleg shrimp heads were obtained from the Vietnam Food Company and transported to the laboratory on dry ice. Upon arrival, the shrimp heads were washed and initially dried at 90 °C for 20 min, followed by further drying at 60 °C until a constant weight was achieved. The dried heads were then ground, packed in sealed polyacrylamide bags, and stored at room temperature. The proximate composition of the shrimp head powder was determined following AOAC (2023) methods and consisted of 5.36 ± 0.12% moisture, 54.5 ± 0.5% protein, 10.5 ± 0.15% lipid, 10.6% carbohydrate, and 19.1 ± 0.23% ash.

Alcalase® 2.5L preparation (2.5 Anson unit/g, optimal performance at pH 7.5 and 55°C) was sourced from Novozymes. All reagents used in this study were of analytical grade, purchased from Sigma-Aldrich, Thermo Scientific Chemicals, and Merck.

Preparation of whiteleg shrimp head protein hydrolysates

Whiteleg shrimp head hydrolysates were prepared following the method of Vo et al. (2025). Shrimp head powder was mixed with distilled water at a 1:6 (w/v) ratio, and the pH of the mixture was adjusted to 7.5 using 1 M HCl or 1 M NaOH. Alcalase® 2.5L was then added at an enzyme-to-substrate ratio of 30 U/g protein, and hydrolysis was carried out at 55 °C for 4 h. After hydrolysis, the mixture was heated at 90 °C for 10 min to inactivate the enzyme, followed by centrifugation to collect the hydrolysate (supernatant). The resulting hydrolysate was lyophilized and stored at −20 °C until further use.

Ultrasound treatment

Ultrasonication was conducted using a probe ultrasonicator (VC505 – Sonics, USA) with a 19 mm diameter probe and a maximum power of 500 W. This device was operated at a fixed frequency of 20 kHz. Amplitude was varied from 30% to 70% in 10% increments, and ultrasonic time ranged from 10 to 30 min with a 5-min interval. A sample-containing beaker was placed on ice during the sonication to maintain low temperature, and the ultrasonicator probe was immersed in the sample at a depth of 2 cm. Three ultrasound-assisted modes were applied in the alcalase hydrolysis: (1) pretreatment (UPr) (ultrasound pretreatment), where the dispersion of whiteleg shrimp head powder in distilled water was sonicated before hydrolysis (before adjusting its pH to 7.5); (2) simultaneous treatment (US) – ultrasonication was applied during hydrolysis (immediately after alcalase addition); and (3) post-treatment (UPo) (ultrasound post-treatment), where ultrasonication was performed after hydrolysis (for the gained Alcalase hydrolysate). The produced hydrolysates were then evaluated for their cholesterol esterase inhibitory activity. For each ultrasound-assisted mode, ultrasonic amplitude and time were investigated to yield the hydrolysates with the highest cholesterol esterase inhibitory activity.

Cholesterol esterase inhibition activity assay

The cholesterol esterase inhibitory activity of whiteleg shrimp head hydrolysates was determined following the method of Alnuaimi et al. (2023) with slight modifications. A sample including 0.25 mL protein hydrolysate (1 mg/mL), 2 mL of sodium phosphate buffer (100 mM, pH 7.2, containing 100 mM NaCl), 0.5 mL of 5 mM p-nitrophenyl butyrate

solution (substrate) and 0.5 mL of cholesterol esterase solution (5 μg/mL) was incubated at 37°C for 30 min. The absorbance at 405 nm of the mixture was then measured. All the protein hydrolysate, substrate, and enzyme solutions were prepared in the sodium phosphate buffer 100 mM, pH 7.2, containing 100 mM NaCl. For the blank, control, and control blank samples, the enzyme solution, protein hydrolysate, and both the components were replaced with the sodium phosphate buffer, respectively.

The cholesterol esterase inhibitory activity (CEIA) of the hydrolysates was then determined using the equation (1):

$$\text{CEIA } (\%) = \left(1 - \frac{C - D}{A - B}\right) \times 100 \quad (1)$$

where A, B, C, and D are the absorbances of the control, control blank, sample, and blank, respectively.

IC50 values of the protein hydrolysates and Simvastatin Stella 20 mg drug (standard) were determined to evaluate their cholesterol esterase inhibitory activity.

