Improved water solubility of myofibrillar proteins by ultrasound combined with glycation: A study of myosin molecular behavior

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A B S T R A C T

The poor water solubility of myofibrillar proteins (MPs) limits their application in food industry, and is directly related to the molecular behavior associated with myosin assembly into filaments. This study aims to explore the effect of high-intensity ultrasound (HIU) combined with nonenzymatic glycation on the solubility, structural characteristics, and filament-forming behavior of MPs in low ionic strength media. The results showed that the HIU (200–400 W) application could promote the subsequent glycation reaction between MPs and dextran (DX) and interfere with the electrostatic balance between myosin rods, suppressing the formation of filamentous myosin polymers. Glycated MPs pretreated by 400 W HIU had the highest solubility, which corresponded to the smallest particle size, highest zeta potential, and optimum storage stability ($P < 0.05$). Structure analysis and microscopic morphology observations suggested that the loss of the MP superhelix and the depolymerization of myosin polymers were the main mechanisms for MP solubilization. In conclusion, HIU combined with glycation can effectively improve the water solubility of MPs by destroying or suppressing the assembly of myosin molecules.

1. Introduction

Meat-derived proteins are generally regarded as "premium nutrients" in the human diet because they exhibit high digestibility and contain all essential and no limiting amino acids [1]. Myofibrillar protein (MPs) are predominant among muscle proteins, and account for approximately 50% of the total protein content [2]. Benefiting from their fibrous flexible structure and abundant side-chain functional groups with high surface activity [3], MPs have great potential as special nutritional ingredients for application in liquid foods. Once successful, the emergence of MP-based beverages or soft foods can meet the meat protein needs of the elderly, infants, and dysphagic patients. Nevertheless, due to their poor solubility in water or low-ionic strength media, MPs have not been utilized as much as plant-derived proteins. Although MPs can be adequately solubilized in a highly concentrated salt solution (0.47–0.68 M NaCl), this is not recommended for a healthy diet [4]. It is thus necessary to explore strategies for improving the solubility of MPs in low-ionic strength media.

The molecular behavior of myosin (one of the main proteins among MPs) in low-salt media is considered responsible for the poor water solubility of MPs [5]. In low-salt media, monomeric myosin regularly assembles into insoluble filamentous polymers (termed myosin filaments) via electrostatic interactions in rod regions, thereby resulting in poor solubility of MPs [6]. To solve this problem, many attempts, including physical, chemical, and enzymatic treatments, have been made to suppress the assembly of myosin filaments in low-ionic strength media [7–9].

One promising strategy to improve the functional properties of proteins is the direct conjugation with a hydrophilic polymer via glycation, which is carried out in mild and safe conditions without any extraneous chemicals [10]. The solubilization effect of glycation was initially attributed to the grafting of hydrophilic residues and the shift of the protein isoelectric point [11], while its effect on the assembly process of myosin molecules in food processing media of low ionic strength has rarely been mentioned. The role of glycation in interfering with the assembly of myosin into filaments is well documented in the field of biomedicine. The changes in myosin behavior due to nonenzymatic glycation in vivo have been suggested to play a major role in diabetes and aging-related muscle degenerative diseases [12]. A reduced rate of muscle contraction resulting from the suppression of myosin assembly is one of the main causes of this condition [13]. However, few studies have addressed this topic in food systems. Hence, in this study we aimed to...
test the hypothesis that glycosyl units may serve as interference factors to suppress the self-assembly of myosin molecules in food systems with low ionic strength.

In a recent study, Li et al. [6] reported that approximately 75 % of rabbit MPs could dissolve effectively in a low-ionic strength medium via glycation under dry-heating conditions (52.4 °C, 23.1 h); however, the shortcomings of this method are that it involves time-consuming procedures and requires precise temperature control. In our previous work [14], we found that the solubility of MPs in water was significantly improved by glycation with dextran (DX) under wet-heating conditions (37 °C, 8 h), which resulted in the solubility of the glycated MPs to reach approximately 50 %. However, it should be noted that the solubility of glycated MPs is still far from complete, due to the low grafting efficiency of traditional wet-heating glycation. On the one hand, the polymeric structure of filamentous myosin limits exposure to most of the active binding sites; on the other hand, most of the non-disaggregated myosin filaments remain in an insoluble state. Thus, ideal techniques should be applied to enhance the efficiency of glycation.

High-intensity ultrasound (HIU) shows great potential in promoting the glycation reaction. The cavitation effect of HIU can increase the flexibility of proteins, which enables the grafting of hydrophilic groups onto the protein molecules [15]. Our previous research showed that the physical force exerted by HIU can disrupt the filamentous myosin structure, thus exposing the internal reactive groups on the myosin surface [6]. However, the subsequent assembly of myosin molecules is still inevitable. An interesting hypothesis is that the destruction of the myosin filament induced by HIU, combined with the suppressing effect of glycation on the myosin assembly process, could further improve the water solubility of MPs. Furthermore, some studies have found that HIU can affect the glycation of proteins, although the conclusions of different investigations were largely inconsistent. It was reported that HIU increased the glycation reaction rate and significantly improved the solubility and foamability of ovalbumin [16]. In contrast, HIU pretreatment was found to induce the aggregation of β-lactoglobulin, partially burying glycation sites [17]. Hence, investigating the enhancement effect of different ultrasonic parameters on the glycation reaction is a highly important task.

