Ultrasonic structural modification of myofibrillar proteins from *Coregonus peled* improves emulsification properties

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**ABSTRACT**

This study evaluated the effects of high intensity ultrasonication (HIU, 100, 150, 200, and 250 W) and treatment time (0, 3, 6, 9, and 12 min) on the structure and emulsification properties of myofibrillar proteins (MPs) from *Coregonus peled*. These investigations were conducted using an ultrasonic generator at a frequency of 20 kHz (ultrasonic probe). Analysis of the carbonyl content and total number of sulfhydryl groups showed that HIU significantly improved the oxidative modification of MPs (*P* < 0.05). SDS-PAGE profiling showed significant degradation of the myosin heavy chain (*P* < 0.05). In addition, Fourier transformed infrared spectroscopy (FTIR) revealed that HIU altered these treated MP secondary structures, this was due to molecular unfolding and stretching, exposing interior hydrophobic groups. Particle size analysis showed that HIU treatment reduced particle sizes. Solubility, emulsification capacity, and emulsion stability were improved significantly, and each decreased with an increase in treatment time (up to 12 min), indicating aggregation with prolonged sonication. These results indicate that HIU could improve the emulsification properties of MPs from *C. peled*, demonstrating a promising method for fish protein processing.

1. Introduction

Ultrasonication is an innovative technology that has applications in both the analysis and the modification of foodstuffs, and it is divided into two categories: high-intensity ultrasonication (HIU, 20–100 kHz) and low-intensity ultrasonication (100 kHz–1 MHz). HIU has gained significant interest of late because it can alter the physical, chemical, and functional properties of foods [1]. In recent years, many studies have reported that the effects of HIU are related to their impact on the structure and functional properties of proteins, for example in soy protein isolates [2], whey protein isolates [3], and chicken myofibrillar proteins [4,5]. It is well known that ultrasonication induces structural changes in protein molecules due to partial unfolding and denaturation resulting in the modification of their functional properties, such as solubility, emulsification and foaming, or gelation [6].

Emulsification, a common function of protein suspensions, is undoubtedly important from the point of view of food technology, because gelation is an important component in the industrial processing of drinks, creams, soups, and others [6]. The emulsification properties of a protein directly depend on its surface hydrophobicity and solubility, and both are strongly affected by the hydrophilic-lipophilic balance and conformational flexibility [7,8]. However, emulsifier systems for proteins are often unstable, and it is necessary to develop new technologies to improve the properties of these systems. At present, HIU is an interesting alternative technology that can improve the emulsifying properties of a protein, as ultrasonication has been shown to have positive effects on both hydrophobicity and solubility [1]. In addition, ultrasonic cavitation can cause disruption and mixing of bubbles, causing collapse at or near oil–water interfaces, further leading to the formation of very fine emulsions [9].

Myofibrillar proteins (MPs) are the main proteins of skeletal muscle, and constitute 55–60% of total muscle proteins [10]. MPs are recognized as the main contributor to the texture-forming properties of muscle tissue foods, including emulsifications and gelations [11]. Generally speaking, MPs rarely show functional properties that are desirable for the food industry. In order to improve these functional properties for
broad applications, modifications of the protein structure are often implemented. Currently, many studies have focused on the structural modification of MPs by ultrasonic treatment related to gelation [5] and rheological properties [4,12]. However, few studies have evaluated the possible effects of ultrasonication on the structural modification of MPs related to emulsification. In addition, the application of HIU may result in quality deterioration through the degradation of the protein, as well as protein oxidation caused by free radicals released from the cavitation caused by ultrasonication [13]. Therefore, the objective of this work was to investigate the effects of HIU treatment on the physicochemical, structural, and emulsifying properties of MPs from Coregonus peled, in order to identify an optimal ultrasonication treatment (intensity and time) that can facilitate applications of HIU in food systems.

2. Materials and methods

2.1. Materials

Fresh C. peled (1000 ± 150 g, n = 20) were purchased from a commercial fisheries processing company (Saihu Fisheries Technology Development Co., Ltd., Xinpjiang, China). After decapitation and evisceration, the dorsal muscle quadrant was excised, and the red muscle tissue was then removed. The samples were cut into uniform sizes (approximately 15 g), packaged, and flash frozen (−80 °C) until use.

2.2. Myofibrillar protein preparation

Myofibrillar proteins (MPs) were extracted as described by Deng et al. [14] with some modifications. The chopped muscle samples were homogenized in four volumes (w/v) of sodium phosphate buffer A (50 mM, at pH 7.5) using a homogenizer (ESB-500X, Scientz Co., Ltd., Ningbo, China) in an ice bath for 60 s, and then centrifuged (X1R, Thermo Fisher Technologies Co., Ltd., Waltham, MA, USA) at 8,000 × g for 15 min at 4 °C. The resulting precipitate was then washed twice with four volumes of the same buffer. Next, the final sediment was homogenized in four volumes of sodium phosphate buffer B (50 mM phosphate buffer, 0.6 M NaCl, at pH 7.5) for 60 s in an ice bath, followed by centrifugation at 8,000 × g for 15 min at 4 °C. The obtained supernatants were used to prepare MP solutions. The protein concentration of MP was determined using the Biuret method [15] with bovine serum albumin as a standard.