Determination of degree of hydrolysis

The formaldehyde titration method described by Vo et al. (2025), was adapted to measure degree of hydrolysis of whiteleg shrimp head hydrolysates. A mixture of protein hydrolysate (5 mL) and distilled water (60 mL) was adjusted to pH 8.2 using 0.05 M NaOH. Subsequently, 10 mL of 36-38% (v/v) formaldehyde solution was added, and the resulting solution was titrated with 0.05 M NaOH solution until its pH reached 9.2. A blank sample (replacing the protein hydrolysate with distilled water) was processed identically. The degree of hydrolysis (DH) of the protein hydrolysate was calculated using the equation (2):

$$\text{DH } (\%) = \frac{(V_1 - V_2) \times C \times M \times V}{m \times P \times 5} \times 100 \quad (2)$$

where V_1 (L) and V_2 (L) depicts volume of 0.05 M NaOH solution used to titrate the sample and the blank sample, respectively; C (mol/L) is the concentration of NaOH solution used for the titration; M (g/mol) is nitrogen's molar mass; V (mL) is the total volume of the protein hydrolysate gained from m (g) of whiteleg shrimp head powder. P (%, w/w) is the total nitrogen content of the whiteleg shrimp head powder; 5 (ml) is the volume of the protein hydrolysate used in this assay.

Determination of solubility and heat stability

The solubility and thermal stability of whiteleg shrimp head hydrolysates were assessed following the method of Vo et al. (2025). Lyophilized hydrolysate powders were re-dissolved in distilled water at a concentration of 10 mg/mL, and the pH of each solution was adjusted to 3, 4, 5, 6, 7, or 8 using 1 M HCl or 1 M NaOH. For solubility determination, the solution underwent a 30-min shake at ambient temperature, then centrifuged (3000 g, 15 min, room temperature) to yield supernatants.

To assess heat stability, the solution was subjected to a heat treatment at either 63°C for 30 min or 93°C for 30s. They were then cooled in an ice-water bath for 10 min, centrifuged (3000 g, 15 min, room temperature) to gain supernatants. The supernatants were then evaluated for their soluble protein content using the Lowry method.

Heat stability was expressed via solubility, which was calculated using the equation (3):

$$\text{Solubility } (\%) = \frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}} \times 100 \quad (3)$$

Total protein content in the sample was the soluble protein content of the solution prepared by dissolving 100 mg of hydrolysate powder in 10 mL of 0.5 M NaOH solution at room temperature.

Determination of oil-holding capacity and water-holding capacity

Procedures detailed in our previous study were employed to estimate the oil-holding capacity (OHC) and water-holding capacity (WHC) of whiteleg shrimp head hydrolysates (Vo et al., 2025).

For the oil-holding capacity assessment, a 50 mL centrifuge tube containing hydrolysate powder (0.5 g) and vegetable oil (10 mL) was placed at 25±1°C. The tube underwent three 30-s agitation cycles, with 10 min intervals between each cycle. After that, the tube was centrifuged (3000 g, 15min, room temperature) and the supernatant volume was recorded.

The water-holding capacity was determined by adding distilled water (20 mL) to hydrolysate powder (0.5 g) in a 50 mL centrifuge tube. The tube was shaken for 30s, then stood at ambient temperature for 6 h. After centrifugation (3000 g, 15min, room temperature), the supernatant was filtered via a Whatman No. 1 filter paper before its volume was measured.

The same protocol was performed for a blank sample (without the hydrolysate powder). The oil-holding capacity (OHC) and water-holding capacity (WHC) were calculated using equations (4) and (5), respectively:

$$\text{OHC (mL oil/g protein hydrolysate powder)} = \frac{V_b - V_s}{m} \quad (4)$$

$$\text{WHC (mL water/g protein hydrolysate powder)} = \frac{V_b - V_s}{m} \quad (5)$$

where V_b (mL) and V_s (mL) are the volumes of the supernatant of the blank and sample, respectively. Casein was used as a standard, and its OHC and WHC were determined identically.

Data analysis

Triplicate experimental data were analysed using Microsoft Excel, and presented as mean±standard deviation. Statistically significant differences were determined, using the Tukey's method with Statgraphics Centurion 18 software.

Results and discussion

Effect of ultrasonic amplitude on cholesterol esterase inhibition activity of the whiteleg shrimp head hydrolysates

As shown in Figure 1, regardless of the ultrasound-assisted mode, increasing the ultrasound amplitude enhanced the cholesterol esterase inhibitory activity of whiteleg shrimp head hydrolysates, reaching a maximum at 60% amplitude before declining, which can be attributed to optimal ultrasound-induced cavitation.