Dextran (DX) is a flexible polysaccharide with high solubility and poor aggregation ability [18]. Compared to glycated proteins with small carbohydrate molecules, the polysaccharides provide strong steric hindrance and low reducibility, preventing excessive color development and protein polymerization [19]. In the present work, the effect of HIU pretreatment on the glycation reaction between MPs and DX was compared with that of the traditional wet-heating method. Moreover, we determined the solubility, structural characteristics, and filament-forming ability of the treated MPs. The objective was to verify the hypothesis discussed above and apply the obtained insights to expand the applications of MPs in food processing media of low ionic strength as well as hydrophilic and colloidal food systems.

2. Materials and methods

2.1. Materials

Fresh porcine longissimus thoracis et lumbarum muscle was collected at 24 h post-mortem from a local supermarket (Harbin, Heilongjiang, China). DX (molecular weight 70 kDa, purity ≥ 99 %) was obtained from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Sodium dodecyl sulphate (SDS), bovine serum albumin (BSA), and γ-leucine were purchased from Sigma-Aldrich (MO, USA). All reagents and chemicals used in the experiment were of analytical grade.

2.2. Extraction of MPs

The MP sample was prepared according to a previous method [6]. Briefly, minced meat was homogenized in 10 volumes of sodium phosphate buffer (20 mM, 25 mM NaCl, 5.0 mM ethylenediaminetetraacetic acid (EDTA), and pH 7.0), then centrifuged (10000 g, 20 min) at 4 °C. The sediment was washed with 10 volumes of sodium phosphate buffer (20 mM, 0.1 M NaCl, 5.0 mM EDTA, and pH 7.0). This step was repeated three more times. Connective tissue and external fat were filtered through four layers of absorbent gauze. The resulting pellet was resuspended and washed twice in 10 volumes of sodium phosphate buffer (5.0 mM, 1.0 mM EDTA, and pH 7.0) and centrifuged. The sediment from the final centrifugation step was used as the MP sample.

2.3. HIU pretreatment combined with glycation

The extracted MPs was dispersed in sodium phosphate buffer (50 mM, 1.0 mM EDTA, pH 7.5) to prepare a 20 mg/mL aqueous MP suspension, then placed in a jacketed beaker and treated with an ultrasound unit (Scientz Biotechnology Co., Ltd., Ningbo, China; frequency 20 kHz) equipped with a titanium probe of 6.0 mm diameter. Samples were treated at 200, 300, 400, and 500 W for 15 min (pulse duration of 5 s on-time and 3 s off-time). The samples were processed in an ice water bath to keep the processing temperature below 25 °C.

After HIU pretreatment, the MP (20 mg/mL) and DX samples were mixed together at a 1:1 (w/w) ratio in sodium phosphate buffer (50 mM, pH 7.5). The protein concentration was adjusted to 10 mg/mL. Then, the solution of MPs and DX was heated at 37 °C for 8 h with constant stirring. Chain reactions were stopped using an ice-water bath after the glycation reaction. Sodium azide (0.02 % w/v) was separately added to each MP sample to retard microbial growth. The unreacted sugars in the solutions were removed through dialysis for 24 h at 4 °C and then stored at 4 °C for further measurements.

In this paper, native (untreated) MPs and MPs subjected only to glycation are referred to as “MP” and “MP-DX”, respectively. The glycated samples pretreated at 200, 300, 400, and 500 W are referred to as “UMP-DX-200”, “UMP-DX-300”, “UMP-DX-400”, and “UMP-DX-500”, respectively.

2.4. Free amino group contents and grafting degree

The degree of MP conjugation was determined by measuring the decrease in free amino group content in the treated MP samples using the o-phthalaldehyde (OPA) method [20]. The OPA solution was freshly prepared by mixing 80 mg of OPA reagent, 50 mL of 0.1 M sodium tetraborate buffer (pH 9.3), 5.0 mL of 20 % (w/w) SDS, and 200 μL of β-mercaptoethanol, followed by dilution to a final volume of 100 mL with distilled water. This reagent should be prepared immediately before use. During the analysis, 200 μL of sample solution (2.0 mg/mL) was mixed with 4.0 mL of OPA reagent and incubated at 35 °C for 2 min. The absorbance value of the solution was measured at 340 nm. The grafting degree (DG) was calculated as follows:

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DG = \frac{C_h - C_i}{C_i} \times 100\%
\]

where \( C_0 \) and \( C_1 \) are the concentrations of free amino groups in the native MPs and conjugate MPs, respectively.

2.5. Fourier transform infrared spectroscopy measurements

Fourier transform infrared (FT-IR) spectra of the MP samples after different treatments were recorded on an ALPHA-T spectrometer (Brucker, Germany). Prior to the analysis, the lyophilized samples (30 mg) were adequately ground and mixed with 3.0 g of dried KBr (1.0 %, w/w), before being compressed to form a thin slice. All spectra between 4000 and 400 cm \(^{-1} \) were acquired through 256 cumulative scans at a resolution of 2.0 cm \(^{-1} \).
2.6. Solubility

The solubility of MP samples was determined using the method described by Fu et al. [9], with some slight modifications. The non-glycosylated or glycosylated samples were diluted to 1.0 mg/mL with 50 mM sodium phosphate (pH 7.5) and then centrifuged at 10,000 g for 15 min. The protein solubility was expressed as the percentage of the protein in the supernatant with respect to the total protein content before centrifugation.