2.3. Ultrasonic treatment

A 100-mL beaker containing 30 mL of MP solution (6 mg protein/mL) was sonicated using an ultrasound processor (VCX 500, Sonics & Materials, Inc., Newtown, CT, USA), equipped with a 20 kHz ultrasonic probe (fused size: 8 amps, slo-blo). Each level of ultrasonic intensity (100, 150, 200, and 250 W) was used for 0, 3, 6, 9, and 12 min (on-time and off-time durations of 2 s and 2 s). Samples were immersed in an ice bath to keep the sample temperature below 10 °C. Samples without ultrasonic treatment served as controls.

2.4. Carbonyl content determination

After ultrasonic treatment, MP solutions were diluted to 2 mg/mL with sodium phosphate buffer B for carbonyl content measurement. Protein carbonyls of MPs were assessed with 2, 4-dinitrophenylhydrazine (DNPH) according to the method in the method from Oliver et al. [16] with minor modifications. Then, 1 mL of 10 mM DNPH solution (diluted in 2 M HCl) or 1 mL of 2 M HCl was added to each MP solution (1 mL, 2 mg protein/mL) and incubated for 1 h at 25 °C in the dark, with mixing every 15 min. The DNPH-reacted samples were precipitated with 20% trichloroacetic acid (TCA) and centrifuged at 8,000 × g at 4 °C for 20 min, and then washed three times with an ethanol/ethy acetate (1:1, w/v) solution. The precipitate was dissolved in 5 mL of 6 M guanidine HCl and incubated for 15 min at 37 °C followed by centrifugation at 8,000 × g for 20 min. The protein carbonyl contents were then measured at 370 nm with a UV spectrophotometer (SP-752PC, Shanghai Spectrum Instrument Co., Ltd., Shanghai, China). An absorption coefficient of 22,000 M⁻¹ cm⁻¹ for protein hydrazones was used to calculate the content of carbonyl and is expressed as nmol carbonyl/mg protein.

2.5. Total sulfhydryl group determination

The total number of sulphydryl groups from samples was determined according to Yongsawatdigul et al. [17] with minor modifications. Briefly, 4 mL of 50 mM sodium phosphate buffer (0.6 M KCl, 10 mM EDTA, and 8 M urea, at pH 7.0) was added to 1 mL of MP solution (2 mg protein/mL) and mixed well. Then, 0.4 mL of Ellman’s reagent (0.2 mM DTNB in 0.1 M sodium phosphate buffer, at pH 7.0) was added to 4 mL of the resulting mixture. The mixture was next incubated in a water bath for 25 min at 40 °C. The absorbance was measured at 412 nm, and the total number of sulfhydryl groups (nmol/mg protein) was calculated using an extinction coefficient of 13,600 M⁻¹ cm⁻¹.

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

MPs samples (2 mg protein/mL) were mixed with 2X loading buffer (20% glycerol, 20% SDS, 1% (w/v) bromophenol blue, and 0.1 M Tris at pH 6.8) with or without 5% β-mercaptoethanol (β-ME), and then boiled for 5 min. Subsequently, 10 μg of each sample was loaded onto 12% polyacrylamide gels, and 4 μL of molecular weight marker (15–250 kDa, Shanghai Ya Enzyme Biotechnology Co., Ltd., Shanghai, China) was used for size estimation. Running voltages were set to 80 V and 120 V for the stacking gel and separation gel, respectively. The SDS-PAGE profiles were observed following the method from Cai et al. [18].

2.7. Surface hydrophobicity determination

The protein surface hydrophobicity of MPs was determined using 8-anilino-1-naphthalene sulfonate (ANS) as described by Wang et al. [4] and Ma et al. [19] with minor modifications. In brief, the MPs samples were adjusted to 0.0, 0.2, 0.4, 0.6, 0.8, or 1.0 mg protein/mL using 20 mM sodium phosphate buffer containing 0.6 M NaCl at pH 7.0. Then, 50 μL of 8 mM ANS was added to 4 mL of each protein solution and mixed well. Samples were kept in the dark at 25 °C for 20 min. The fluorescence intensity was then determined using a fluorescence spectrophotometer (970CRT, INESA Analytical Instrument Co., Ltd., Shanghai, China) at 374 nm (excitation) and 485 nm (emission), with excitation and emission slits set at 5 nm. The slope of the relative fluorescence versus protein concentration (mg/mL) was used to calculate the surface hydrophobicity of MPs dilutions.