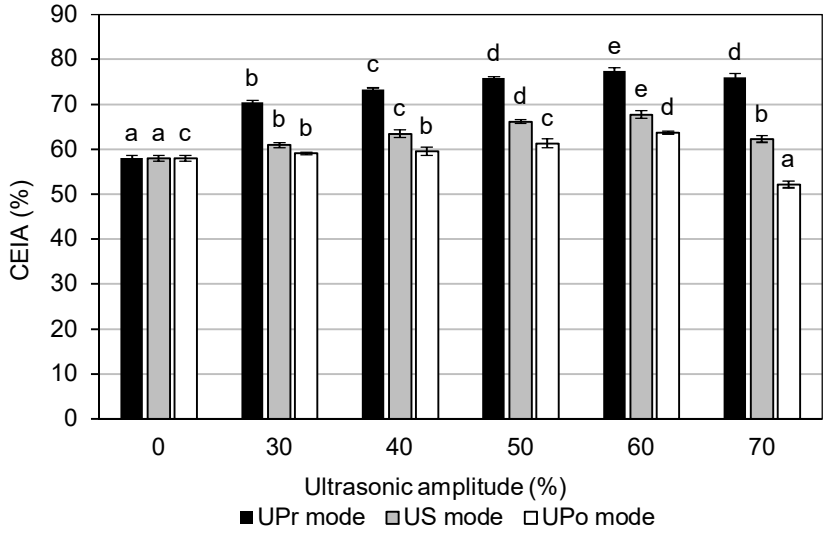

Figure 1. Effect of ultrasonic amplitude on cholesterol esterase inhibitory activity (CEIA) of hydrolysates prepared using three ultrasound-assisted modes
Bars of the same color with different letters indicate significant differences ($p<0.05$)

This cavitation created mechanical, thermal, and/or chemical effects, which might alter the shrimp head proteins by unfolding their molecular structure, reducing their size, or disrupting their non-covalent interactions (hydrogen bonding, Van der Waals forces, and dipole attractions) (Fadimu et al., 2022; Hong et al., 2022). These changes ameliorated peptide bond susceptibility to alcalase, thus improving the protein hydrolysis and heightening the bioactivity of resulting hydrolysates. Additionally, sonication at 60% amplitude possibly aided in unmasking hydrophobic amino acids at an appropriate level, which favoured cholesterol esterase inhibitory activity (Chen et al., 2024; Zhang et al., 2024).

The reduced cholesterol esterase inhibitory activity observed at low ultrasonic amplitudes (30, 40, and 50%) may be attributed to insufficient cavitation effects. Liu et al. (2022) reported that the turbulent forces and microstreaming generated by low-intensity ultrasonic cavitation could induce peptide collisions and aggregation, thereby decreasing the bioactivity of hydrolysates. Conversely, excessively high ultrasonic amplitudes may cause overexposure of non-polar groups, enhancing hydrophobic interactions among peptides and leading to the formation of large peptide aggregates. Such aggregates may obstruct the active sites of hydrolytic enzymes, limiting the release of bioactive sequences from native proteins (Yan et al., 2024). Furthermore, Justino et al. (2024), underscored peptide denaturation induced by excessive heat and mechanical force during high-amplitude ultrasound application. Thus, the declined cholesterol esterase inhibitory activity of the hydrolysates at an amplitude of 70% were observed (Figure 1). Our findings are in line with the findings of Hu and Li (2022), and Siewe et al. (2020), which indicated that there was an increase in antioxidant activity of soy protein isolate and fish (*Labeo rohita*) head hydrolysate, respectively, as ultrasound amplitude augmented up to specific thresholds. Similarly, Chen et al. (2024) observed a decline in cholesterol esterase inhibitory activity of soy protein hydrolysate when it underwent ultrasonic processing at excessively high power. Therefore, for further investigations, an amplitude of 60% was set for all ultrasound treatment modes.

Effect of ultrasonic processing time on cholesterol esterase inhibition activity of the whiteleg shrimp head hydrolysates

Ultrasonication applied for an appropriate duration can enhance protein hydrolysis by increasing the susceptibility of peptide bonds to enzymatic cleavage, thereby improving enzyme accessibility to catalytic sites. Hong et al. (2022) similarly reported that optimal ultrasound treatment refines protein particles and facilitates enzyme–substrate complex formation, leading to enhanced hydrolysis efficiency. Consequently, bioactive peptide sequences are more effectively released into the resulting hydrolysates, thereby increasing their biological activities. However, excessive ultrasonication may promote the formation of large peptide aggregates and reduce enzyme catalytic efficiency, ultimately diminishing hydrolysis performance (Pacheco et al., 2023; Rao et al., 2024). Moreover, as highlighted by Thongrattanatrai et al. (2023) and Bhetwal et al. (2024), prolonged exposure of protein hydrolysates to ultrasound can induce protein reassembly and structural rearrangements that mask peptide bonds and amino acid residues, thereby hindering enzymatic hydrolysis and negatively affecting the bioactivities of the obtained hydrolysates.

