Food Chemistry 320 (2020) 126665

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Enhancing tilapia fish myosin solubility using proline in low ionic strength solution

Yige Zhou[a,b], Hongshun Yang[a,b,*]

[a] Department of Food Science & Technology, National University of Singapore, Singapore 117542, Singapore
[b] National University of Singapore (Suzhou) Research Institute, 377 Lin Quan Street, Suzhou Industrial Park, Suzhou, Jiangsu 215123, PR China

ARTICLE INFO

Keywords:
Myosin
Solubilisation
Amino acid
Aggregation
Protein
Secondary structure
Aquatic food
Molecular interaction

ABSTRACT

The effects of using proline to solubilise fish myosin under low ionic strength conditions were studied. After solubilising myosin in 0.1 M NaCl containing 5, 10, 15, and 20 mM proline, respectively, it was observed that more than 80% of the myosin was effectively solubilised using 10 mM proline. The addition of 10 mM proline lowered the surface hydrophobicity of myosin from 18.25 to 8.22 mg/g, increased the amount of β-sheet structure from 33.87% to 46.88%, both of which facilitated solubilisation. As revealed by transfer free energy measurements, the interactions between proline and tyrosine and tryptophan residues were more favourable. Furthermore, the ability of proline to shield hydrophobic sites of myosin and to partially break disulphide bonds helped to form myosin oligomer aggregates. Transmission electron microscopy images verified the effects of proline on myosin proteins. A solubilisation mechanism based mainly on chemical interactions between myosin and proline was proposed.

1. Introduction

Fish is a popular food worldwide, not only because of its low lipid content and delicious taste, but also the high digestibility of fish protein provides humans with premium protein. In addition to fresh fish fillets, processed fish meat has become more prevalent; for example, fish balls, fish cakes, and fish sticks are major fishery products. To assure good product texture, fish proteins are the main concern. Myosin is the major muscle protein in fish, and NaCl at a concentration of 2–3% (0.47–0.68 M) is required to facilitate adequate myosin solubilisation (Hayakawa et al., 2012): better solubilisation leads to a better final product (Chen, Zou et al., 2016). However, the excessive intake of salt can result in numerous health problems, thus there is an emerging trend to seek ways to improve myosin solubilisation under low salt conditions. What's more, for the sake of people with mastication and swallowing difficulties, which is usually the elderlies and infants, it would be of great help for them if meat with fibrous hard texture can be readily solubilised in low salt condition and has very soft and tender texture (Nieuwenhuizen, Weenen, Rigby, & Hetherington, 2010; Tokifuji, Matsushima, Hachisuka, & Yoshioka, 2013).

Methods including high pressure processing (Chen, Xu et al., 2016), the pH shift method (Park, Yongsawatdigul, Choi, & Park, 2008; Zhou & Yang, 2019), substituents of Na^+ (Tahergorabi & Jaczynski, 2012), and the addition of small amounts of amino acids (Arakawa et al., 2007; Chen, Zou et al., 2016; Hayakawa, Ito, Wakamatsu, Nishimura, & Hattori, 2010) have been developed and confirmed to effectively increase the solubility of myosin.

The utilisation of amino acids, as universally popular and generally safe food additives, could be expanded. Chen, Zou et al. (2016) reported that the imidazole moiety in histidine could impede the assembly process of filaments and increase the solubility myosin. In addition, transmission electron microscopy (TEM) revealed that histidine could elongate light meromyosin, which inhibited filaments formation (Hayakawa et al., 2010). Furthermore, the interaction with hydrophobic amino acid residues also played an important role (Li, Zheng, Xu, Zhu, & Zhou, 2018). Takai, Yoshizawa, Ejima, Arakawa, and Shiraki (2013) reported that L-lysine induced no structural changes in porcine myosin; however, the activation energy of the self-association of monomeric myosin increased. Myosin is a pH sensitive protein (Liu et al., 2010); therefore, Zhou, Li, and Tan (2014) proposed that the pH changes caused by lysine and arginine were related to the increased solubility of myosin. However, according to Schiffman and Dackis (1975), although the above studied amino acids showed excellent ability in solubilising myosin, histidine was "definitely not foodlike"; arginine had a repulsive and complex taste, and even made people feel like they were being poisoned; and people described lysine to be both salty and bitter. These taste attributes could be detrimental in food processing, even though they were proved to efficiently increase

* Corresponding author at: Department of Food Science & Technology, National University of Singapore, Science Drive 2, Singapore 117542, Singapore.
E-mail address: fstynghs@nus.edu.sg (H. Yang).

https://doi.org/10.1016/j.foodchem.2020.126665
Received 25 October 2019; Received in revised form 19 March 2020; Accepted 20 March 2020
Available online 21 March 2020
0308-8146/

myosin solubility. These disadvantages mentioned above have prompted researches to seek other sources to fulfil the requirements in reducing salt usage.