2.8. Intrinsic fluorescence determination

The intrinsic emission fluorescence spectra of MPs were measured using the method of Cai et al. [18] with diluted MP suspensions (0.1 mg protein/mL). The excitation wavelength was 283 nm. Emission spectra from 300 to 400 nm were obtained with a constant slit of 10 nm for both excitation and emission.

2.9. Fourier transformed infrared spectroscopy (FTIR)

A sample pretreatment method was performed according to the method from Kang et al. [20] with minor modifications. Flakes of KBr (100 mg) containing sample powder (1 mg) were used for FTIR measurements. All of the FTIR spectra in the region of 4,000–500 cm⁻¹ were obtained with an average of 16 scans on a spectrophotometer (Nicolet 6700, Thermal Fisher Scientific, WI, USA) at a resolution of 4 cm⁻¹. Triplicate measurements were carried out. PeakFit version 4.0 software
was used to analyze the secondary structure of MPs, and a Gaussian peak-fitting algorithm was used.

2.10. Particle size measurement

MPs solutions (1 mg protein /mL) were injected into clear zeta cells for particle size measurement using a Zeta potentiometer (Nano-plus, Micromeritics Co., Ltd., USA) in triplicate according to the description in Li et al. [21].

2.11. Protein solubility measurement

The solubility of MPs solutions was measured as described by Chen et al. [22]. MP solutions (2 mg protein /mL) were centrifuged at 8,000 × g at 4 °C for 10 min. The protein concentration in the supernatant was measured according to the Biuret method. Protein solubility (%) was expressed as the ratio of the protein concentration of the supernatant with that of the original MP solution.

2.12. Emulsifying property measurement

The emulsifying activity index (EAI) and emulsion stability index (ESI) of the MPs were determined according to the method in Chen et al. [23] with minor modifications. Pure soy oil and MP solution (v/v, 1:4) were homogenized using a homogenizer at 12,000 rpm for 2 min. Aliquots of the freshly prepared emulsion (50 μL each) were pipetted from the bottom of the emulsion into 5 mL of sodium dodecyl sulfate solution (0.1%, w/v) at 0 and 10 min after homogenization. The absorbances of these emulsions were immediately measured at 500 nm in a spectrophotometer (SP-752PC, Shanghai Spectrum Instruments Co., Ltd., Shanghai, China). The absorbances measured at 0 min and 10 min were used to calculate [EAI (m²/g)] and [ESI (%)] based on Li et al. [24].

2.13. Statistical analysis

One-way analysis of variance (ANOVA) was carried out using SPSS 25.0 (IBM Corporation, USA). The correlation was estimated with the Pearson correlation coefficient option of SPSS 25.0. Data significance differences (P < 0.05) from three replicates were analyzed and compared using Duncan’s multiple range test.

### 3. Results and discussion

#### 3.1. Carbonyl content and total sulfhydryl groups

Previous studies have shown that the cavitation produced by ultrasonication can generate free radicals, and these radicals can cause hydrolysis or oxidation of proteins [20]. Therefore, similar reactions may occur during the HIU process. As shown in Table 1, the carbonyl content of MPs was significantly affected by HIU treatment (P < 0.05). A similar trend was reported by Kang et al. [20] for beef proteins during power ultrasonic treatment. In this study, the initial carbonyl content of MPs was about 1.26 nmol/mg protein and this increased continuously with increasing HIU intensity and treatment time. When the HIU intensity was ≥ 200 W, the carbonyl content increased significantly, and the carbonyl contents at 12 min were 5.48-fold and 6.40-fold higher in the 200 W and 250 W groups than the values of controls, respectively. These results indicated that HIU induced oxidative modification of MPs. Previous studies have demonstrated that reactive oxygen species (ROS) can modify or oxidize the proteins in muscle [32]. Thus, the free radicals produced by HIU treatment might contribute to the increased protein carbonyl contents found in our work. Carboxylation is the most salient oxidative modification of MP by fragmentation of backbones through the α-amidation pathway and β-scission, or by direct oxidation of the side chains of lysine, arginine, threonine, and proline [25]. Increased carbonyls of MPs can be attributed to the conversion of some amino acid residues to carbonyl derivatives or to oxidative peptide scission [26].

When ultrasonication was applied, the total numbers of sulfhydryl groups declined progressively with increasing HIU intensity and treatment time (P < 0.05, Table 1). However, with 250 W and 6 min of sonication, the intensity of the HIU treatments did not result in any significant changes (P > 0.05). These results are similar to those of Gülseren et al. [27]. MPs are rich in thiol groups, which are sensitive to attack by ROS and subsequently convert to intra- and intermolecular disulfide bond linkages [28]. It is well known that the majority of thiol groups are buried in the interior zones of protein molecules, and they are not readily accessible to the oxidants [29]. The cavitation caused by HIU unfolds the integrated myofibril structure, and exposes the reactive sulfhydryl groups [20]. Those changes make SHs in MPs vulnerable to attack by ROS, resulting in the intense loss of SH groups (Table 1). Decreases in the number of thiol groups are essential for the formation of aggregated structures of muscle proteins [30], and this may be linked to functional properties modification [25].