In this study, within the investigated ultrasound processing times (10–30 min), ultrasound pretreatment and ultrasound-simultaneous treatment produced the highest cholesterol esterase inhibitory activities of 77.74±1.46% and 67.21±0.60%, respectively, at 20 min (Figure 2).

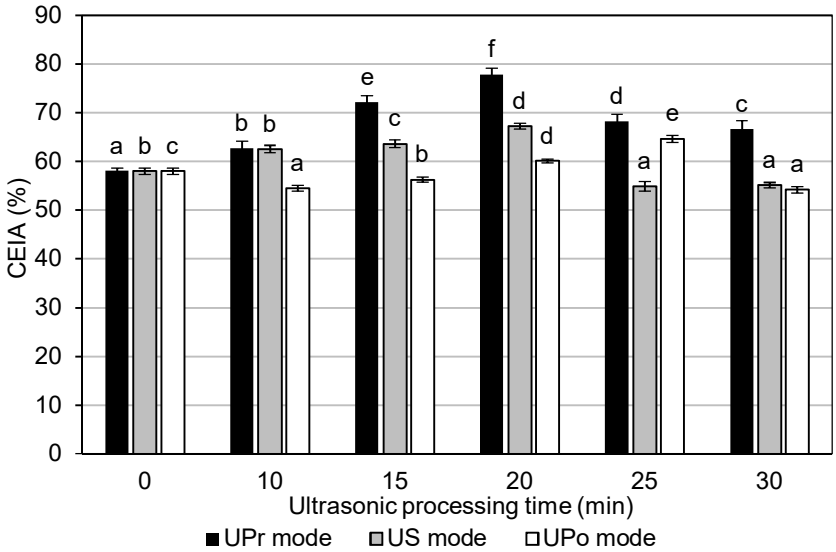

Figure 2. Effect of ultrasonic processing time on cholesterol esterase inhibitory activity (CEIA) of hydrolysates prepared using three ultrasound-assisted modes
Bars of the same color with different letters indicate significant differences ($p<0.05$)

In contrast, the ultrasound post-treatment hydrolysate exhibited its maximum inhibitory activity (60.12±0.43%) after 25 min of sonication. This is consistent with the results of Rao et al. (2024), which pointed out there was an increase in cholesterol esterase inhibitory activity of millet protein hydrolysate as the sonication time increased from 5 to 15 min, followed by a decline. Besides, Fathi et al. (2021) indicated that the ultrasound time beyond 10 min diminished both antioxidant and lipase inhibition activity of rice bran hydrolysate.

Similarly, anti-inflammatory and anti-diabetic activities of edible bird's nest protein hydrolysates increased when ultrasound processing time ranging from 15 to 30 min and decreased thereafter, as highlighted by Tang and Koh (2023). Thus, for more experiments, both ultrasound pretreatment and ultrasound-simultaneous treatment modes were performed with a duration of 20 min, while ultrasound post-treatment mode was conducted for 25 min.

Hydrolysis degree and half inhibition concentration of cholesterol esterase inhibition activity of the whiteleg shrimp head hydrolysates

A higher degree of hydrolysis corresponds to a greater release of free amino groups and short peptides in the hydrolysate (Jafar et al., 2018). Meanwhile, lower IC50 denotes stronger cholesterol esterase inhibition activity of inhibitors. As illustrated in Figure 3, all three ultrasound-assisted patterns promoted both the degree of hydrolysis and cholesterol esterase inhibitory activity of hydrolysate, with ultrasound pretreatment type expressing the highest effectiveness, followed by ultrasound-simultaneous treatment and ultrasound post-treatment models.

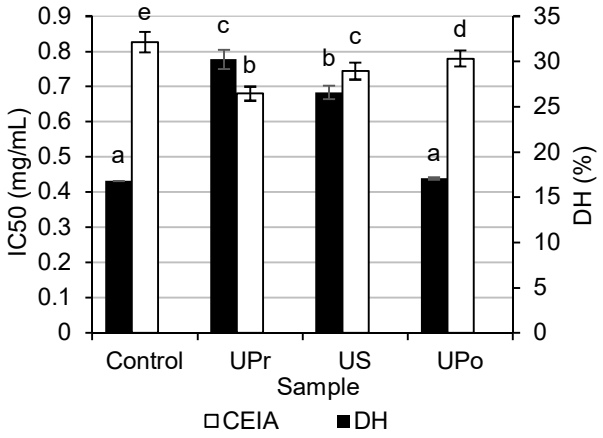

Figure 3. Degree of hydrolysis (DH) and IC50 of cholesterol esterase inhibitory activity (CEIA) of hydrolysates prepared using three ultrasound-assisted modes
Bars of the same color with different letters indicate significant differences ($p<0.05$)

