Assessment of Lipase Inhibition Activity of Whiteleg Shrimp (*Litopenaeus vannamei*) Head Protein Hydrolysate

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Abstract: *This study investigated lipase inhibition activity (a new bioactivity relating to managing diseases of obesity) of whiteleg shrimp (*Litopenaeus vannamei*) head (WLSH) protein hydrolysate and its peptide fractions, contributing to enhance value for the shrimp processing by-product. Firstly, single-factor tests were performed to select appropriate levels for three hydrolysis parameters, encompassing WLSH powder-to-water ratio, enzyme-to-substrate (E:S) ratio and hydrolysis time. The best hydrolysis condition included a WLSH powder-to-water ratio of 1:5 (w/v), an E:S ratio of 40 U/g protein, and a hydrolysis time of 4 h, yielding the protein hydrolysate with the highest lipase inhibition activity of 31.60 ± 0.58%. Notably, the hydrolysate retained over 93% its native activity after pH (1–9) or heat (100 °C, 30 min) treatment. Moreover, it was rich in essential amino acids, comprising 39.95% of its total amino acid content. In addition, the hydrolysate was fractionated using ultrafiltration with sequential molecular weight cutoffs of 30, 10, 3 and 1 kDa. This process yielded the < 1 kDa peptide fraction with elevated lipase inhibition activity (33.67 ± 0.28%). These findings could be beneficial for the application of the WLSH hydrolysate and its < 1 kDa peptide fraction in the development of functional foods or nutraceuticals.*

Keywords: *lipase inhibition activity; whiteleg shrimp head; protein hydrolysate*

■ INTRODUCTION

During the production of frozen beheaded whole whiteleg shrimp (*Litopenaeus vannamei*), the whiteleg shrimp heads (WLSH), accounting for approximately 33% of total shrimp weight [1], are discarded as a by-product. In Vietnam, WLSH's quantity reaches 450,000 tons in 2025. The WLSH contained 55.9–57.9% crude protein, 2.6–4.3% crude lipid, and 23.1–30.5% ash (on dry weight basis) [2-3]. Protein hydrolysates from WLSH, with various bioactivities (comprising antioxidant capacity [3], copper chelation activity [4], anti-freeze potential [5]; α-amylase, α-glucosidase, and angiotensin-I converting enzyme inhibition activities [6-7]) were reported by many researchers, showing the application potential of WLSH as a source of bioactive protein hydrolysates production.

Furthermore, the exploration of anti-obesity agents substituting for orlistat (tetrahydrolipstatin), the only drug approved by the Food and Drug Administration for obesity management, has recently emerged because the long-term consumption of the drug triggers different adverse effects such as hives, gastrointestinal irritation, steatorrhea, dyspnea, and proctalgia [8]. Orlistat effectively controls the human body weight via inhibiting the pancreatic lipase, suppressing the digestion and absorption of lipids [9]. In this context, previous studies have underscored the potency of protein hydrolysates in delaying the lipase activity, indicating their prospects as alternative lipase inhibitors [10-11]. Various protein hydrolysates with lipase inhibition activity have been produced from canary seeds [12], baby clam meat [13], *Rosa roxburghii* seeds [14], and cow and camel casein [15].

Alcalase, an endo-protease produced by *Bacillus licheniformis*, has a broad substrate range and specificity, which releases small and medium-size peptides [16]. It is known to preferentially cleave peptide bonds containing His, Phe, Trp, Tyr, Glu, Met, Leu, Ala, Ser, Thr and Lys, particularly those between Leu-Tyr, Ser-His and Tyr-Thr [16-17]. The alcalase hydrolysis creates peptides with these amino acids at either their C- or N-terminus. These enhance the lipase inhibition activity of hydrolysates or peptides [18-20]. Lipase inhibition Alcalase hydrolysates have been successfully generated from different sources, including sea cucumber [21], Mueller's pearlside (*Maurolicus muelleri*) [22], porcine blood [23], camel skin gelatin [11], watermelon seeds [24], and olive seeds [25]. This study also employed Alcalase to create a lipase inhibition protein hydrolysate from the WLSH.