2.7. Particle size and zeta potential analyses

The average particle size (d₄,₃), size distribution, and zeta potential of the samples were measured using a laser particle analyzer (Microtrac S3500, Florida, USA) and a Zeta Sizer Nano ZS 90 instrument (Malvern Instruments, Ltd., Great Malvern, UK), following a previously described method [21]. The samples were diluted to 0.1 mg/mL with 50 mM phosphate buffer (pH 7.5) and subsequently injected into a cuvette for analysis.

2.8. Confocal laser scanning microscopy (CLSM)

Images of the MP samples were assessed using confocal laser scanning microscopy (Leica TCS SP5, Heidelberg, Germany). A staining solution containing 1.0 g/L of Nile blue was mixed with 1.0 mL of the aqueous MP suspension. To inspect the morphological characteristics of the MPs, 10 μL of the stained samples was dropped on glass slides with coverslips. Representative fluorescent images of each sample were obtained using a 40-fold objective lens [7].

2.9. Circular dichroism (CD) spectroscopy

CD spectra of the MP samples were obtained at 25 °C using a J-715 (Jasco Corp., Tokyo, Japan) spectrometer, using the method reported by Wen et al. [22]. The measurements were performed by scanning aqueous MP suspensions (0.3 mg/mL) from 190 to 260 nm using quartz cuvettes (1.0 mm path length). The protein secondary structure content was calculated using the secondary structure estimation program.

2.10. Fluorescence spectroscopy

Each sample was continuously diluted with 50 mmol/L phosphate buffer (pH 7.5) to obtain the 1.0 mg/mL protein solution. After thorough stirring, the intrinsic fluorescence spectra were recorded on an F-4500 fluorescence spectrophotometer (Hitachi Co., Tokyo, Japan). The excitation wavelength was set to 283 nm, and the emission spectrum was recorded between 300 and 400 nm. Both the excitation and emission slits widths were 5.0 nm, and the scanning speed was 1000 nm/min.

2.11. Surface hydrophobicity

The hydrophobicity of the MP samples was determined using 8-anilino-1-naphthalenesulfonic acid (ANS) as a hydrophobic probe, following the method reported by Liu et al. [2] with minor modifications. Each sample was diluted to concentrations ranging from 0.2 to 1.0 mg/mL in 50 mM phosphate buffer. Subsequently, 4.0 mL of each diluted sample was mixed with 20 μL of 8.0 mM ANS solution and incubated in the dark for 20 min. The fluorescence intensity of the mixed solution was measured using an F-4500 spectrophotometer (Tokyo, Japan) at wavelengths of 380 nm (excitation) and 410-570 nm (emission), with excitation and emission slit widths set at 5.0 nm. The hydrophobicity of the MP samples was determined as the initial slope of the linear regression equation of a fluorescence intensity vs protein concentration plot.

2.12. Simulation of thick filament assembly process

The MP, MP-DX, and UMP-DX-400 samples suspended in 0.6 M NaCl (pH 7.5) were centrifuged at 20,000 g for 20 min, and the obtained supernatant was denoted as salt-soluble MPs. To clearly investigate the effects of the HIU–glycation combination on the assembly ability of thick filaments at low ionic strength, each salt-soluble MPs (0.6 M NaCl, pH 7.5) was dialyzed at medium (0.3 M NaCl, pH 7.5) and low (1.0 mM, pH 7.5) ionic strength to simulate the assembly process of myosin molecules.

2.12.1. Turbidity

The turbidity of non-dialyzed and dialyzed samples was measured according to the method of Xu et al. [23], with minor modifications. MP samples were diluted to 1 mg/mL and analyzed with a UV-visible spectrophotometer (UT1810, Beijing Purkinje General Instrument, ltd., China) at 370 nm and 25 °C.

2.12.2. Transmission electron microscopy (TEM)

The micromorphology of MPs was examined using a transmission electron microscope (HT7800, HITACHI, Japan) at an acceleration voltage of 100 kV. A 5.0 μL aliquot of MP samples (1 mg/mL) was deposited on a carbon-coated copper grid for 2 min. The excess liquid was removed by filter paper, and the samples were stained with 0.2 % phosphotungstic acid for 1 min before drying at room temperature. Then the morphology of the samples was observed under ×4000 magnification.

2.13. Statistical analysis

Three batches of MP samples were prepared, and each of the above measurements was performed in triplicate. The results were reported as mean values ± standard error (SE) and analyzed using the Statistic 8.1 software package (Analytical Software, St Paul, MN, USA). One-way analysis of variance (ANOVA) was used to assess the significance (P < 0.05) of differences among calculated means using Tukey’s multiple comparison procedure.