Ultrasound pretreatment mode aided protein hydrolysis by preparing substrate molecules for the hydrolytic enzymes, involved in conformational changes of protein molecules and creation of micropores on the substrate particles' surfaces, promoting their susceptibility to hydrolytic enzymes (Bing et al., 2024; Hong et al., 2022). Moreover, the ultrasound-simultaneous treatment may enlarge enzymatic cleavage sites, thereby enhancing the accessibility of bulky substrate molecules. In addition, this mode improves mass transfer within the reaction mixture by generating turbulence and microstreaming in the liquid phase (Rao et al., 2024).

However, certain amino acid sequences generated during hydrolysis may be incompatible with the active sites of hydrolytic enzymes or may competitively occupy these sites, thereby hindering the binding of more suitable substrate segments (Tang and Koh, 2023). Consequently, the degree of hydrolysis of the ultrasound-simultaneous treatment

hydrolysate was 1.14-fold lower than that of the ultrasound pretreatment sample, indicating that ultrasound pretreatment was more effective than ultrasound-simultaneous treatment in enhancing alcalase-mediated protein hydrolysis. Consistent with the findings of Indriani et al. (2022) and Pacheco et al. (2023), ultrasound pretreatment was more effective than ultrasound-simultaneous treatment in enhancing the enzymatic hydrolysis of Asian bullfrog skin and pumpkin seed proteins, respectively. In contrast, Yan et al. (2024) reported that sheep hoof collagen hydrolysates produced using ultrasound-simultaneous treatment exhibited a higher degree of hydrolysis than those obtained by ultrasound pretreatment. These discrepancies may be attributed to differences in protein sources and hydrolysis conditions. With respect to cholesterol esterase inhibitory activity, the lower degree of hydrolysis observed in the ultrasound-simultaneous treatment sample may explain its reduced inhibitory activity, as evidenced by a 1.09-fold higher IC_{50} value compared with the ultrasound pretreatment sample (Figure 3). This observation is consistent with the findings of Ashraf et al. (2024), who reported that increasing the degree of hydrolysis enhanced the cholesterol esterase inhibitory activity of lentil and mung bean protein hydrolysates. Furthermore, Wang et al. (2024) demonstrated that peptides with lower molecular weights exhibit superior cholesterol esterase inhibitory activity.

On the other hand, the increase in cholesterol esterase inhibitory activity of the ultrasound post-treatment sample, compared to the non-ultrasonicated hydrolysate (Figure 3), might result from the cavitation-induced fragmentation of bioactive peptide aggregates and modifications in the peptide structure, thereby exposing additional binding sites for cholesterol esterase (Liu et al., 2022). This ultrasound-assisted mode may not affect peptide chains, since there was a statistically insignificant change in the degree of hydrolysis of the ultrasound post-treatment sample compared to the non-ultrasonicated hydrolysate (Figure 3). In addition, the ultrasound post-treatment type displayed the lowest cholesterol esterase inhibitory activity among the three ultrasound-assisted patterns, with its IC50 surpassing that of the ultrasound pretreatment and ultrasound-simultaneous treatment modes by 1.15 and 1.05 times, respectively. Similarly, Lopes et al. (2023) reported that the ultrasound pretreatment mode improved the antioxidant activity of common bean and lentil protein hydrolysates more efficiently than the ultrasound post-treatment type did.

All hydrolysates in this study demonstrated a mild cholesterol esterase inhibitory activity with their IC50 ranging from 0.68±0.02 to 0.83±0.03 mg/mL, which were significantly higher than that of simvastatin drug (IC50 of 0.083±0.001 μg/mL), a drug used for managing blood cholesterol level. However, these IC50 values were comparable to those of hydrolysates derived from date seed protein (Mostafa et al., 2022) and camel skin gelatin (Fawale et al., 2023), and significantly lower than that of *Rosa roxburghii* seeds (Yin et al., 2025). For that reason, it could be suggested that hydrolysate could be considered as a natural potential cholesterol-lowering agent.

Solubility of whiteleg shrimp head hydrolysates

Solubility is an effective indicator of assessing protein aggregation and denaturation. This characteristic not only impacts other functional properties of protein but also significantly influences sensory qualities of fortified foods (Cropotova et al., 2024). In this study, as illustrated in Figure 4, all three ultrasound-assisted modes improved the solubility of the hydrolysate across the tested pH range of 3 to 8.