Hydrolysis conditions significantly impact amino acid sequence and molecular weight of peptides in the hydrolysate, influencing its bioactivities [25]. Previous studies of Fawale et al. [11], Lin et al. [26], and Vo et al. [13] have reported on the notable effect of hydrolysis parameters (including solid-to-liquid ratio, E:S ratio, and hydrolysis time) on lipase inhibition activity of protein hydrolysates from baby clam meat, camel skin gelatin, and *Spirulina*, respectively. Thus, these parameters would be investigated to obtain the hydrolysate with the highest lipase inhibition activity. Hence, this study aimed to exploit the WLSH to generate a lipase inhibition protein hydrolysate. Firstly, hydrolysis parameters—ratio of WLSH powder to water, E:S ratio, and hydrolysis time—were investigated to maximize lipase inhibition activity of the resulting hydrolysate. The gained hydrolysate was further analyzed for its degree of hydrolysis (DH), amino acid profile, and activity stability across various pH and thermal conditions. Moreover, the hydrolysate underwent a peptide fractionation using centrifugal ultrafiltration devices equipped with various molecular weight cut-off membranes of 30, 10, 3, and 1 kDa. The obtained peptide fractions were determined for their lipase inhibition activity, paving a way for further research regarding the separation and *de novo* sequencing of bioactive peptides.

■ EXPERIMENTAL SECTION

Materials

WLSHs, supplied by the Vietnam Food company, were washed, dried at 90 °C for 20 min, then dried at 60 °C until their weight remained constant. The resulting WLSHs were ground and stored in sealed polyacrylamide bags at ambient temperature in the dark. Alcalase® 2.5 L preparation was procured from Novozymes, while other analytical grade chemicals were obtained from Sigma-Aldrich, Merck, and Biobasic.

Instrumentation

This study made use of a UV-vis spectrometer (UV-vis 752, China), a water bath (Memmert WB14, Germany), and a freeze-dryer (Alpha 1–2/Ldplus, UK) to conduct the experiments.

Procedure

Analysis of chemical composition and heavy metal contents of WLSH powder

Standard methods from the Association of Official Analytical Collaboration (AOAC) [27] were used for the determination of moisture (AOAC 950.46B), protein (AOAC 981.10), lipid (AOAC 920.39), carbohydrate (AOAC 986.25), ash (AOAC 938.08), cadmium (AOAC 999.11), and lead (AOAC 999.11) contents of the WLSH powder.

For moisture content assessment, the WLSH powder was dried at 105 °C until a constant weight was achieved. The moisture content of the sample was then calculated using Eq. (1):

$$\text{Moisture content } (\%) = \frac{m_1 - m_2}{m_1} \times 100\% \quad (1)$$

where m_1 and m_2 denote the weight of the sample before and after the drying process.

Crude protein content of the WLSH powder was determined using the Kjeldahl method, including three main steps: (i) the sample was digested with concentrated H_2SO_4 in the presence of H_2O_2 until a clear solution was obtained; (ii) the digested sample was alkalized with 0.1 N NaOH solution to release NH_3, which was distilled and absorbed by a known volume of 0.1 N H_2SO_4 solution;

and (iii) the excess H_2SO_4 was titrated with standardized 0.1 N NaOH solution. Crude protein was then calculated in Eq. (2):

$$\text{Crude protein content } (\%) = \frac{(a-b)\times 0.0014\times 6.25}{m}\times 100\% \quad (2)$$

where a (mL) is volume of 0.1 N H_2SO_4 used to absorb NH_3 vapor; b (mL) is volume of 0.1 N NaOH used for the titration of the excessive H_2SO_4; m represents the sample weight (g); 0.0014 (g) depicts nitrogen weight related to the reaction with 1 mL of 0.1 N H_2SO_4; and 6.25 is the coefficient related to total nitrogen percentage in a protein.

For the determination of lipid content, the WLSH powder was dried at 105 °C until its weight remained unchanged. The dried WLSH powder was subsequently subjected to lipid extraction using petroleum ether solvent under reflux for 8 h. After extraction, the residue was placed under the fume hood at room temperature for solvent evaporation, followed by drying at 105 °C until its weight remained constant. Lipid content of the sample was then calculated in Eq. (3):

$$\text{Lipid content } (\%) = \frac{M_1 - M_2}{M_1}\times 100\% \quad (3)$$

where M_1 (g) is the initial weight of the dried WLSH powder, and M_2 (g) denotes the residue weight after drying.

Total carbohydrate content of the WLSH powder was estimated by difference, calculated as 100% minus the sum of its moisture, protein, lipid, and ash contents. Regarding ash content quantification, the WLSH powder was burnt at 550 °C until white or grey ash with constant weight was obtained. The ash content was expressed as the percentage of the ash weight relative to the initial weight of the WLSH powder.