Surprisingly, little attention has been devoted to proline. Proline is a non-essential, proteinogenic and hydrophobic amino acid with molecular weight of 115.1 g/mol. It contains a second alpha amino group and has the ability to form associates in water solutions (Borzova, Markossian, Kara, & Kurganov, 2015). Proline has very high solubility (162 g/L) and has sweet, salty, and sour tastes (Schiffman & Dackis, 1975); however, its effects on myosin remain unknown. The main reason of selecting proline was indeed due to its high efficiency in improving the solubility and its better sensory attributes. On the one hand, there are many controversial discussions that using sweeteners may bring some unpleasant aftertastes even though it reduces sugar intake, thus we tried to avoid this problem by selecting ingredient that doesn't bring unpleasant aftertastes while using this ingredient to substitute salt. On the other hand, we were also very interested in the mechanism of the solubilisation effects of myosin by adding proline. During our literature review, only His showed very high efficiency with very simple lab work, other methods might solubilise myosin up to around 80% but their procedure could be rather complicated. The high solubility of proline suggests its good potential as an effective additive in food processing.

The objective of this study was to test the hypothesis that proline would lower the myosin surface hydrophobicity and increase the hydrophilicity of the protein, thus increase the myosin solubility in low ionic strength solution. To clarify the solubilisation mechanism, interactions among the solubilisation medium, myosin protein, and amino acid additives were studied. The secondary structure and surface hydrophobicity of proteins were examined to determine the structural changes of myosin. In addition, TEM was used to study the morphology of proteins before and after treatments. Collectively, a solubilisation mechanism was proposed based on our observations.

2. Materials and methods

2.1. Extraction of myosin

Live black tilapia fish *(Oreochromis niloticus)* was sacrificed, gutted, skin removed, and filleted at a local supermarket in Singapore. Red muscle was manually excised and discarded. Only white muscle from the dorsal part of the fish was used for further processing (Kristinsson & Hultin, 2003). After washing with tap water, tilapia fish fillets were transferred to the laboratory within 30 min and kept on ice during the whole transfer period.

Myosin was extracted immediately after having delivered from above mentioned dorsal fish muscle according to a previous method, with modifications (Duan, Zhang, Xing, Konno, & Xu, 2011). Fish muscle was minced and mixed with 10 volumes of solution A (0.10 M NaCl, 0.02% sodium azide, and 20 mM Tris-HCl buffer, pH 7.5). After homogenisation and stirring at 4 °C for 15 min, the mixture was centrifuged at 10000 × *g* for 5 min. The sediment was resuspended in five volumes of solution B (0.45 M NaCl, 5 mM β-mercaptoethanol, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA, and 20 mM Tris-HCl buffer, pH 6.8). ATP was added to a final concentration of 5 mM, the mixture was kept at 4 °C for 60 min, and then centrifuged at 10000 × *g* for 10 min. The supernatant was diluted five times using 1 mM $KHCO_3$ and kept at 4 °C for 15 min. After centrifugation at 10000 × *g* for 10 min, the pellet was resuspended in 2.5 volumes of solution C (0.5 M NaCl, 5 mM ß-mercaptoethanol, and 20 mM Tris-HCl buffer, pH 7.5). The mixture was incubated at 4 °C for 15 min and diluted with 2.5 volumes of 1 mM $KHCO_3$, which was then supplemented with $MgCl_2$ to a final concentration of 10 mM. After overnight incubation at 4 °C, the solution was centrifuged at 10000 × *g* for 15 min to obtain myosin.

The myosin pellets were either resuspended in 0.6 M NaCl (50 mM phosphate buffer, pH 7.0, high salt buffer) as a control group (high salt myosin (H)) or in 0.1 M NaCl (50 mM phosphate buffer, pH 7.0, low salt buffer) as low salt myosin (L), and then homogenised. All protein solutions were stored at 4 °C and used within 2 days (Zhuang et al., 2020). All experiments were carried out on ice.

In addition to H and L, four other myosin solutions were prepared: 5P, 10P, 15P, and 20P, which comprised L with 5, 10, 15, or 20 mM L-proline, respectively. After the addition of proline, all solutions were mixed well and stored at 4 °C overnight to allow thorough solubilisation.