#### Table 1

| Treatment (min) | Ultrasound power (W) | SE  | p-Values |
|----------------|----------------------|-----|----------|
|                | 100                  | 150 | 200      | 250 |
| Carboxyl content (nmol/mg protein) | | | | |
| 0              | 1.26 ± 0.17 g<sup>ab</sup> | 1.26 ± 0.17 g<sup>ab</sup> | 1.26 ± 0.17 g<sup>ab</sup> | 1.26 ± 0.17 g<sup>ab</sup> | 0.22 | < 0.001 | < 0.001 | < 0.001 |
| 3              | 4.07 ± 0.11<sup>ab</sup> | 4.27 ± 0.08<sup>ab</sup> | 6.03 ± 0.07<sup>ab</sup> | 6.15 ± 0.07<sup>ab</sup> |
| 6              | 4.53 ± 0.07<sup>ab</sup> | 4.87 ± 0.05<sup>ab</sup> | 6.17 ± 0.02<sup>ab</sup> | 6.73 ± 0.31<sup>ab</sup> |
| 9              | 4.93 ± 0.11<sup>ab</sup> | 5.90 ± 0.05<sup>ab</sup> | 6.50 ± 0.23<sup>ab</sup> | 7.73 ± 0.39<sup>ab</sup> |
| 12             | 5.09 ± 0.08<sup>ab</sup> | 5.24 ± 0.05<sup>ab</sup> | 6.91 ± 0.12<sup>ab</sup> | 8.07 ± 0.29<sup>ab</sup> |
| Total sulfhydryl groups (nmol/mg protein) | | | | |
| 0              | 48.78 ± 2.21<sup>ab</sup> | 48.78 ± 2.21<sup>ab</sup> | 48.78 ± 2.21<sup>ab</sup> | 48.78 ± 2.21<sup>ab</sup> | 0.79 | 0.001 | < 0.001 | < 0.001 |
| 3              | 48.16 ± 4.57<sup>ab</sup> | 41.31 ± 3.38<sup>ab</sup> | 41.31 ± 3.38<sup>ab</sup> | 37.83 ± 3.38<sup>ab</sup> |
| 6              | 46.95 ± 0.54<sup>ab</sup> | 43.14 ± 1.38<sup>ab</sup> | 36.96 ± 0.74<sup>ab</sup> | 33.56 ± 1.18<sup>ab</sup> |
| 9              | 44.38 ± 0.56<sup>ab</sup> | 42.06 ± 0.85<sup>ab</sup> | 35.26 ± 0.95<sup>ab</sup> | 33.27 ± 0.86<sup>ab</sup> |
| 12             | 40.61 ± 0.40<sup>ab</sup> | 39.61 ± 0.38<sup>ab</sup> | 32.89 ± 0.38<sup>ab</sup> | 31.44 ± 0.38<sup>ab</sup> |

<sup>a</sup>-<sup>b</sup> different letters in the same row indicate statistically significant differences at P < 0.05. <sup>A</sup>-<sup>B</sup> different letters in the same column indicate statistically significant differences at P < 0.05. TT means treat time. UI means ultrasound intensity.
3.2. SDS-PAGE

Non-reducing (−βME) and reducing (+βME) SDS-PAGE analyses were next conducted to observe the changes in cross-linkage and Mw-distribution of MPs. Fig. 1 presents the typical pattern for MPs consisting of the two major bands of myosin heavy chain (MHC, > 220 kDa) and actin (42 kDa) and several low-intensity bands, including tropomyosin β-chain (37 kDa), troponin T (35 kDa), tropomyosin-α-chain (33 kDa), troponin C (19 kDa), and myosin light chain 2 (MLC2, 16 kDa) [31]. The reduced SDS-PAGE patterns showed that MHC was the protein most affected by HIU treatments, resulting in substantial MHC loss with increasing HIU intensity, especially at longer treatment times and higher HIU intensities (Fig. 1A and B). The results were similar to those of Cai et al. [32] who reported that the MHC showed less intensity after ultrasound treatment. Sriket et al. [33] indicated that MHC was more likely to cause protein degradation than other proteins. Thus, it is most likely that the turbulent flow and shear force associated with ultrasonic cavitation dissociated the complex structure of MPs [2,34] and increased their accessibility. This makes MHC in MPs vulnerable to fragmenting into small particles, in effect leading to protein degradation in the present study. Furthermore, the protein aggregates at the top of stacking gels (band 1) in non-treated MP groups may be attributed to cross-linking of oxidation during MP extraction [35]. Other protein bands were less affected by ultrasonic treatment.