For the measurement of Cd and Pb contents, the WLSH powder (approximately 20 g) was incinerated at 450 °C for 8 h. Then, 1–3 mL of distilled water was added, and the mixture was evaporated on an electric stove before being incinerated at 450 °C for an additional 2 h. This process was repeated until white or gray ash with a constant weight was obtained. Subsequently, the ash was mixed with 2 mL of 6 M HCl solution and evaporated again on an electric stove. The resulting residue was then dissolved in 10 mL of 0.1 M HNO_3 solution, remained at room temperature for 2 h, and analyzed by atomic absorption spectrophotometry (AAS) at 228.8 nm for Cd and 283.3 nm for Pb. The concentrations of these metals in the sample were determined based on the calibration curves between absorbances of standard solutions, measured at the corresponding wavelengths, and their concentrations. Standard solutions with a series of Cd or Pb concentrations were prepared by diluting stock solutions obtained by dissolving Cd or Pb in concentrated nitric acid with distilled water.

Hg content of the WLSH powder was determined using a direct mercury analyzer (MA-3000, Nippon Instruments Corporation), following the U.S. Environmental Protection Agency (EPA) method 7473 [28]. The WLSH powder was thermally and chemically decomposed in an oxygenated decomposition furnace. The resulting gas was then carried to a gold amalgamator that selectively trapped Hg vapor. Subsequently, the amalgamator was rapidly heated to liberate the Hg vapor, which was quantified by AAS at 253.7 nm. The Hg content of the sample was calculated using the calibration curve between absorbances of standard solutions ($Hg(NO_3)_2$ solutions) and their Hg concentrations.

Alcalase hydrolysis of WLSH powder

Following the protocol detailed in our previous publication [28], a dispersion of WLSH powder in distilled water was exposed to a heat treatment at 90 °C for 10 min to deactivate enzymes existing in the WLSH. The dispersion was then brought to its pH to 7.5 using HCl 1 M or NaOH 1 M solution while its temperature was maintained at 55 °C using a water bath. Subsequently, Alcalase® 2.5 L was added to initiate the hydrolysis of the WLSH proteins. After a required time, the mixture was heated at 90 °C for 10 min to inactivate the Alcalase, centrifuged to gain the WLSH protein hydrolysate (the supernatant). The hydrolysate was lyophilized and kept at −20 °C until used.

In this hydrolysis process, the WLSH powder-to-water ratio was first varied from 1:3 to 1:7 (w/v), while the E:S ratio and hydrolysis duration were set at 40 U/g protein and 4 h, respectively. The produced hydrolysates were then tested for their lipase inhibition activity. Based on the results, an appropriate ratio of WLSH powder to

water yielding the hydrolysate's highest lipase inhibition activity, was chosen. A similar procedure was implemented to determine suitable levels of E:S ratio and hydrolysis time in the tested arrays from 20 to 60 U/g protein and from 2 to 6 h, in order. After these investigations, the hydrolysate gained under the chosen hydrolysis condition was ascertained for its DH using the formaldehyde titration method [13], amino acid profile, and bioactivity stability, as well as subjected to peptide fractionation.

Determination of lipase inhibition activity

The lipase inhibition assay was performed following the procedure of Vo et al. [13]. The hydrolysate (1 mg/mL in distilled water, 0.5 mL) was mixed with *p*-nitrophenyl butyrate solution (5 mM, 0.5 mL) and maintained at 37 °C for 5 min. Following the addition of pancreatic lipase solution (5 mg/mL, 0.4 mL) and sodium phosphate buffer (0.1 M, pH 7.3, 1.1 mL), the mixture was kept at 37 °C for a further 30 min before its absorbance at 405 nm was recorded. Both the *p*-nitrophenyl butyrate solution and the enzyme solution were prepared in 0.1 M sodium phosphate buffer pH 7.3. Lipase inhibition activity of the WLSH hydrolysate was then calculated in Eq. (4):

$$\text{Lipase inhibition activity } (\%) = \left(1 - \frac{C - D}{A - B}\right) \times 100\% \qquad (3)$$

where A, B, C and D are the absorbances of the control (distilled water was employed in lieu of the hydrolysate), control blank (the hydrolysate was replaced with distilled water and the sodium phosphate buffer was used instead of the lipase solution), sample and blank (the sodium phosphate buffer was substituted for the enzyme solution), respectively. For the determination of the half-inhibition concentration (IC_{50}) value for the lipase inhibition activity of the WLSH hydrolysate, a graph of the activity against the hydrolysate concentration was plotted. Accordingly, a regression equation was derived from the graph and used to calculate the IC_{50} value. The Orlistat 120 mg STELLA drug was employed as the standard.