2.2. Determination of proline solubilisation effects

2.2.1. Protein solubility

After overnight incubation, all protein solutions were mixed well and centrifuged at 12000 × *g* for 1 min. The supernatants were collected as the solubilised protein solutions. The protein concentration was determined using the Bradford method (Bradford, 1976).

2.2.2. Transfer free energy of proline

Transfer free energy (TFE) was determined according to the method of Arakawa et al. (2007). Briefly, selected amino acids were solubilised in 1 M proline solution until saturated and the solubility of the amino acids was recorded. The TFE was calculated according to the following equation:

$$\Delta G_{\text{Transfer}} = -RT\ln\frac{S}{S_{\text{water}}}$$

$\Delta G_{\text{Transfer}}$: TFE of amino acids from water to additive solution.R: gas constant ($J{\bullet}mol^{-1}{\bullet}K^{-1}$).$T$: absolute temperature ($K$).$S$ and S_{water}: solubility of amino acid in solution with additive and in water ($g{\bullet}mL^{-1}$), respectively.

2.2.3. Myosin amino acid content

The total amino acid content was analysed using an amino acid analyser (ARACUS, MembraPure, Berlin, Germany). Before analysis, 100 mg of the freeze-dried myosin sample was hydrolysed with 4.9 mL of 6 M HCl for 24 h at 110 °C. After filtration, 200 μL of the hydrolysed mixture was heated to dryness at 130 °C and mixed with 1 mL of sample dilution buffer (MembraPure). The mixture was further diluted 10 times with sample dilution buffer and filtered through a 0.2-μm syringe filter and then subjected to analysis.

2.2.4. Surface hydrophobicity

To 1 mL of myosin solution (3 mg/mL), 200 μL of 1 mg/mL bromophenol blue (BPB in water) was added and mixed well. A control sample was prepared using 1 mL of 20 mM phosphate buffer (pH 6.0) instead of the myosin solution. Protein and control samples were kept under agitation at room temperature for 10 min. After centrifugation at 2000 × *g* for 15 min, the supernatants were collected and diluted 10 times. The absorbance was read at 595 nm. The amount of BPB bound was calculated using the following expression (Li et al., 2020):

$$\text{BPB bound } (\mu g) = 200\,\mu g \times (A_{control} - A_{sample})/A_{control},$$

A = absorbance at 595 nm.

2.2.5. Total sulfhydryl and disulphide bonds

Content of total –SH groups examined according to previously published method (Zhou & Yang, 2019). Briefly, every 1 g sample was homogenised with 10 mL of 0.6 M NaCl and 8 M urea mixture. After centrifugation, 0.5 mL of supernatant was added to 4.5 mL buffer A (0.2 M Tris-HCl, 8 M urea, 3 mM EDTA, 1% SDS, pH 8.0). Another 0.625 mL buffer B (10 mM Tris-HCl, 10 mM DTNB, pH 8.0) was added and the mixture was under incubation at 40 °C for 25 min. After cooling to room temperature, the absorbance was read at 412 nm. The concentration of -SH bonds content was calculated using the extinction

Table 1
Effects of different proline concentrations and salt concentrations on myosin.

Sample	Concentration (%)	Protein surface hydrophobicity (BPB bound mg/g)	Particle size (nm)	Zeta potential (mV)	Hydrophobic interactions (mg/g)
H	100.00^{d}	18.25 ± 1.15^{c}	567.88 ± 45.32^{b}	−13.54 ± 2.28^{a}	8.08 ± 1.04^{b}
L	30.48 ± 7.06^{a}	13.10 ± 2.67^{b}	419.97 ± 25.07^{a}	−6.44 ± 0.57^{b}	5.77 ± 1.29^{a}
5P	62.12 ± 3.44^{b}	12.21 ± 0.84^{b}	505.73 ± 10.68^{b}	−7.66 ± 2.02^{b}	7.61 ± 1.55$^{a, b}$
10P	83.36 ± 4.07^{c}	8.22 ± 1.78^{a}	560.50 ± 61.37^{b}	−11.58 ± 1.51^{a}	12.77 ± 2.24^{b}
15P	82.07 ± 2.73^{c}	9.11 ± 1.14^{a}	549.14 ± 55.01^{b}	−11.57 ± 0.92^{a}	12.01 ± 1.33^{b}
20P	80.55 ± 1.70^{c}	10.17 ± 1.50^{a}	621.17 ± 52.65^{b}	−13.48 ± 1.13^{a}	12.87 ± 1.17^{b}

Different letters in the same column indicate significant differences at $P < 0.05$. Note: H: control group, myosin solubilised in 0.6 M NaCl; L: myosin in 0.1 M NaCl; 5, 10, 15, 20P: L added with 5, 10, 15, 20 mM proline.

coefficient of 13,600 M^{-1} cm^{-1}.