3. Results and discussion

3.1. Degree of glycation

Fig. 1A illustrates the free amino group content and grafting degree of the glycoconjugates between MPs and DX. During glycation, the carbonyl groups of carbohydrate molecules are covalently attached to the ε-NH₂ moiety of the amino groups in the MPs to generate glycoconjugates [24]. Therefore, the decreased amount of free amino group content in glycated MP samples indicated that DX was successfully conjugated to MPs by glycation. Correspondingly, the grafting degree gradually increased with decreasing free amino group content. Compared to native MP and MP-DX samples, HIU pretreatment (200–400 W) promoted the subsequent glycation reaction between MPs and DX, which significantly improved the grafting degree of the conjugates (P < 0.05). The maximum grafting degree was observed in glycated MP samples pretreated by 400 W ultrasound (P < 0.05). Consequently, we speculate that the cavitation effect of ultrasound provided an intense physical force to expose more reactive groups for the grafting of MPs and DX [25]. However, the grafting degree of glycated MPs pretreated by 500 W ultrasound decreased significantly (P < 0.05), presumably because excessive power caused protein molecules to agglomerate through internal thermal effects, which in turn reduced the amount of exposed free amino groups in MPs and suppressed the glycation reaction [20].
3.2. Fourier transform spectroscopy analysis

The FT-IR spectra of the native and glycated MPs at different ultrasound powers (0, 200, 300, 400, and 500 W) are shown in Fig. 1B. The absorption peaks at 1653 and 1544 cm⁻¹ corresponded to the amide I (C–O stretching) and amide II (N–H bending and C–N stretching) bands, which are the most significant spectral features of proteins [26]. The amide I and amide II peaks of glycated MPs exhibited an increasing trend, which was attributed to the glycation reaction, because this process involves the formation of new compounds (C–N, C–O, C–N) [22]. A new peak (2933 cm⁻¹) corresponding to C–H stretching appeared in the spectra of glycated MPs, consistent with the formation of glycation products and confirmed by previous reports [20]. In addition, the absorption peak of the free hydroxyl groups was observed in the 3700–3200 cm⁻¹ region. Compared with the native MPs, glycation resulted in an increase in the absorption peak intensity in the range of 3700–3200 cm⁻¹, indicating an increased hydroxyl group content in the glycated MPs. These results show that the DX molecules were covalently bonded to the MPs. In addition, ultrasound pretreatment promoted the grafting reaction between MPs and DX, and the absorption intensity of the UMP-DX-400 conjugates at 2933 and 3700–3200 cm⁻¹ was higher than that of the native MPs and other conjugated MPs.

3.3. Solubility

The changes in the solubility of the native and glycated MP samples are shown in Fig. 2A. As expected, the solubility of the native MPs in water was relatively low (3.7 %). This was consistent with previous reports; the adjacent myosin monomers tend to form self-assembled filamentous polymers in low-ionic strength media, which make it insoluble and unstable [27]. After glycation with DX, the solubility of MPs showed a significant ($P < 0.05$) increase to 50.62 % (MP-DX). This improvement may be attributed to the incorporation of more hydrophilic groups, facilitating the protein–water interaction, and to the grafting of DX, which suppressed the formation of filamentous myosin [15,21]. It is interesting to observe that HIU pretreatment further improved the solubility of glycated MPs. According to Liu et al. [6], the intense physical forces generate by ultrasound lead to the depolymerization of filamentous polymers, thereby improving the solubility of MPs in water. Consequently, we speculate that the HIU pretreatment may expose more reactive sites that were originally located inside the filamentous polymeric structure, increasing the subsequent glycation degree of the protein; in this way, the electrostatic repulsion and steric hindrance caused by the grafted DX would effectively hinder the polymer formation. Notably, UMP-DX-400 showed the highest solubility (78.21 %), which was attributed to the fact that the higher DG provided better solubility, in agreement with the results of the DG analysis. However, with the ultrasound power was further increased to 500 W, the solubility of glycated MPs decreased significantly ($P < 0.05$), likely due to the relatively high ultrasound power causing a reaggregation of protein particles [28]. Correspondingly, the aggregation of proteins...
could bury a fraction of the glycation sites inside the aggregates, which may reduce their accessibility to polysaccharide molecules in the subsequent glycation reaction.

The macroscopic properties of the samples were inspected to monitor the stability of MPs in water, as shown in Fig. 2B. After storage at 4 °C for 7 days, the MP samples showed different delamination levels. The control suspensions migrated to the bottom of the bottle and exhibited aggregation and precipitation effects, reflecting the instability of the native MPs in water. It is evident that the dispersibility of MPs was improved to varying degrees after different glycation treatments, consistent with the solubility results. The UMP-DX-400 conjugate, in particular, showed a more homogeneous and transparent appearance, without marked flocculation and stratification.