Obtaining peptide fractions from the WLSH protein hydrolysate

Centrifugal devices (Macrosep, Pall Laboratory, USA) equipped with 30, 10, 3, and 1 kDa molecular weight cut-off membranes were used to collect five peptide fractions (< 1, 1–3, 3–10, 10–30, and > 30 kDa) from the WLSH protein hydrolysate. Their lipase inhibition activity was then determined.

Determination of amino acid profile

The protocol detailed in our previous study was performed to determine the amino acid profile of the WLSH hydrolysate [13]. A complete hydrolysis of the hydrolysate was implemented using a 6 M HCl solution at 110 ± 2 °C for 23 h. After that, ion-exchange chromatography was employed for the separation of free amino acids, which were then detected via the Ninhydrin reaction. Free amino acid contents of the sample were quantified using standard amino acid solutions, with absorbance measurement taken at 440 nm for Pro and 570 nm for all other amino acids.

Thermal and pH stability of lipase inhibition activity of the WLSH protein hydrolysate

Bioactivity stability against pH and thermal treatments of the WLSH hydrolysate was assessed using the methods of Vo et al. [13]. Thermal stability was determined by heating 5 mL of the redissolved hydrolysate (40 mg/mL in distilled water) at 100 °C for various durations from 15 to 180 min, followed by quick cooling in an ice water bath and determination of lipase inhibition activity of the heat-treated sample.

For pH stability assessment, 5 mL of rehydrated hydrolysate (40 mg/mL in distilled water) was adjusted to a pH of 1.0–11.0 using HCl 6 M or NaOH 6 M solution, and held at ambient temperature for 30 min. The sample was then brought to pH 7.0 using 1 M phosphate buffer pH 7.0 and diluted to 20 mL with distilled water before testing its lipase inhibition activity. Both thermal and pH stability of the hydrolysate's bioactivity were expressed as relative activity (%), representing the ratio of the remaining lipase inhibition activity of the pH or heat-treated sample to the initial activity of the hydrolysate.

Data analysis

Each experiment was implemented in triplicate. Data presented as mean ± standard deviation were processed using the Microsoft Excel 2024, while the

Tukey method was performed via the Statgraphics Centurion 18 software to determine statistically significant differences ($p < 0.05$).

■ RESULTS AND DISCUSSION

Chemical Composition of WLSH Powder

Literature review showed that protein sources with protein content ranging from 8.25 to 47.98%, including colocynth seeds (8.25%) [10], pork and chicken skins (15.21–28.32%) [29], okra seed (26.42%) [19], and apple pomace (41.31–47.98%) [18], were utilized to produce lipase inhibition protein hydrolysates. Accordingly, the WLSH powder with protein content of 54.5 ± 0.5% (Table 1), could be a promising material for the generation of protein hydrolysates with lipase inhibition activity.

Furthermore, the safety of the WLSH powder was highlighted via the analysis results of its heavy metal contents. Specifically, Cd content in the WLSH powder was determined to be 0.35 mg/kg, which was below its maximum levels in crustacean set by the Vietnamese Technical Regulations 8-2:2011/Ministry of Health (Vietnam) (2.0 mg/kg) and Commission Regulation (Europe) 2023/915 (1.0 mg/kg). Additionally, Pb and Hg were not detected in the WLSH powder with the method quantification limit of 0.2 mg Pb/kg and 0.03 mg Hg/kg, respectively.

Influence of Hydrolysis Condition on Lipase Inhibition Activity of WLSH Protein Hydrolysate

In this study, as shown in Fig. 1, a 1:5 (w/v) WLSH powder to water ratio yielded the WLSH hydrolysate with the greatest lipase inhibition activity of 29.28 ± 0.63%. The ratio of WLSH powder to water influenced the solubility of WLSH proteins and their accessibility to the active sites of protease molecules, thereby impacting the lipase inhibition activity of the obtained hydrolysates. Particularly, increasing the ratio to a suitable level reduced the reaction mixture's viscosity and facilitated the solubilization of intact proteins from the WLSH powder, promoting the enzyme-substrate complexation and the release rate of bioactive peptides [30]. In addition, an appropriate solid-to-liquid ratio could minimize the protease-inhibition effects of accumulated hydrolyzed products [31].