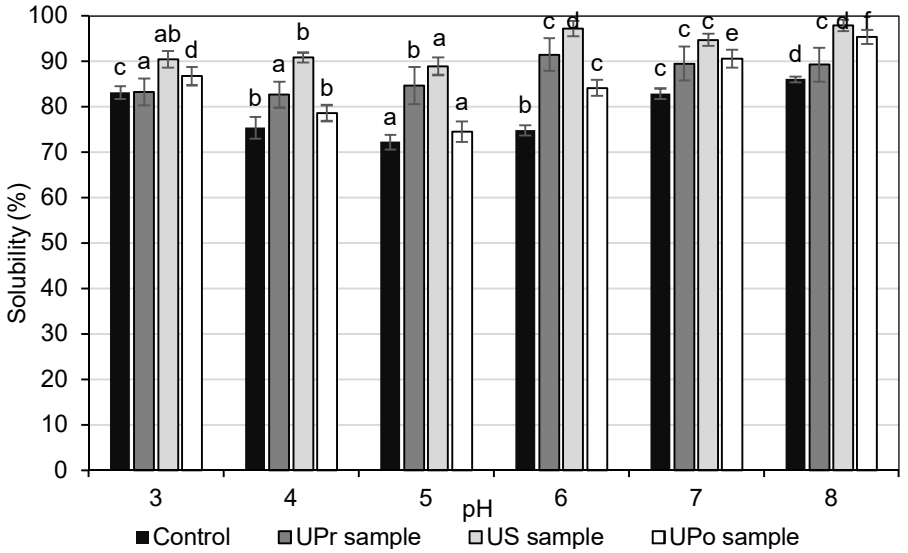

Figure 4. Solubility of hydrolysates prepared using three ultrasound-assisted modes
Bars of the same color with different letters indicate significant differences ($p<0.05$)

This increment could be ascribed to acoustic cavitation-induced unfolding of peptide molecules and disruption of their non-covalent linkages, including hydrogen bonds, electrostatic, and hydrophobic interactions. These changes enhanced the availability of hydrophilic amino acid side chains for hydrating, thereby boosting the peptide solubility (Saran et al., 2024; Yang et al., 2024). Similar observations were revealed by Gautam et al. (2025), who recorded the increase in solubility of shrimp shell and housefly larvae protein hydrolysates produced using ultrasound pretreatment and ultrasound-simultaneous treatment modes, respectively.

Within the tested pH range, among the three ultrasound-assisted modes, the ultrasound-simultaneous treatment pattern was the most effective type for elevating the shrimp head hydrolysate solubility, with an increase of 1.09 to 1.29-folds compared to the control (Figure 4). This might be attributed to the appropriate DH of the ultrasound-simultaneous treatment sample, which increased the number of hydrophilic groups, sufficiently forming hydrogen bonds with water molecules, thus facilitating its solubility (Vo et al., 2022). Conversely, lower degree of hydrolysis of the ultrasound post-treatment sample indicated the presence of a high quantity of large peptides, which may inefficiently expose their ionisable amino and carboxyl groups, mitigating their hydration and solubility (Vo et al., 2022). Meanwhile, decreased solubility of the higher DH hydrolysate (the ultrasound pretreatment sample) could be associated with the excessive exposure of hydrophobic amino acids, which favored hydrophobic interactions, leading to peptide aggregations (Barea et al., 2024).

Regarding the influence of pH on the solubility of hydrolysates, all samples demonstrated their peak solubility at pH 8, exceeding 80% of solubility (Figure 4). As reported by Xu et al. (2021), alkaline conditions imparted a strong net negative charge to peptides, fostering their interactions with the aqueous environment and thereby increasing their solubility. Besides, Vo et al. (2022) and Dhanabalan et al. (2020) found that solubility of hydrolysates from small shrimp (*Acetes japonicus*) and non-penaeid shrimp elevates at alkaline pH.

Overall, all hydrolysates in this study exhibited over 70% solubility across a wide pH range from 3 to 8, highlighting their potential to be incorporated into various food formulations.

Heat stability of whiteleg shrimp head hydrolysates

Thermal treatment influences the functional characteristics of proteins due to their intrinsic heat sensitivity. Heat-induced denaturation alters protein structure, resulting in the exposure of hydrophobic residues, which promotes protein-protein interactions and reduces protein solubility (Vo et al., 2020b). In this study, within the tested pH range from 3 to 8, all the hydrolysates remained greater than 70% solubility following heat treatment at 63 °C for 30 min or 93 °C for 30 s (Figure 5).