3.4. Particle diameter and size distribution analyses

To further characterize the particle properties in protein suspensions after different treatments, we carried out CLSM, particle size distribution, average particle size ($d_{4,3}$), and zeta potential measurements of the MP samples (Fig. 3). As shown in Fig. 3A, in which green fluorescence areas represent the proteins, several intact and linear structures with relatively large particle size were observed in the native MP sample, which were attributed to the self-assembly of myosin, consistent with our previous report [6]. For MP-DX conjugates, the intact fibrous protein structure was decomposed into small filamentous fragments, and the particle size was significantly lower than that of the native MPs ($P < 0.05$). These results suggest that the introduction of the glycosyl units could suppress the formation of ordered filamentous myosin structures to a certain extent, thus reducing the particle size of MPs. This could be attributed to the changes in surface hydrophobicity, electrostatic interactions, and steric hindrance induced by glycation [21]. In the case of glycated MPs pretreated with HIU, smaller aggregates and shorter fibrils were observed with increasing ultrasound power. The particle size distribution peaks measured for all treatments shifted to the left, and $d_{4,3}$ decreased. These results were consistent with the appearance of the samples (Fig. 2B), indicating that the combination of HIU pretreatment with glycation could further improve the solubility and stability of MPs in water. The most uniform distribution and smallest particle size were observed for the UMP-DX-400 conjugate ($P < 0.05$), probably because it exhibited the highest grafting degree, causing a larger steric hindrance and suppressing self-assembly to the largest extent. Notably, the UMP-DX-500 conjugate showed a marked increase in particle size, and a large peak appeared in the corresponding distribution, which may be due to the “over-processing” effect at higher power leading to protein reaggregation [29].

The particle size distributions of all samples presented an obvious bimodal distribution (Fig. 3B). The native MP samples exhibited peaks corresponding to relatively small (6–18 μm) and larger (18–74 μm) particles. Interestingly, the intensity of the large- and small-particle peaks appeared to decrease and increase, respectively, in the glycated MP samples, particularly in the UMP-DX-400 conjugate. The small-particle peak was ascribed to oligomer or monomer species in the MPs, whereas the large-particle one was probably due to the filamentous myosin polymers [30]. In general, the formation of filamentous myosin polymers in low-ionic strength media are mediated by charged clusters periodically distributed in the bipolar tail of myosin [7,15]. It is...
therefore reasonable to speculate that the introduction of polysaccharide units interferes with the assembly of myosin molecules and suppresses the formation of filamentous myosin polymers, causing a decrease in the peak area corresponding to large particles. Furthermore, the present results suggest the occurrence of a moderate synergistic effect between HIU and glycosylation, and the MPs subjected to the combination of the two treatments showed a smaller particle size and a more uniform distribution (Fig. 3B, C). However, no further increase in the small-particle peak was observed when the HIU power increased to 500 W. On the contrary, the peak area corresponding to the large particles increased, which could be attributed to the aggregation of protein particles, as discussed in section 3.1.

3.5. Zeta potential analysis

The zeta potential is an important parameter reflecting the stability of MPs, which can be used to characterize the dispersion or aggregation of protein particles [31]. As shown in Fig. 3D, all MP suspensions exhibited a net negative charge, because of the deprotonation of amino acids contained in the MPs at neutral pH [7]. Notably, the MP-DX samples possessed a significantly higher net negative charge than the native MPs ($P < 0.05$). This was consistent with a previous study reporting that the net negative charge of whey proteins increased after glycation [32]. The increased negative charge can be attributed to the introduction of negatively charged sugar units and the consumption of positively charged lysine after glycation, resulting in a more negative charge on the particle surface. After the combined treatment, the net negative charge of glycated MP samples showed a further increase. The highest zeta potential value (−24.68 mV) was obtained for glycated MP samples pretreated by 400 W ultrasound ($P < 0.05$), in agreement with the results of particle size (Fig. 3B). A reasonable interpretation of this result is that HIU pretreatment induced the dissociation of myosin filaments and the unfolding of proteins, culminating in the exposure of more charged residues and glycation sites, whose combination promoted the observed increase in negative charge [28].

The myosin contained in MPs is characterized by bipolar rod domains that, driven by electrostatic interactions, tend to stack up against each other, assemble in a regular fashion, and eventually form filamentous polymers in low-ionic strength media [28]. Changes in the MP charge thus reflect a broken electrostatic balance between myosin rods, suppressing the self-assembly of myosin. The higher negative zeta potential of the UMP-DX-400 conjugate indicates a stronger electrostatic repulsion, which is expected to effectively suppress the molecular assembly behavior of myosin by hindering rod–rod electrostatic interactions [27]. As a result, the UMP-DX-400 conjugate had a more uniform particle size distribution and higher solubility in water (Figs. 2 and 3).

3.6. Changes in the secondary structure of MPs

The above results indicate that HIU pretreatment can promote the subsequent glycation reaction and further improve the dispersion stability of MPs, which may be related to the depolymerization of filamentous polymers and the unfolding of the protein structure. Further analysis focused on determining the effect of the combination of HIU pretreatment with glycation on the conformation of MPs. The secondary structure of the protein was assessed using CD spectroscopy (Fig. 4A). The CD spectrum of MPs displayed two negative bands corresponding to the $\alpha$-helix structure near 208 and 222 nm, reflecting the predominance of the $\alpha$-helix structure of myosin rods [33]. Obviously, the intense negative troughs of each of these two peaks were present in the native MPs, due to the superhelix conformation formed by the aggregation of

Fig. 4. Structural characteristics of myofibrillar proteins (MPs) subjected to different treatments: (A) far-UV circular dichroism spectra, (B) calculated secondary structure content, (C) intrinsic emission fluorescence spectra, and (D) surface hydrophobicity. Different letters (a–e) indicate significant differences among different treatments ($P < 0.05$).
myosin rods, and indicating the presence of many myosin filaments in the aqueous MP suspension [6]. After glycation, varying degrees of intensity attenuation of the peaks at 208 and 222 nm were observed in the CD spectrum; this effect was more pronounced after ultrasound pretreatment, denoting significant disruption and loss of the helical structure of filamentous myosin [30]. The absence of α-helix structures in the myosin rods may lead to changes in intermolecular interactions, thereby suppressing the assembly of myosin molecules, which is consistent with our previous hypothesis [6].