Investigations on the effect of E:S ratio on bioactivities of protein hydrolysates usually reveal a regular pattern that activities increase and peak at a specific E:S ratio, then decrease afterwards [28,30,32]. This pattern was also reported by Ketprayoon et al. [33] and Fawale et al. [11] for the relationship between E:S ratio and lipase inhibition activity of rice bran and camel

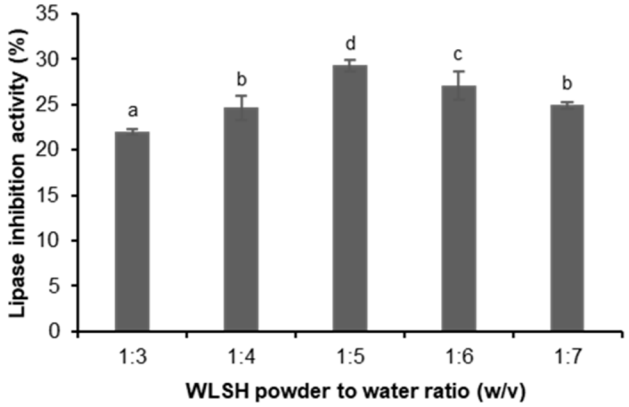

Fig 1. Impact of WLSH powder to water ratio on lipase inhibition activity of WLSH protein hydrolysate. Bars with different letters show significant differences ($p < 0.05$).

Table 1. Chemical composition and heavy metal contents of WLSH powder

Parameters	Content
Moisture	5.36 ± 0.12%
Protein	54.50 ± 0.50%
Lipid	10.50 ± 0.15%
Carbohydrate	10.60%
Ash	19.10 ± 0.23%
Cadmium (Cd)	0.35 mg/kg
Lead (Pb)	Not detected (Method detection limit = 0.20 mg/kg)
Mercury (Hg)	Not detected (Method detection limit = 0.03 mg/kg)

skin gelatin hydrolysates, respectively. The increase in E:S ratio elevates the enzyme-substrate complexation level, releasing an abundance of active sequences into the hydrolysates, thereby enhancing their biological activities [13]. However, overused E:S ratios might induce detrimental conversion of bioactive peptides into inactive fragments, diminishing the obtained hydrolysate's bioactivities [30]. Notably, this study found a decrease in lipase inhibition activity of WLSH hydrolysate when increasing E:S ratio from 20 to 30 U/g protein, before the typical correlation between the activity and E:S ratios in the range from 30 to 60 U/g protein was observed (Fig. 2). The reduced lipase inhibition activity of the hydrolysate at the E:S ratio of 30 U/g protein could be explained that the enzymes mainly released soluble protein fragments with minor or no lipase inhibition activity, which might obstruct the contact between highly bioactive peptides and the lipase's active site [30].

Fig. 3 indicated that within the tested hydrolysis time ranging from 2 to 6 h, lipase inhibition activity of the WLSH hydrolysate peaked at 4 h of hydrolysis. This observation was consistent with the results published by Fawale et al. [11] and Gonzalez-Noriegá et al. [29]. Generally, extended hydrolysis breaks down proteins into small peptides, which easily enter and interact with the lipase's active site, thus elevating the hydrolysate's inhibition activity against the lipase [18]. However, further hydrolysis for up to 6 h led to the loss of the hydrolysate's lipase inhibition activity, probably due to extensive peptide degradation and back reaction of accumulated hydrolysis products [34]. To summarize, suitable hydrolysis parameters were determined as a WLSH powder to water ratio of 1:5 (w/v), E:S ratio of 40 U/g protein and 4 h period of hydrolysis. The resulting hydrolysate exhibited a DH of 41.32 ± 0.92%, a lipase inhibition activity of 31.60 ± 0.58% and an IC_{50} for this activity of 4.48 mg/mL. The activity of the hydrolysate surpassed that of unhydrolyzed samples by 5.5 times, indicating the effectiveness of Alcalase hydrolysis in enhancing the hydrolysate's bioactivity. This increment could be attributed to the Alcalase-induced exposure of aromatic, charge, and hydrophobic amino acids from the WLSH protein core, supporting the peptide binding to lipase's catalytic site [12].