Content of disulphide bonds was examined according to Zhao et al. (2019) using 2-nitro-5-thiosulphobenzoate (NTSB) with modifications. To 0.5 mL of myosin sample solution, 3.0 mL of freshly prepared NTSB assay solution were added. The mixture was at room temperature for 25 min. A sample blank was prepared with distilled water instead of NTSB assay solution. The absorbance was read at 412 nm. The disulphide bond content was calculated using the extinction coefficient of 13,900 M^{-1} cm^{-1}.

2.2.6. Protein secondary structure

Fourier transform infrared spectroscopy (FTIR) was used to examine the secondary structure changes of all protein samples (Sow, Toh, Wong, & Yang, 2019). Before the analysis, about 4 mg of freeze-dried proteins were thoroughly mixed with 150 mg KBr and pressed into a thin pellet using a hydraulic press. The sample pellets were then subject to scanning in the range of 450 and 4500 cm^{-1}, at a resolution of 4 cm^{-1} for 64 scans using a Spectrum One FTIR spectrometer (PerkinElmer, Waltham, MA, USA). The obtained spectra were deconvoluted in the amide I region (1600–1700 cm^{-1}) using Origin Pro 9 software (OriginLab, Northampton, MA, USA). The contents of protein structural components (α-helix, β-sheet, β-turn, and random coil) were determined by the area of the individual component peak divided by the overall area of the amide I region after deconvolution (Sow, Tan, & Yang, 2019).

2.2.7. Protein particle size and zeta potential

Particle size and zeta potential were measured using a NanoBrook Omni Particle Size and Zeta Potential analyser (Brookhaven Instruments, Holtsville, NY, USA) in the dynamic light scattering (DLS, λ = 633) and phase analysis light scattering modes, respectively. Protein solutions were diluted to 0.5 mg/mL using the appropriate buffers and placed in a 1 cm path-length quartz cuvette (Chen, Zou et al., 2016). The detection angle was fixed at 90°, and the Smoluchowski model was applied to assess the zeta potential (Sow et al., 2019). All procedures were conducted at 25 °C.

2.2.8. Protein patterns of dissolved myosin

Before the analysis, protein solutions were adjusted to the same concentration using the appropriate buffers. To determine the existence of myosin aggregates and proline's effects on myosin, protein solutions were treated with same volume of Laemmli sample buffer (Bio-Rad Laboratories Inc., Hercules, CA, USA) with (reducing SDS-PAGE) or without (non-reducing SDS-PAGE) 5% β-mercaptoethanol (β-MCE) and incubated at 100 °C for 4 min. The electrophoretic analysis was performed using 4–20% Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories Inc.) (Feng, Hang, Zhou, Liu, & Yang, 2018). Each well was loaded with 35 μL of samples and the electrophoresis condition was set at 120 V for 1 h.

2.2.9. Protein morphology

After being diluted to 0.05 mg/mL, 20 μL of protein solution was fixed on a carbon-coated copper 400-mesh grid for 2 min, after blotting off excess solutions, a drop of 3% tungsphosphoric acid was dropped onto the grid to stain the proteins for 3 min. After blotting dry with filter paper strips, the copper grid was further washed with deionised water until the grid was clean (Hayakawa et al., 2010). The specimens were then visualised using a JEOL JEM-3011 transmission electron microscope (JEOL Ltd., Tokyo, Japan) (Yu et al., 2019).

2.3. Statistical analysis

At least three independent samples were prepared for each experiment, and the experiment was conducted in triplicate independently. Data were analysed statistically using analysis of variance (ANOVA), and means were compared using the least significant difference (LSD) method with computer software IBM SPSS Statistics Version 23 (International Business Machines Co., Armonk, NY, USA). Differences with a P value ≤ 0.05 were considered significant.

3. Results and discussion

3.1. Effects of proline addition on myosin solubilisation in low ionic strength solution

During pre-experiments, 19 common amino acids were tested as additives at different concentrations (1, 5, and 10 mM). Most amino acids improved the solubility of myosin in low ionic strength solutions (data not shown). The reasons for choosing proline were mainly because of its high solubility in water and slightly sweet taste. High solubility could ensure the addition of low amounts of additives and when surimi is further processed into fishery products, i.e., fish balls or fish cakes, sugar is usually added as a seasoning.