The percentages of secondary structure of the MPs were calculated from the CD Pro program, as shown in Fig. 4B. Both the α-helix and β-sheet contents decreased significantly after different glycation treatments, while the β-turn and random coil contents increased (P < 0.05), which is consistent with the circular dichroism results reported by [24]. In general, the α-helix structure is stabilized by intramolecular hydrogen bonds between the carbonyl oxygen (–CO) and amino hydrogen (NH–) of single polypeptide chains, while the β-sheet structure is supported by hydrogen bonds between peptide chains [31]. Therefore, the decrease in the α-helix and β-sheet contents revealed that the grafting of polysaccharides induced protein unfolding and denaturation, possibly disrupting both intramolecular and intermolecular hydrogen bonds and consequently altering the secondary structure of the protein. The samples in the combined treatment groups (UMP-DX) showed more extensive changes in the secondary structure of MPs than those in the individual treatment groups (MP-DX). This may be attributed to the intense physical force induced by sonic cavitation, which destroyed the protein–protein interactions, resulting in the dissociation of more hydrogen bonds. Furthermore, the glycation reaction might introduce complex groups into the hydrogen bonding sites of protein molecules, and ultrasound pretreatment further enhanced this phenomenon. In addition, the steric hindrance caused by the grafted DX could further shield the intermolecular hydrogen bonds [31]. However, a further increase in ultrasound power to 500 W resulted in a significant increase in α-helix and β-sheet contents, at the expense of the β-turn and random coil contents (P < 0.05). Excessive ultrasound-induced internal thermal effects resulted in protein particles reaggregating rather than staying in the unfolded state, with their structure transforming from disordered and flexible to ordered and rigid.

3.7. Changes in the tertiary structure of MPs

3.7.1. Intrinsic tryptophan fluorescence

As a method for assessing the tertiary structure of proteins, intrinsic tryptophan fluorescence is sensitive to the polarity of the microenvironment surround tryptophan molecules [34]. Generally, tryptophan residues are located inside the protein structure and have a high fluorescence intensity. When the protein structure unfolds, the tryptophan residues are exposed to hydrophilic solvent, resulting in reduced fluorescence intensity [35]. In this study, the fluorescence intensity of MP-DX samples was lower than that of the native MP samples (Fig. 4C), indicating that the protein structure stretched and unfolded after glycation, which exposed tryptophan residues previously buried in the hydrophobic inner regions of the protein. The HIU pretreatment, especially at 400 W power, led to a further reduction in fluorescence intensity, indicating further protein unfolding and DX grafting onto MPs. On the other hand, as a water-soluble polysaccharide, the presence of DX would enhance the polar environment and partially shield the fluorescence signal. In addition, the maximum emission wavelength (λmax) of the native MPs was 331.0 nm, while MP-DX, UMP-DX-200, UMP-DX-300, UMP-DX-400, and UMP-DX-500 were red-shifted to 332.4, 332.8, 334.2, 336.4, and 333.6 nm, respectively. This further proved that the microenvironment of the tryptophan residues became more hydrophilic in the presence of a more flexible structure [36].

3.7.2. Surface hydrophobicity

Fig. 4D showed that glycated MP samples had a higher surface hydrophobicity than the native MP samples, reflecting the exposure of previously buried hydrophobic residues induced by protein unfolding [22]. Most hydrophobic amino acids in MPs are known to be negatively charged in a neutral environment. The exposure of the hydrophobic groups could lead to a relatively high net negative charge of MPs, which would increase the electrostatic repulsion between proteins and consequently enhance their solubility in water [6]. This aspect was consistent with the zeta potential results and further supported the solubility analysis. It should be noted that the surface hydrophobicity of glycated MPs pretreated by ultrasound was markedly lower than that of MPs subjected only to glycation. Moreover, the surface hydrophobicity of glycated MPs gradually decreased with increasing ultrasonic power. This may be due to the promoting effect of the HIU pretreatment on the subsequent glycation reaction, allowing more hydrophilic hydroxyl groups to be effectively grafted onto the protein, thereby reducing the hydrophobicity of the MP conjugates as a whole [37]. Another reason may be that the presence of DX increased the steric hindrance on the glycated MP surface, thereby preventing ANS molecules from approaching hydrophobic groups [17].

3.8. Evaluation of filament-forming ability

To explore how the changes in MP conformation induced by the combination of HIU and glycation affected the ability to form myosin filaments in low-salt media, we designed models with different NaCl contents to simulate the assembly process of myosin molecules. We examined the solubility, turbidity, and microscopic morphology of high-salt media-soluble MP, MP-DX, and UMP-DX-400 after dialysis in 0.3 M and 1 mM NaCl.