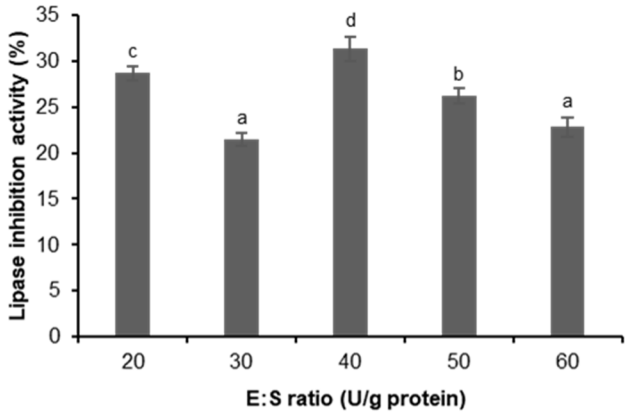

Fig 2. Impact of E:S ratio on lipase inhibition activity of WLSH protein hydrolysate. Bars with different letters show significant differences ($p < 0.05$)

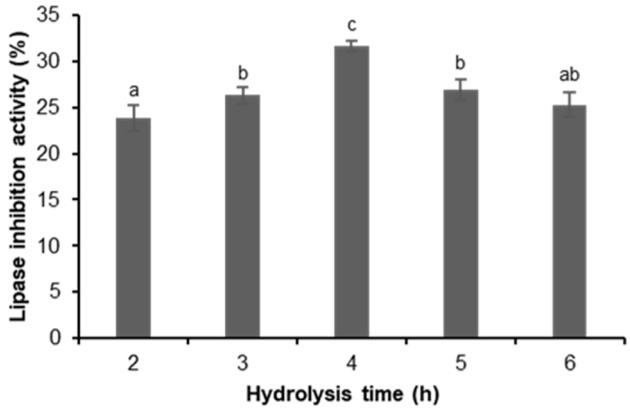

Fig 3. Impact of hydrolysis time on lipase inhibition activity of WLSH protein hydrolysate. Bars with different letters show significant differences ($p < 0.05$)

In addition, Alcalase hydrolysis further alters the secondary structure of peptides, enhancing the peptide's flexibility, thus elevating their lipase inhibition activity [18]. Furthermore, the WLSH hydrolysate demonstrated superior lipase inhibition activity compared to hydrolysates derived from tuna dark muscle (IC_{50} = 5.14 mg/mL) [20] and pork skin collagen (IC_{50} = 4.33 mg/mL) [29], but it was less potent than camel skin gelatin hydrolysate (IC_{50} = 0.5 mg/mL) [11]. This discrimination can be ascribed to the differences in amino acid composition, sequence and length of the peptides in these hydrolysates [29]. Compared to the drug Orlistat 120 mg – STELLA, the WLSH protein hydrolysate exhibited a moderately reduced lipase inhibition activity, with its IC_{50} for this activity being 2.8-

fold higher than that of the drug. These results suggested that the WLSH protein hydrolysate may be an alternative lipase inhibitor.

Amino Acid Composition of the WLSH Hydrolysate

Table 2 indicates that acidic amino acids (Asp, Glu) comprise approximately 25% of the overall amino acid content of the WLSH protein hydrolysate. Their side chains were confirmed to be involved in strong electrostatic interactions with the lipase's catalytic residues [35]. These interactions were further strengthened by side chains of His, Thr, Ser, Met, and Tyr [34], which represented 17% of the hydrolysate's amino acid profile. Besides, lipophilic amino acids, accounting for 43.91% the hydrolysate's total amino acids, promoted hydrophobic forces between the containing peptides and lipase, potentially hindering the reposition of the N-terminal domain flap, thus restricting the lipase catalytic activity [36]. Furthermore, hydrophobic amino acids aided the insertion of peptides into the hydrophobic cavity of lipase, impeding substrate accessibility to the lipase's active site, thus improving the hydrolysate's lipase inhibition activity [13].

pH and Thermal Stability of Lipase Inhibition Activity of the WLSH Hydrolysate

As seen in Fig. 4, the relative activity of the WLSH was over 100% at acidic pH from 1 to 5 and mildly alkaline pH (pH 8). This increase could be ascribed to strengthened electrostatic interactions among charged amino acids at these pH conditions, promoting peptide disaggregation [37], thus improving accessibility of the peptides to the lipase active site. Furthermore, these pH values may positively influence the charge of peptide's N- and C-terminal, boosting their electrostatic interactions with the lipase's catalytic residues [35], thereby improving the peptide's lipase inhibition activity. However, low relative activities (< 42%) of the hydrolysate were observed at extremely alkaline pH levels (pH 10 and 11). This result was aligned with the study of Zhang et al. [38], who attributed the reduced antioxidant activity of salmon skin protein hydrolysates