Table 1 shows the solubility of myosin in different solutions. If the solubility of myosin in the high ionic strength solution was regarded as 100%, the solubility of myosin in the low ionic strength solution was only 30.48%, which was in agreement with a previous report (Chen, Zou et al., 2016). At 5 mM proline, the solubility of myosin in the low ionic strength solution increased to 62.12% and reached a maximum of 83.36% solubility in 10 mM proline. When 15 and 20 mM proline were added to L, no significant solubility changes were observed.

This result was unsurprising, because a number of previous reports showed that certain amino acids could effectively increase myosin solubility (Chen, Zou et al., 2016; Fu, Zheng, Lei, Xu, & Zhou, 2017; Li et al., 2018). No prior discussion about the effects of proline has been reported; however, for histidine addition, the imidazole ring played an important role (Chen, Zou et al., 2016). Elongated myosin rods and loss of α-helix content synergistically impeded myosin filaments formation (Chen, Zou et al., 2016). For lysine and arginine, in addition to inhibiting the formation of filaments, they interacted with the acidic amino acid residues of myosin (Arakawa et al., 2007).

3.2. Transfer free energy of proline

To clarify the specific solubilisation location and interactions between myosin and the additives, the TFE was determined and 13 amino

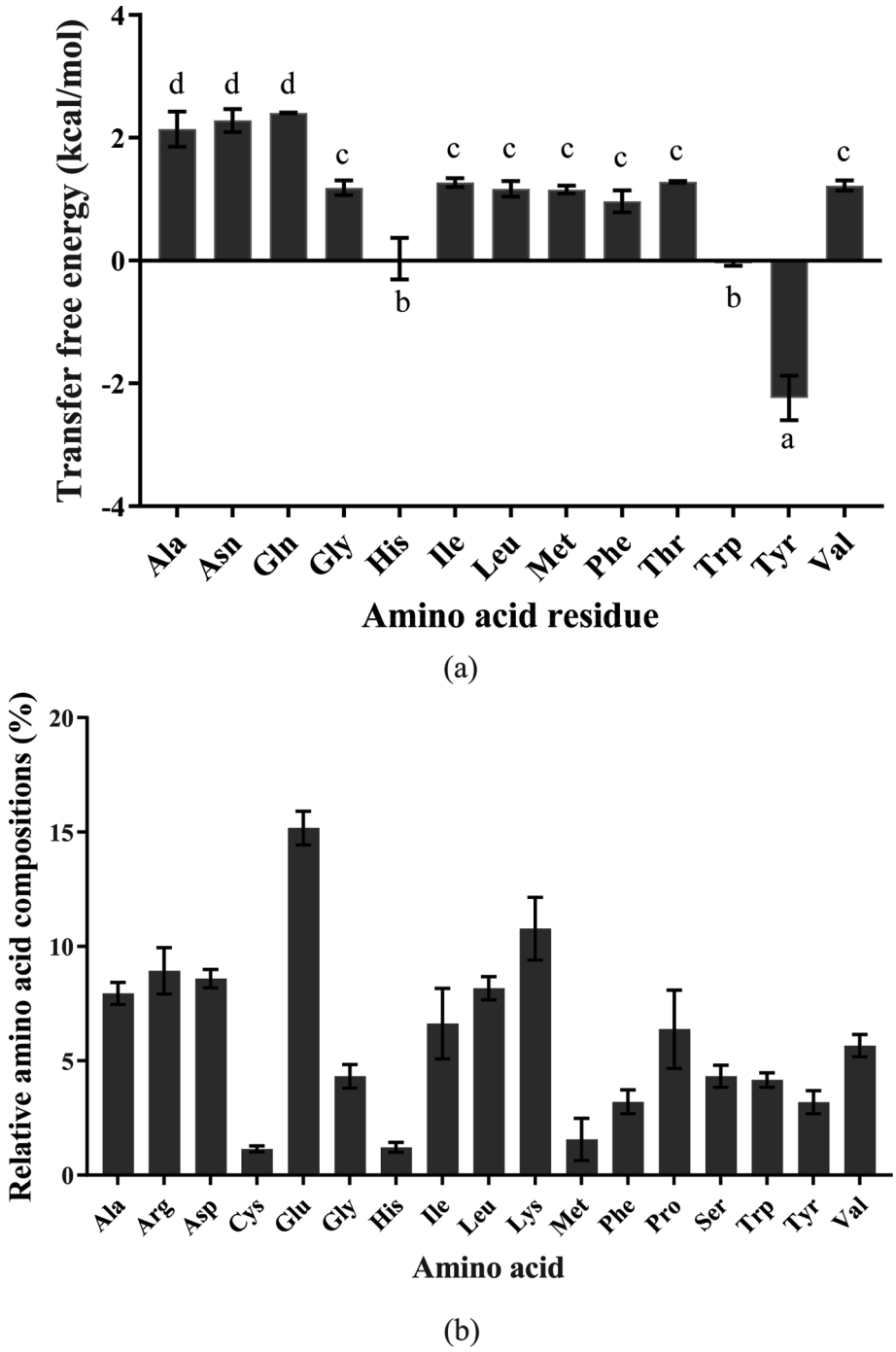


Fig. 1. (a): Transfer free energy (TFE) of 1 M proline; (b) relative amino acid compositions of tilapia myosin.

acids were chosen as representatives of the main amino acid composition of myosin. The results for the solubility of the 13 amino acids in 1 M proline are summarised in Fig. 1(a). The overall amino acid content of hydrolysed myosin is shown in Fig. 1(b). Although the content of amino acids could not precisely indicate the amount of amino acid residues exposed, it provided a view of the interactions between the residues and the additive. If the reactions of the transferring amino acids from water to the 1 M additive solution are favourable, the TFE value should be negative, and vice versa (Arakawa et al., 2007).

Histidine, tryptophan, and tyrosine had negative TFE values in the 1 M proline solution, which means their solubility in 1 M proline was higher than that in water. From the perspective of chemical structure, the imidazole ring in histidine is aromatic at all pH values (Mrozek, Karolak-Wojciechowska, & Kieć-Kononowicz, 2003), thus histidine and tryptophan are both aromatic. In water, they show rather low solubility and the addition of proline increased their solubility.

In the case of proline addition, according to Schobert and Tschesche (1978), proline residues form aggregates in solution in which their hydrophobic backbones are stacked, which resulted in the exposure of hydrophilic groups. Similarly, when interacting with proteins, the same interactions could occur between proline and hydrophobic surface residues, thus increasing the hydrophilic area. In the 1 M proline solution, histidine and tryptophan showed TFE values close to 0, whereas the TFE of tyrosine was around −2 kcal/mol, indicating that the hydrophobic portion of protein was shielded by proline addition and the increased hydrophilic portion could interact with the surrounding