Based on our non-reduced SDS-PAGE profiles (Fig. 1C, D and E), the band intensity of MHC became lesser with increasing ultrasonic intensity (100–250 W), and there was more protein aggregation on the tops of the stacking gels (band 1). β-ME is a strong reducing agent that possesses the ability to cleaving inter and/or intra-molecular disulfide covalent bonds in proteins [36]. This observation indicated that

![Fig. 1. SDS-PAGE of MPs prepared with (A, B) or without (C, D) β-mercaptoethanol (βME). Pixel intensity of myosin heavy chain (MHC) (E) for MPs samples. Letters (a–e) denote significant difference (P < 0.05) amongst MHC for different treat time. Error bars represent the standard deviations. MHC: myosin heavy chain.](image-url)
molecular cross-linking occurred and aggregations were formed in MPs during HIU-treatment. These results were similar with those of Kang et al. [20], who reported that ultrasonic treatment increased protein aggregation through disulfide cross-linking.

Myosin is also affected by protein oxidation. Xiong et al. [37] reported that the rod of myosin was the preferred target for free radicals. In the current study, the results may be attributed to the free radicals produced by cavitation through HIU [20]. The decrease of SH groups is indispensable to the formation of aggregated structures of MPs [30]. In the present study, the results were consistent with those of the total sulphydryl analysis (Table 1), which further confirms that the protein oxidation contributes to MP aggregation during HIU treatment. Therefore, the results indicated that the treatment of MPs with HIU could initiate cavitation causing hydrolysis, aggregation or oxidation of MPs.

3.3. FTIR spectroscopy analysis

Fig. 2 (A-D) displays the normalized FTIR spectra of HIU-treated and nontreated MPs. All samples showed a strong band centered between 3,600 and 3,200 cm⁻¹ (Amide A), attributable to intermolecular H-bonded N–H and O–H stretching vibration. In Fig. 2, this peak at 3,424 cm⁻¹ was shifted to lower wave numbers after HIU treatment, suggesting that intra- and intermolecular interactions were altered in these MPs. In addition, HIU treatment increased the intensity of the absorption peak at 2,933 cm⁻¹ to some extent, which could be ascribed to C–H stretching vibration. Typical protein bands centered approximately between 1,650 and 1,540 cm⁻¹ were related to Amide I (C = O stretch and C–N stretch) and Amide II (N–H bend, C–N stretch and C–C stretch), respectively [8,38]. Compared with controls, the HIU-treated MPs showed a lower absorption band at 1,081 cm⁻¹. It has been suggested that HIU pretreatment possibly promotes the C–H stretching vibration [39]. It is well known that N–H and O–H are involved in the formation of hydrophobic bonds and the hydrogen bonds within proteins [23]. Thus, it can be inferred that the intense mechanical forces (turbulence, shearing, and cavitation) disrupted the hydrogen bonding and hydrophobic interactions during the HIU process, inducing unfolding of the protein conformation of MPs [40].

As the amide I band (1,700–1,600 cm⁻¹) is based primarily on C = O stretching vibration (approximately 80%) of amide groups, it can be used to determine protein secondary structures [41]. The α-helix, β-sheet, β-turn, and random coil contents of MPs are presented in Fig. 2E. Upon 100 W ultrasonic treatment, the secondary structure in MPs was not significantly changed. As the HIU intensity increased to 150 W, HIU treatment (3–12 min) caused a significant decrease in α-helix content,
with increases in β-sheet and β-turn (P < 0.05). The proportion of random coil content showed little change, reaching a maximum at 6 min and then decreasing. This result may have been due to the cavitation bubbles and microstreaming effects promoting unfolding of α-helical regions [5]. Meanwhile, the increase in β-sheet content implied that increased protein–protein interactions caused the formation of intermolecular β-sheet structures [42,43]. A similar trend was reported by Jiang et al. [44] for black-bean protein isolates. The 200 W ultrasonic treatment resulted in an increase in the β-sheet and random coil proportions, and a remarkable decrease in the α-helix and β-turn content of MPs (P < 0.05). This phenomenon may be attributed to the mechanical vibration generated by the collapse of cavitation bubbles, which generated strong microstreaming, leading to the disruption of the protein secondary structure [6]. As HIU intensity increased, protein molecules unfolded and partial hydrogen bonds were weakened, resulting in the disruption of the ordered structure of α-helices and β-turns and the formation of β-sheets and random coils. In other words, 200 W HIU-treatment tended to induce a conformational transition from ordered structures to unordered structures. However, when increasing the dose level to 250 W, the distribution of secondary structures subjected to different treatment times showed a similar trend to that of the 150 W treatment. In general, α-helices were stabilized by the intra-hydrogen bonds of peptide chains, while β-sheet structures relied on the interhydrogen bonds between peptide chains [45]. From this point, 250 W ultrasonication could further contribute to the reconstruction of inter/intra hydrogen bonds inside MPs, and was conductive to reversed conformational transitions from unordered structures to ordered structures.