Table 2. Amino acid composition of the WLSH hydrolysate

Amino acids	Content (mg/100mL)	Ratio to total amino acids (%)
His	0.16	2.19
Ile	0.35	4.79
Leu	0.56	7.66
Lys	0.61	8.34
Met	0.15	2.05
Phe	0.35	4.79
Thr	0.33	4.51
Val	0.41	5.61
Ala	0.50	6.84
Arg	0.51	6.98
Asp	0.72	9.85
Glu	1.16	15.87
Gly	0.49	6.70
Pro	0.40	5.47
Ser	0.34	4.65
Tyr	0.27	3.69
Essential amino acids (Ile, Leu, His, Lys, Phe, Thr, Val, Met)	2.92	39.95
Hydrophobic amino acids (Ile, Leu, Val, Phe, Met, Ala, Pro, Gly)	3.21	43.91
Total amino acids	7.31	100.00

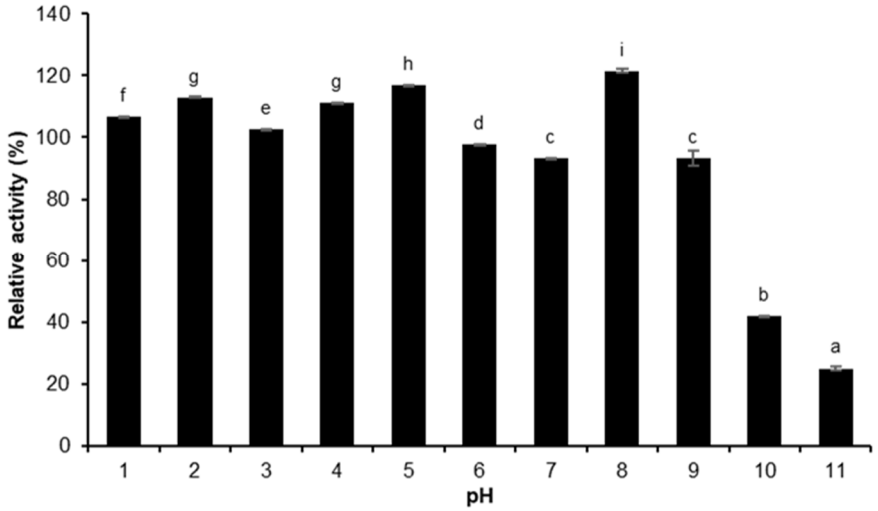

Fig 4. pH stability of lipase inhibition activity of the WLSH hydrolysate. Bars with various letters show significant differences ($p < 0.05$)

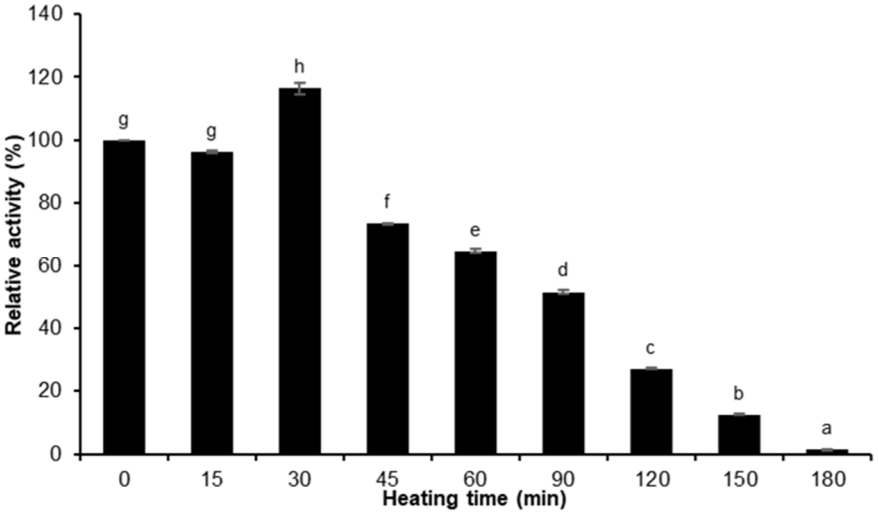

Fig 5. Thermal stability of lipase inhibition activity of the WLSH hydrolysate. Bars with various letters show significant differences ($p < 0.05$)

under alkaline conditions to undesirable reactions such as deamination and isomerization.