solvent.

From Fig. 1(a), we concluded that proline tended to interact with histidine, tryptophan, and tyrosine. However, based on the amino acid content information from Fig. 1(b), the content of histidine in myosin is quite low (lower than 1%), while tyrosine was relatively abundant (around 4%), and proline helped to lower the TFE of tyrosine, which might be one of the main reasons for the increase in myosin solubility.

3.3. Surface hydrophobicity

Surface hydrophobicity is widely used to evaluate changes in protein conformation. When more hydrophobic sites are exposed in the protein structure, more bromophenol blue (BPB) dye can bind to the protein molecules (Panpipat & Chaijan, 2017). As shown in Table 1, the highest binding at 18.25 mg/g was obtained in H, and the lowest were 8.22, 9.11, and 10.17 mg/g obtained by 10P, 15P, and 20P, respectively. In general, the surface hydrophobicity is inversely proportional to solubility.

In the absence of proline, the only solute for solubilisation was NaCl and Na^+ only served as an agent to manipulate protein folding. When a higher NaCl concentration was applied, myosin was unfolded more thoroughly, and more hydrophobic sites were exposed, thus allowing more BPB to bind. Similarly, caused by the low ionic strength, myosin proteins were not able to unfold to the same extend as in H, only 13.10 mg/g BPB could bind to L myosin. Similarly, Guo, Peng, Zhang, Liu, and Cui (2015) observed that the protein surface hydrophobicity was directly proportional to the concentration of salt used, which was in accordance with the results mentioned above.

The relationship between solubility and hydrophobicity remains debatable. Generally, it was believed that the more hydrophobic surface exposed, the lower the solubility of myosin, as confirmed by You, Pan, Shen, and Luo (2012) and Li et al. (2018). However, according to Chen, Xu et al. (2016), the high value of surface hydrophobicity suggested increased unfolding of the myosin structure, which would allow water or the solvent to interact with the interior of myosin or even disturb the tertiary structure, thus increasing solubility. Based on the results above, we presumed that the solubilisation mechanisms of salt and amino acids are different and might be multifactorial. Under high ionic strength conditions, the added salt broke the intermolecular ionic bonds to dissociate the highly insoluble myosin filaments (Wang et al., 2018). Under low ionic strength conditions, the low salt content could not break the bonds, thus the filaments were formed and solubility was low. However, when amino acids were added, decreased filament formation might not be the main reason for the increased solubility. For proline addition, the BPB bound was even lower than that in L. According to Schobert and Tschesche (1978), hydrophobic interactions of proline would occur with hydrophobic surface residues of proteins to increase their hydrophilic area. However, proline solutions are hydrotropic, which could help to solubilise hydrophobic compounds (Troitzsch, Tulip, Crain, & Martyna, 2008). Based on the discussion related to TFE, we postulated that proline interacts with tyrosine, tryptophan, or other hydrophobic residues to decrease the surface hydrophobicity of myosin, which increased its solubility directly.