Previous studies have confirmed that oxidation can change the secondary structures of proteins, so the oxidative modifications of protein structure should be taken into consideration. Sun et al. [46] reported that the secondary structures of porcine muscle proteins were changed after treatment with an oxidizing agent, with a decrease in α-helix and an increase in β-sheet content. Similarly, Liu et al. [47] indicated that all of the soy protein isolates showed a decrease in α-helix and β-turn structures, and an increase in β-sheet and random coil structures after oxidative modification with peroxyl radicals. In this study, protein oxidation occurred during the HIU process (Table 1), and this may also have contributed to the changes in the secondary structures of MPs.

3.4. Fluorescence spectroscopy

Intrinsic fluorescence is another common method for exploring tertiary structural changes of proteins. As shown in Fig. 3A, the control group had the highest fluorescence intensity, suggesting that tryptophan residues were mostly enclosed inside the core (a hydrophobic environment) of MPs. After 100 W treatment, there were no significant differences between treatment times (Fig. 3A, 100 W). For the 150 W treatment, the intrinsic fluorescence of MPs decreased for the first 6 min and then kept constant afterwards. However, as the HIU intensity increased, the intrinsic fluorescence of MPs significantly decreased with increasing HIU treatment during the initial stages (3–9 min) and then increased with further treatment (12 min) (Fig. 3A, 200–250 W). The decrease of fluorescence intensity implied protein unfolding. In addition, oxidation of tryptophan and protein aggregation with protein unfolding may also decrease fluorescence intensity [47]. However, the intrinsic fluorescence increased with further treatment (12 min), possibly because of the aggregation of proteins and specific modification of tryptophan residues in MPs, resulting in lesser exposure of tryptophan residues to the aqueous environment [48].

The maximum fluorescence emission wavelength (λmax) can reflect changes in protein tertiary structure. The greater the red shift, the greater the conformational change of a protein during a treatment. In turn, the degree of blue shift of a protein λmax can be used to predict the change in its overall conformation [32]. From the λmax, there was some red shift (to longer wavelengths) after HIU treatment (Fig. 3B), which may be related to the exposure and microenvironment of the tryptophan becoming more polar, possibly due to protein unfolding [32], resulting in a looser tertiary conformation of MPs. Further treatment (200 W, 12 min; 250 W, 9 and 12 min) inhibited this structural change. This indicated that further HIU treatment reduced the damage to the tertiary structure of MPs.

3.5. Particle size distributions

The particle size distribution of HIU-treated and nontreated MPs was investigated. As shown in Fig. 3C, there were two major peaks in native MPs, distributed mainly in a small zone (100–1,000 nm) followed by a larger zone (>1,000 nm). After 100 W treatment (Fig. 3C), the two peaks

![Fig. 3. Intrinsic fluorescence (A), maximum fluorescence emission wavelength (B) and particle size distributions (C), diameter (D) of MPs treated by 100–250 W ultrasound. Letters (a-d) indicates that the different letters are significantly different (P < 0.05). Error bars represent the standard deviations.](image-url)
were slightly left-shifted, indicating a size decline. This in turn indicated that the protein subunits might be rearranged by the limited protein molecules due to the hydrophobic interactions that resisted electrostatic repulsion [49]. Nevertheless, the 100–1,000 nm peak appeared to strengthen, and the > 1,000 nm peak appeared to attenuate after HIU treatment; this could be explained by the loss of MHC (Fig. 1E). It is noteworthy that 150 W and 200 W treatment influenced the particle size in a similar but more pronounced way in MPs than in the 100 W treated sample (Fig. 3C). The increase in particle size demonstrated the agglomeration of MPs after prolonged sonication treatment (> 9 min), similar to results reported by Gülseren et al. [27] for bovine serum albumin. However, HIU treated MPs at an ultrasonic power of 250 W (Fig. 3C) showed unimodal particle size distributions, indicating that these proteins could evenly spread in water.

As shown in Fig. 3D, the particle sizes of MPs decreased continuously with increasing HIU intensity and treatment time. The decrease in the particle sizes of MPs could be attributed to the ultrasonic cavitation during HIU treatment along with microstreaming and turbulent flow [1]. Similar results were also reported for other protein isolates. Hu et al. [50] reported that the particle size of HIU-treated soy protein isolates decreased after 400 W sonication treatment. Zhang et al. [51] reported a similar observation for peanut protein isolate. The current results indicated that dispersion and small aggregation are two types of HIU damage that occurred in HIU-treated MPs.