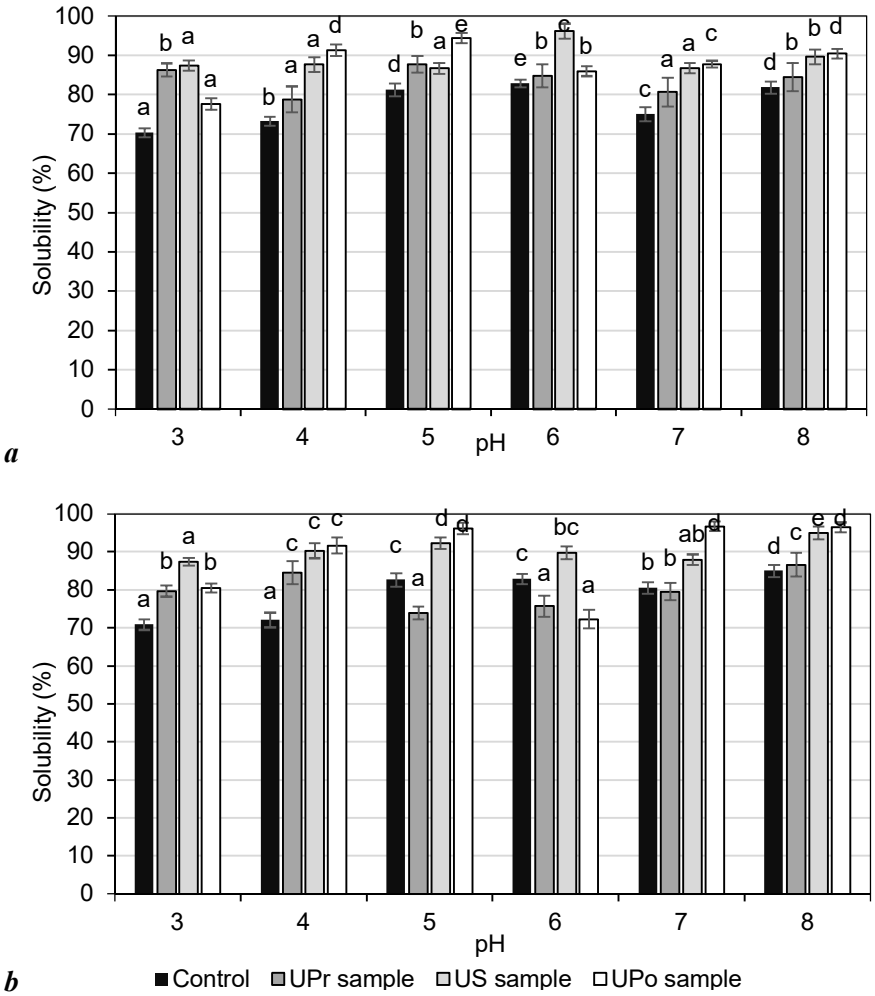

Figure 5. Heat stability ((a) 63 °C for 30 min and (b) 93 °C for 30 s) of whiteleg shrimp head hydrolysates prepared using three ultrasound-assisted modes
Bars of the same color with different letters indicate significant differences ($p<0.05$)

The results indicated that three ultrasound-assisted modes strengthened the heat stability of the hydrolysates under both thermal treatment conditions. It could be due to the fact that acoustic cavitation might disrupt noncovalent interactions between peptide molecules, making their amino acid side chains available for water molecules to form hydrogen bonds, thus impeding peptide aggregation (Luo et al., 2024).

Water-holding capacity of whiteleg shrimp head hydrolysates

As seen in Figure 6, the ultrasound pretreatment, ultrasound-simultaneous treatment, and ultrasound post-treatment modes decreased the water holding capacity of whiteleg shrimp head hydrolysates by 7.3, 2.5, and 1.3 times, respectively.

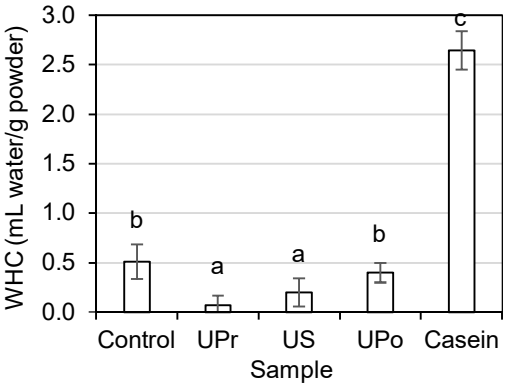

Figure 6. Water-holding capacity (WHC) of whiteleg shrimp head hydrolysates prepared using three ultrasound-assisted modes
Bars of the same color with different letters indicate significant differences ($p<0.05$)