Fig. 5 indicated that the first 30-min of heating at 100 °C enhanced the lipase inhibition activity of the WLSH protein hydrolysate by 16.48% compared to its initial activity. This enhancement was most likely due to the fact that an appropriate period of heat treatment induced disruption of peptide aggregates and exposure of additional bioactive sites on the peptide surface [39]. Furthermore, substantial thermal stability of the hydrolysate's activity may correlate with its high hydrophobic amino acid content, comprising approximately 44% of the total amino acid composition (Table 2). Peptides which were rich in hydrophobic amino acid residues exhibited increased resistance to high-temperature aggregation, as noted by Vo et al. [32]. However, extending the heating duration from 45 min to 180 min reduced the hydrolysate's relative activity from 73.41 ± 0.20% to 1.27 ± 0.04% (Fig. 5). Prolonged heat exposure can negatively impact peptide secondary structure, potentially leading to peptide aggregate reformation, thus reducing their bioactivity [40-41].

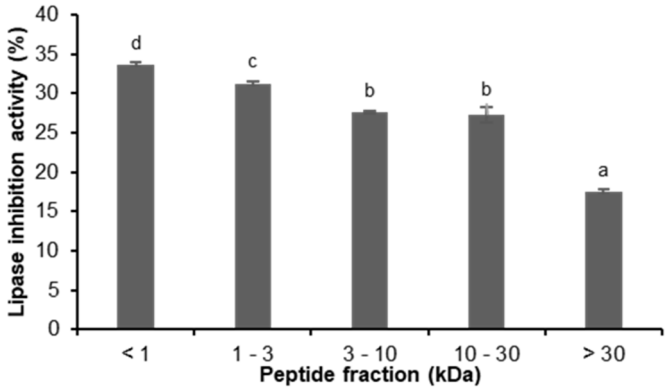

Fig 6. Lipase inhibition activity of peptide fractions separated from the WLSH protein hydrolysate. Bars with different letters show significant differences ($p < 0.05$)

Lipase Inhibition Activity of Peptide Fractions Separated from the WLSH Hydrolysate

Peptide molecular weight is a critical factor influencing its bioactivity, with lower molecular weight peptides exhibiting enhanced efficacy [42]. Consistent with the suggestion, this study found that the < 1 kDa peptide fraction demonstrated the highest lipase inhibition of 33.67 ± 0.28% (Fig. 6). This observation was also corroborated by the findings of Ketprayoon et al. [33] and Kuptawach et al. [43], who reported that the lowest molecular weight peptide fraction exhibited the greatest lipase inhibition activity. The enhanced activity of small peptides may be due to their improved accessibility to lipase binding sites [13]. As revealed by Zhao et al. [44], low molecular weight peptides could avoid steric hindrance from the lipase flap and enable direct interaction with the active site. Furthermore, the enhanced exposure of aromatic and hydrophobic residues on the surface of small peptides enhanced their interactions with lipase [12].

■ CONCLUSION

This study provides a background for utilizing WLSH as a source of lipase inhibition protein hydrolysate. An appropriate Alcalase hydrolysis condition was established, including pH of 7.5, temperature of 55 °C, WLSH powder to water ratio of 1:5 (w/v), E:S ratio of 40 U/g protein, and hydrolysis time of 4 h. The obtained WLSH hydrolysate exhibited lipase inhibition activity of 31.60 ± 0.58%. Notably, the activity was further enhanced after the hydrolysate was exposed to a pH from 1 to 5 or heat treatment at 100 °C for 30 min. Besides, future studies focusing on purification, *de novo* sequencing and *in silico* analysis of lipase inhibition peptides could be performed on the < 1 kDa peptide fraction from the WLSH hydrolysate.

■ ACKNOWLEDGMENTS

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■ CONFLICT OF INTEREST

The authors have no conflict of interest.

■ AUTHOR CONTRIBUTIONS

Conceptualization, Funding acquisition, Project administration, Supervision, Resources, Writing-review and editing: Tam Dinh Le Vo; Visualization, Writing-original draft preparation, Data curation: Bao Chi Vo; Investigation: Tam Dinh Le Vo, Bao Chi Vo, Hieu Trung Ma, Thao Huynh Ngoc Nguyen, Vy Thuy Pham, Vy Le Thao Vo, Huong Le Ngoc Nguyen, Nhan Minh Nguyen, Tien Thuy Dang, Thy Lam Anh Nguyen; Formal analysis, Validation and Methodology: Tam Dinh Le Vo, Bao Chi Vo. All authors agreed to the final version of this manuscript.

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