3.6. Surface hydrophobicity (S\textsubscript{0}-ANS)

Changes in S\textsubscript{0}-ANS have been shown to reflect changes in protein tertiary and quaternary structures [45,52], and they can be used to monitor conformation changes in proteins. As shown in Fig. 4A, the S\textsubscript{0}-ANS of all HIU-treated samples were significantly higher than those of the controls (P < 0.05). This phenomenon might be due to HIU-promoted MP molecule unfolding and stretching, that exposed the partially buried interior hydrophobic groups, leading to an increase in surface hydrophobicity [4]. The results were consistent with a previous study by wang et al. [4] who reported similar results for chicken myofibrillar proteins.

In addition, at the same HIU intensity, the S\textsubscript{0}-ANS of MPs significantly increased with increasing ultrasonic treatment in the initial stages (3–9 min) and then decreased with further treatment (12 min) (Fig. 4A). This trend indicated that the exposure of MPs groups caused by the 9 min treatment was more conducive to the unfolding of the hydrophobic groups in MP molecules, which has been shown to be beneficial for the bond formation between protein molecules [53]. As the treatment time increases, partial hydrophobic residues approach each other, leading to weakening of the hydrophobic interactions of MP molecules [4]. This might be due to sonication destroying internal hydrophobic interactions and to tryptophan side chains moving to the outside of protein molecules [54]. Moreover, oxidative modification alters the protein surface hydrophobicity [36]. Lu et al. [55] reported that treatment with H\textsubscript{2}O\textsubscript{2} increased protein surface hydrophobicity of bighead carp proteins. Nyaisaba et al. [56] observed a similar result in MPs from Alaska pollock exposed to hydroxyl radicals. These findings are consistent with the results of the current study.

3.7. Protein solubility

The solubility of MPs as affected by HIU treatment is shown in Fig. 4B. HIU was able to enhance the solubility of MPs by up to 71%, and 200 W, 9 min were the optimal ultrasonic conditions. As shown in Fig. 4B, there was a significant increase at 9 min (P < 0.05) with increasing of HIU power, while a prolonged processing time (12 min) slightly decreased the solubility of MPs. The increase of protein solubility was consistent with the results for particle size (Fig. 3C and D). This
result was supported by a similar study reported by Wang et al. [4]. Arzeni et al. [57] also reported that ultrasonic treatment increased the protein solubility of soy protein isolates, possibly due to ultrasonication reducing the protein particle size, which strengthened the protein-water interaction. In addition, the increasing solubility might be attributable to internal hydrophilic group exposure with increasing processing time as well as conformational changes and formation of soluble protein aggregates [58]. Previous studies have shown that the disruption of hydrophilic interactions resulting from cavitation including turbulence and shear force initiated by ultrasonication, can promote the intramolecular association of protein molecules, thereby improving solubility [59]. Furthermore, the unfolding of protein molecules was improved during sonication processing as observed in the FTIR; this promotes exposure of internal hydrophilic groups and facilitates the protein–water interaction, thereby leading to an increase in solubility [44]. However, the solubility of MPs was decreased by ultrasonication for 12 min, which might be due to disulfide cross-linking or hydrophobic interactions [4] leading to the reformation of macromolecular aggregates, ultimately causing the loss of protein solubility. Overall, these results indicated that the changes in protein structure, molecular size and exposed hydrophilic groups induced by HIU influence the sensitive balance between repulsive and attractive intermolecular forces regulate the solubility of proteins [23].

3.8. Emulsification properties

Next, the emulsifying activity index (EAI) and the emulsion stability index (ESI) were adopted to evaluate the emulsification properties of MPs from C. peled. As shown in Fig. 4C, HIU processing had a positive effect on both EAI and ESI. The emulsification properties of MPs after 9 min HIU treatment were the highest. Among the tested HIU intensities, ≥200 W processing rendered MPs with the greatest emulsifying properties (Fig. 4C). This was in accordance with previous studies reported by Uluko et al. [60] for milk protein concentrate hydrolysates and Malik et al. [1] for sunflower protein isolates. Emulsifying properties of proteins are strictly affected by solubility, hydrophobicity, and conformational flexibility [1,8]. Ultrasonication has been reported to partially unfold and expose the interior hydrophobic groups of MPs from chicken breast, and this structural state may enhance the surface activity and protein adsorption at the oil–water interface [5]. This increased hydrophobicity favors the formation of a more stable and rigid film. In addition, the increases of EAI and ESI could be attributed to cavitation during HIU processing, allowing a greater integration of bubbles in the oil phase of an emulsion. However, the decrease in both EAI and ESI at a higher treatment time (12 min) might be associated with the unfolded-structure of MPs with slight decreases in the surface hydrophobicity (Fig. 4A) and partly weakened conformational flexibility due to minor protein aggregation [6]. Previous research has shown that denatured proteins will expose more hydrophobic groups, leading to protein aggregation [61]. Therefore, there must be a balance between the exposure of hydrophobic groups and the aggregation of protein molecules.