3.4. Secondary structures of myosin

The major secondary structure (α-helix and β-sheet) contents of all myosin samples are shown in Fig. 2. Low salt conditions were unable to thoroughly unfold the protein (Guo et al., 2015); therefore, it was believed that myosin was mostly similar to its original protein structure state in L. It comprised 54.50% α-helix and 37.14% β-sheet. After solubilisation in the high ionic strength solution, H had less α-helix, 42.83%, and less β-sheet, 33.87%. By disturbing the bonds, loss of the helix structure would also expose more hydrophobic sites (Chen, Xu et al., 2016), which was supported by the increased amount of BPB bound in H (Table 1).

The addition of 5 mM proline had little impact on myosin: the α-helix and β-sheet contents remained almost the same, at 54.52% and 35.36%, respectively, which indicated an insufficient concentration of proline to effectively disturb myosin's structure. With the addition of more proline (10P), the α-helix content decreased markedly to 38.61%, and the β-sheet content increased to 46.88%. It was found that proline was the most potent alpha-helix breaker, at least in globular proteins soluble in aqueous media, which could be the main reason to explain the significant decrease of the helix structure in 10P (Biedermannova, Riley, Berka, Hobza, & Vondrasek, 2008).

Based on the solubility results shown in Table 1, 5 mM proline slightly increased the solubility, whereas 10 mM proline nearly tripled the solubility compared with that of L. We presumed that 5 mM proline was unable to thoroughly unfold the α-helix structure, thus the solubility did not increase significantly, while 10 mM proline could unfold the helix structure to form a greater extent of sheets and random coils. With an increased β-sheet content at the expense of the α-helix content (Cando, Herranz, Borderías, & Moreno, 2015), myosin was better hydrated by the surrounding solution and had better solubility (Guo et al., 2015). As shown in Fig. 2, 10P had a lower α-helix content and higher β-sheet content compared with those in H, indicating a very strong interaction between proline and the myosin structure. Notably, although the secondary structure change induced 10P was promising, in terms of solubility, it was still much lower than that of H, which consistent with our hypothesis that the solubilisation mechanism of proline addition was multifactorial, and the structural changes caused by proline addition was only one of the parameters.

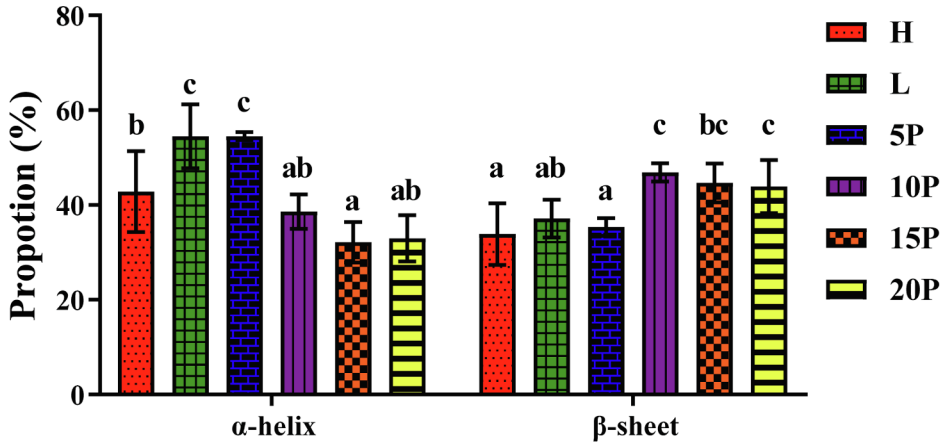
3.5. Particle size, zeta potential, and hydrophobic interactions of myosin and their relations

All samples were subjected to DLS particle size and particle zeta potential analysis and the results are shown in Table 1. Surprisingly, although H had the highest solubility, the particle size of H was 567.88 nm, which was neither the largest nor the smallest. L showed the smallest particle size, 419.97 nm. The largest particle, 621.17 nm, was found in 20P; however, no significant difference was found among all the proline added groups.