The 0.6 M NaCl solution of MPs exhibited a transparent and clear appearance, corresponding to the expected high solubility and low turbidity (Fig. 5). As the ionic strength decreased, the native MP samples became turbid and white-colored due to polymerization, reflecting a decrease in solubility and increase in turbidity (Fig. 5A, D). A similar trend was observed for the solubility and turbidity of MP-DX samples; however, the changes were less dramatic than those of the native MP samples (Fig. 5E). The MP-DX samples were slightly turbid and translucent at each dialysis stage (Fig. 5B). Compared to the native MP and MP-DX samples, the UMP-DX-400 samples displayed a more transparent appearance during dialysis (Fig. 5C). Notably, after dialysis against 1 mM NaCl, the solubility of the UMP-DX-400 samples remained at a high level (Fig. 5F), which indirectly indicated an impaired filament-forming ability. Thus, we speculated that the ultrasound pretreatment combined with glycation might influence the myosin monomer–filament transition, thereby improving the solubility of MPs in low-salt solutions. Finally, the microscopic morphology of MPs in solutions of different ionic strength was investigated by transmission electron microscopy (Fig. 6). Before dialysis, the native MP, MP-DX, and UMP-DX-400 samples were predominantly present as monomers in high-salt solutions (0.6 M NaCl) [8,15]. As the NaCl concentration decreased from 0.6 M to 1 mM, the native MP monomers polymerized and eventually formed highly ordered filamentous polymers, confirming that in low-ionic strength media the myosin in the native MPs would assemble into myosin filaments via rod–rod electrostatic interactions [27]. Notably, MP-DX displayed a more dispersed microstructure in 0.3 M NaCl solution, while some shortened, disordered, and irregular filamentous polymers were observed in 1 mM NaCl solutions; this suggested that the glycation suppressed the formation of integrated filament structures, forming some small filamentous fragments instead. Interestingly, no filamentous polymers were observed in the UMP-DX-400 samples at both 0.3 M and 1 mM NaCl concentrations. Some myosin monomers, as well as their dimers and oligomers, were clearly identified in 1 mM NaCl solution, in good agreement with previous results based on particle size and CLSM measurements (Fig. 3A). These observations confirmed that the combination of HIU pretreatment and glycation could improve the solubility of MPs by destroying or suppressing the assembly of myosin.
filaments at low ionic strength.

3.9. Proposed mechanism for enhancement of MP solubility in water by ultrasound–glycation treatment

Based on the above results, we proposed a possible mechanism for the enhancement of the solubility of MPs by the combination of HIU pretreatment and glycation (Fig. 7). Myosin is at the center of this mechanism, as the main protein in MPs and thus responsible for their solubility. According to previous studies, myosin has a rod-like structure, with two spherical head regions and a tail region consisting of a coiled helix [30]. Native myosin is usually polymerized in low-salt media to form insoluble filamentous polymers (Fig. 3A and 6) with large particle size (Fig. 3A) and low absolute zeta potential (Fig. 3B). After glycation, the net negative charge (Fig. 3B) and electrostatic repulsion increases, due to the covalent binding of positively charged lysine residues of the protein with dextran (Fig. 1A, B). As a result, the electrostatic balance of the myosin tail is broken, and the assembly process of filamentous polymers is disrupted (Fig. 3A and 6), which improves the solubility of MPs in water (Fig. 2). After HIU pretreatment, the grafting degree of the glycated MPs increases (Fig. 1A). This is because HIU pretreatment destroys the filamentous polymerization structure, induces protein unfolding, and exposes more residues and glycation sites. Under this condition, ultrasound combined with glycation can provide a stronger electrostatic repulsion to suppress filament assembly at low ionic strength (Fig. 6). In addition, the grafting of more hydrophilic residues can provide a stronger steric hindrance, which may effectively prevent subsequent aggregation of MPs. Thus, the combination of HIU pretreatment with glycation further improves the solubility and stability of MPs in water.

4. Conclusions

In conclusion, HIU pretreatment promoted the subsequent glycation between MPs and DX. The HIU pretreatment, especially at 400 W power, significantly improved the solubility of glycated MPs in water, as evidenced by their increased grafting degree, reduced particle size, and more uniform size distribution. In particular, the 400 W ultrasound pretreatment significantly increased the net charge of glycated MPs. The stronger inter/intramolecular electrostatic repulsion was responsible for the loss of α-helix structures and the suppress of myosin filament formation in low-salt media. In addition, the steric hindrance produced by the grafted polysaccharides would destroy existing filamentous myosin aggregates and hinder further aggregate formation, resulting in the
solubilization of MPs. Overall, the covalent bonding of MPs with DX through ultrasound pretreatment combined with glycation might be a promising method to improve the water solubility of MPs; this approach opens a new avenue for the development of muscle protein-based products with desirable properties.
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.

Data availability

No data was used for the research described in the article.

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References

[1] Y. Hu, L. Zhang, Y. Yi, I. Solangi, L. Zan, J. Zhu, Effects of sodium hexametaphosphate, sodium tripolyphosphate and sodium pyrophosphate on the ultrastructure of beef myofibrillar proteins investigated with atomic force microscopy, Food Chem. 358 (2021) 128146.

[2] H. Liu, Z. Wang, I.H. Batdar, Q. Liu, Q. Chen, B. Kong, Combination of high-intensity ultrasound and hydrogen peroxide treatment suppresses thermal aggregation behaviour of myofibrillar protein in water, Food Chem. 367 (2022) 130756.