3.9. Relationship between HIU-induced structural changes and emulsification

Correlation analysis for the structural (secondary structure, surface hydrophobicity, $\lambda_{\text{max}}$) and emulsifying (EAI and ESI) parameters of treated MPs is shown in Table 2. The correlations between the emulsifying parameters and protein oxidation (carbonyl and total sulphydryl), degradation parameters (the intensity of MHC bands), particle size, protein solubility were also analyzed. EAI and ESI were significantly correlated with structural parameters ($\alpha$-helix, $\beta$-sheet, $S_0$-ANS) ($P < 0.01$ or $P < 0.05$); however, they were not correlated with $\beta$-turn or $\lambda_{\text{max}}$ ($P > 0.05$). For protein degradation, EAI and ESI were negatively correlated with the MHC band gray ($P < 0.01$). As for protein oxidation, EAI and ESI were both positively correlated with carbonyl content while being negatively correlated with total sulphydryl content ($P < 0.01$). Moreover, EAI and ESI were positively correlated with protein solubility, and negatively correlated with particle size ($P < 0.01$).

Based on the above results, molecular flexibility and surface hydrophobicity increased with positive consequences for emulsification that was attributable to the structural modification of MPs by the denaturation and polydispersability induced by HIU treatment. We propose that HIU induced changes in intra- and intermolecular interactions and promoted MP unfolding that facilitated the exposure of hydrophobic groups and increased the conformational flexibility, ultimately causing increased surface activity and protein adsorption enhancement at the oil–water interface [23,24]. Wang et al. [4] detected changes in chicken myofibrillar protein molecules’ tertiary and quaternary structures after ultrasonic treatment due to ultrasonication facilitating the exposure of thiol groups and hydrophobic groups in protein molecules.

In addition, HIU treatment induced the hydrolysis of MHC, and molecular cross-linking occurred with aggregation forming in MPs, as verified by the particle size data. Few studies have been carried out on the possible effects of HIU treatment on the proteolysis of MPs. It has been suggested that HIU treatment denatures MPs, dissociating the protein quaternary structure [4]. Muscle myofibril structure is primarily comprised of thin and thick filaments. The most abundant proteins in the thick filaments are composed of myosin, which can more easily cause protein degradation than other proteins [23]. Thus, it is most likely that the turbulent flow and shear force associated with ultrasonic cavitation had already dissociated the complex macromolecular structure of MPs and increased their accessibility. This makes MHC in MPs vulnerable to fragmentation into small particles, in effect leading to the greater solubility observed in the present study.

Furthermore, protein oxidation is induced by HIU treatment, and this was highly correlated with emulsification properties. It is well known that protein oxidation-induced structural modification can change protein functionality and thus influences product quality [25]. However, such chemical processes are not always associated with deleterious effects on protein functionality. Previous studies have reported that appropriate oxidation-induced improvement of the emulsifying properties of proteins is largely attributable to structural changes as well as increased surface hydrophobicity [47,62]. In this case, the improvement of the emulsifying properties of MPs was partially attributed to structural changes as well as the increased surface hydrophobicity induced by

| Emulsifying properties | Structural parameters | Particle size | Protein solubility | Protein degradation MHC | Protein oxidation Carbonyl content Total sulphydryl groups |
|------------------------|-----------------------|--------------|-------------------|-------------------------|----------------------------------------------------------|
| EAI                    | 0.653*                | 0.443*       | 0.259             | 0.329*                  | 0.846**                                                  | 0.057                                                    | 0.526**        | 0.825**        | 0.668**        | 0.655**        | 0.780**        | 0.812**        |
| ESI                    | 0.327*                | 0.316*       | 0.118             | 0.111                  | 0.358**                                                  | 0.194                                                    | 0.408**        | 0.534**        | 0.446**        | 0.518**        | 0.612**        | 0.447**        |

* P < 0.01*, P < 0.05* EAI: Emulsifying activity index; ESI: Emulsion stability index. MHC: myosin heavy chain. $S_0$-ANS: Surface hydrophobicity.
free radicals after HIU treatment.

In all, HIU produces a variable degree of conformational structural changes that depend on the HIU intensity and the treatment time and these factors in turn led to changes in the emulsification properties of MPs from C. peled. Data presented in this work suggest that 200 W and 9 min were the optimum ultrasonic conditions for modifying MP conformation to improve emulsification properties.

4. Conclusions

The present study revealed that HIU alters the structure and emulsification properties of MPs from C. peled. HIU treatment changed the MP secondary structure, promoting the exposure of the tertiary MHC and a decrease in particle size, thereby promoting an increase in classification properties of MPs from C. peled. These results demonstrate a promising method in the technology of meat protein processing, but further studies are required to understand the detailed mechanism of ultrasonic treatment on fish proteins.

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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