As the degree of hydrolysis increased, the hydrolysates became more flexible, resulting in lower water-holding capacity (Qoms et al., 2023). This inverse proportion relationship between the degree of hydrolysis and WHC of protein hydrolysates was also published in our previous study (Vo et al., 2022). Compared to casein, all the ultrasonication-treated hydrolysates displayed significantly lower WHC, equivalent to 2.6–15.1% casein's WHC (Figure 6). Ghalamara et al. (2024) and Qoms et al. (2023) proposed that high solubility of protein hydrolysates led to reduction of their water-holding capacity, because their peptide molecules were highly dispersed in the aqueous medium. The findings of our present study was aligned with the study of Bhetwal et al. (2024), which indicated that the ultrasound-simultaneous treatment mode decreased the WHC of the hemp protein hydrolysate by 1.3 times compared to the control sample. In addition, Thongrattanatrai et al. (2023) found that the ultrasound pretreatment mode reduced the water-holding capacity of eri silkworm pupa hydrolysate from 0.33 to 0 ml water/g hydrolysate powder. However, Rawat and Saini (2023) published that there was an increase in water-holding capacity of sunnhemp protein hydrolysate prepared with the ultrasound pretreatment mode. This discrimination was probably due to different protein sources, hydrolysis and ultrasound conditions.

Oil-holding capacity of whiteleg shrimp head hydrolysates

Oil-holding capacity (OHC), which reflects the ability of peptides to interact with oil molecules, plays an important role in maintaining the acceptability and quality of food products. The retention of oil within the peptide matrix is primarily attributed to interactions between the hydrophobic side chains of peptides and the hydrocarbon chains of the oil (Saran et al., 2024). Ultrasound boosts the OHC of protein hydrolysates, owing to its cavitation effects that aids in exposing hydrophobic groups of the component peptides, as revealed by Saran et al. (2024). A 22% increase in OHC of eri silkworm pupa protein hydrolysate was observed by Thongrattanatrai et al. (2023), when applying ultrasound pretreatment mode. Similarly, as recorded by Bhetwal et al. (2024), and Saran et al. (2024), ultrasound pretreatment mode improved OHCs of hydrolysates derived from hemp seeds and rice bran by 1.5 and 1.2 times compared to that of non-ultrasonic treated samples, respectively. Furthermore, Bing et al. (2024), reported that ultrasound post-treatment mode significantly ameliorated the OHC of mung bean protein hydrolysate. The findings of this study are consistent with the above results. The OHC of whiteleg shrimp head hydrolysates increased from 0.98±0.12 to 1.69±0.23, 2.13±0.17, and 3.18±0.19 ml oil/g hydrolysate powder, when the ultrasound pretreatment, ultrasound-simultaneous treatment, and ultrasound post-treatment patterns were employed, respectively (Figure 7). Compared to casein, the ultrasound pretreatment, ultrasound-simultaneous treatment, and ultrasound post-treatment samples demonstrated superior OHC, surpassing by 1.2, 1.5, and 2.2 times, respectively (Figure 7). Accordingly, whiteleg shrimp head hydrolysates could be utilized to retard phase separation as well as improve palatability and taste retention of some fortified food products.

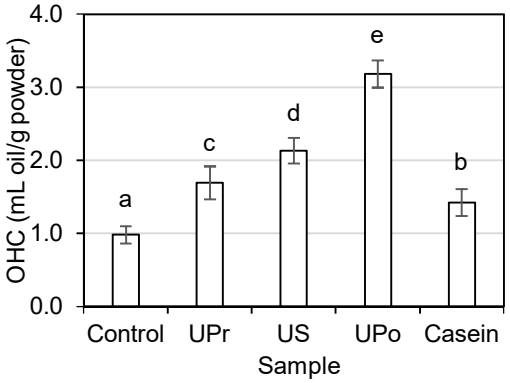

Figure 7. Oil holding capacity (OHC) of whiteleg shrimp head hydrolysates prepared using three ultrasound-assisted modes

Bars of the same color with different letters indicate significant differences ($p<0.05$)

Conclusions

This study is the first to systematically investigate the effects of three distinct ultrasound-assisted modes – ultrasound pretreatment, ultrasound-simultaneous treatment, and ultrasound post-treatment – on cholesterol esterase inhibitory activity and functional properties of protein hydrolysates derived from whiteleg shrimp (*Litopenaeus vannamei*) heads. All three ultrasound-assisted modes significantly enhanced inhibitory activity, solubility, thermal stability, and oil-holding capacity of the hydrolysates, while concomitantly reducing their

water-holding capacity. These findings provide preliminary evidence supporting further research into ultrasound-induced modifications of protein structure, process scale-up, and the potential clinical application of whiteleg shrimp head protein hydrolysates as functional ingredients for dietary management or supportive treatment of hypercholesterolemia.

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