[3] X. Chen, Q. Qiu, K. Chen, D. Li, L. Liang, Water-soluble myofibrillar protein-pcet complex for enhanced physical stability near the isoelectric point: Fabrication, rheology and thermal property, Int. J. Biol. Macromol. 142 (2020) 615–623.

[4] K. Wang, Y. Li, Y. Zhang, X. Luo, J. Sun, Improving myofibrillar proteins solubility and thermostability in low-ionic strength solution: A review, Meat Sci. 189 (2022).

[5] X. Chen, R.K. Tume, X. Xu, G. Zhou, Solubilization of myofibrillar proteins in water or low ionic strength media: Classical techniques, basic principles, and novel functionalities, Crit. Rev. Food Sci. Nutr. 57 (15) (2017) 3260–3280.

[6] H. Liu, H. Zhang, Q. Liu, Q. Chen, B. Kong, Solubilization and stable dispersion of myofibrillar proteins in water through the destruction and inhibition of the assembly of filaments using high-intensity ultrasound, Ultrason. Sonochem. 67 (2020) 105160.

[7] X. Chen, X. Xu, G. Zhou, Potential of high pressure homogenization to solubilize chicken breast myofibrillar proteins in water, Innovative Food Sci. Emerg. Technol. 32 (2016) 167–179.

[8] E. Takai, S. Yoshizawa, D. Ejima, T. Arakawa, K. Shiraki, Synergistic solubilization of porcine myosin in physiological salt solution by arginine, Int. J. Biol. Macromol. 33 (2016) 170–179.

[9] X. Chen, X. Xu, M. Han, G. Zhou, C. Chen, P. Li, Conformational changes induced by high-pressure homogenization inhibit myosin filament formation in low ionic strength solutions, Food Res. Int. 85 (2016) 1–9.

[10] C. Su, Z. He, Z. Wang, D. Zhang, H. Li, Aggregation and deaggregation: The effect of high-pressure homogenization cycles on myofibrillar proteins aqueous solution, Int. J. Biol. Macromol. 189 (2021) 567–576.

[11] X. Xu, Y. Zhao, Z. Wei, H. Zhang, M. Dong, M. Huang, M. Han, X. Xu, G. Zhou, B. Zhu, L. Chen, Binding of aldehyde flavour compounds to beef myofibrillar proteins and the effect of nonenzymatic glycation with glucose and glucosamine, LWT-Food Sci. Technol. 144 (2021) 111198.

[12] X. Zhang, X. Yue, B. Ma, X. Fu, H. Ren, M. Ma, Ultrasonic pretreatment enhanced the glycation of ovotransferrin and improved its antibacterial activity, Food Chem. 346 (2021) 128905.

[13] L. Liu, X. Li, L. Du, X. Zhang, W. Yang, H. Zhang, Effect of ultrasound assisted heating on structure and antioxidant activity of whey protein peptide grafted with galactose, LWT-Food Sci. Technol. 109 (2019) 130–136.

[14] X. Chen, X. Xu, M. Han, G. Zhou, C. Chen, P. Li, Conformational changes induced by high-pressure homogenization inhibit myosin filament formation in low ionic strength solutions, LWT-Food Sci. Technol. 137 (2021) 110387.

[15] H. Liu, J. Zhang, H. Wang, Q. Chen, B. Kong, High-intensity ultrasound improves the physical stability of myofibrillar protein emulsion at low ionic strength by destroying and suppressing myosin molecular assembly, Ultrason. Sonochem. 74 (2021) 105554.

[16] Z. Li, Y. Zheng, Q. Sun, J. Wang, B. Zheng, Z. Guo, Structural characteristics and emulsifying properties of myofibrillar protein-dextran conjugates induced by ultrasound Maillard reaction, Ultrason. Sonochem. 72 (2021) 105458.

[17] G. Liu, Q. Zong, Thermal aggregation properties of whey protein grafted with various saccharides, Food Hydrocolloids 32 (1) (2013) 87–96.

[18] Y. Zhong, P. Han, S. Sun, N. An, X. Ren, S. Lu, Q. Wang, J. Dong, Effects of apple polyphenols and hydroxypropyl-β-cyclodextrin inclusion complexes on the oxidation of myofibrillar proteins and microstructures in lamb during frozen storage, Food Chem. 375 (2022) 131874.

[19] Y. Cao, X. Xiong, Chlorigenic acid mediated gel formation of oxidatively stressed myofibrillar protein, Food Chem. 180 (2015) 235–243.

[20] S. Li, Z. He, M. Li, B. Li, L. Li, A. study of the physicochemical properties of rabbit glycated myofibrillar protein with high solubility in low ionic strength medium, Int. J. Biol. Macromol. 147 (2021) 241–249.

[21] M. Mohammadian, M. Salami, Z. Eman-Djomeh, S. Momem, A. Moosavi-Movahedi, Gelation of oil-in-water emulsions stabilized by heat-denatured and nanoencapsulated whey proteins through ion bridging or citric acid-mediated cross-linking, Int. J. Biol. Macromol. 120 (2018) 2247–2258.

[22] J. Liu, C. Fang, X. Xu, Q. Su, P. Zhao, Y. Ding, Structural changes of silver carp myofibrillar protein with high solubility glyca- tion, Food Hydrocolloids 91 (2019) 275–282.