In general, most reports showed that a higher solubility would lead to a smaller particle size (Chen et al., 2017; Chen, Zou et al., 2016). However, the present study showed the opposite: myosin with higher solubility possessed larger particles. Higher surface hydrophobicity is beneficial to hydrophobic interactions between proteins (An et al., 2018) and might be the main factor causing the large particle size in H. In addition, according to confocal laser scanning images obtained by Wang et al. (2018), myosin gradually became swollen when concentration of the solubilisation medium increased from 0.1 M to 0.6 M, which is in agreement with our findings.

The zeta potential is used to evaluate protein stability and the larger the absolute value, the more stable are the particles. Without proline addition, bigger particles tended to be more stable. For the larger particles observed in H, the zeta potential was −13.54 mV which could be considered as quite stable (Sow et al., 2018). For the particles observed in L, the zeta potential was −6.44 mV, indicating markedly reduced stability compared with H. Comparing the zeta potentials between NaCl and proline addition, proline seemed to act as a stabiliser, similar to a high content of NaCl. Even when NaCl concentration was low, the addition of proline significantly lowered the zeta potential value compared with that in L.

In addition to particle size and zeta potential, the hydrophobic interactions of myosin were studied to further explain the above results. The formation of hydrophobic interactions is usually responsible for protein aggregation (Chen et al., 2018). Concordantly, the particle size and hydrophobic interactions of myosin under different treatments showed direct relations. When the particles were bigger, the number of hydrophobic interactions increased accordingly. The highest-level



Sample	α−helix (%)	β−sheet (%)
H	42.83±8.52^{b}	33.87±6.48^{a}
L	54.50±6.70^{c}	37.14±3.98$^{a, b}$
5P	54.52±0.84^{c}	35.36±1.89^{a}
10P	38.61±3.62$^{a, b}$	46.88±1.92^{c}
15P	32.14±4.31^{a}	44.67±4.10$^{b, c}$
20P	32.97±4.87$^{a, b}$	43.88±5.58^{c}

Fig. 2. Major secondary structure content changes of myosin protein under different treatments. H: myosin in 0.6 M NaCl solution; L: myosin in 0.1 M NaCl solution; 5P: L with 5 mM proline addition; 10P: L with 10 mM proline addition; 15P: L with 15 mM proline addition; 20P: L with 20 mM proline addition. Different letters in the same column indicate significant differences at $P < 0.05$.

hydrophobic interaction was found in myosin treated with 10, 15, and 20 mM proline, which also had largest particles, at 560.50, 549.14, and 621.17 nm, respectively. We presumed that myosin tended to aggregate after proline addition, and with the increase in proline, more and larger aggregates were formed, thus leading to more hydrophobic interactions. As mentioned in section 3.2, proline could form aggregates in solution to expose hydrophilic sites and same rule applies to proline-protein interactions: the results of the present study suggested that proline addition effectively induced myosin aggregation, which in turn indicated that proline has a tendency to shield the hydrophobic sites of a protein and increase the intramolecular hydrophobic interactions.

3.6. Protein patterns of myosin and its relations to total sulfhydryl and disulphide bonds

In addition to normal SDS-PAGE in the presence of β-MCE, protein patterns were also observed in the absence of β-MCE in the "non-reducing mode". Usually, β-MCE is used to reduce disulphide bonds, which is a major bond that maintains the protein structure promotes the formation of aggregates. In order to clearly demonstrate the changes of –SH/–S–S– bonds caused by proline addition, contents of the two bonds were shown in Fig. 3(a). When myosin was solubilised by low ionic strength solution, 5.00 mol of –SH and 2.96 mol of –S–S– were found in 10^5 g of proteins, respectively. In high ionic strength solution, 4.83 and 2.47 mol of –SH and –S–S– were found in 10^5 g of proteins, respectively. Although L group showed a very slight increase for both bonds, no significant difference was found between H and L. After solubilised with proline added solution, when more proline was added, higher –SH content was found. Meanwhile, the changes of disulphide bonds showed a reverse trend. The two reverse trends indicated that there was formation of sulfhydryl bonds due to loss of disulphide bonds. During the solubilising procedure, all myosin was homogenised with proline-contained solution, it was possible that the intense mechanical forces caused the structural changes and exposed the –SH groups (Chen, Xu et al., 2016; Chen, Zou et al., 2016). On the other hand, we have found that proline has a relatively strong affinity to hydrophobic sites on protein surface, during the homogenisation of the protein solution, there could be interactions between proline and the newly formed hydrophobic surface which inhibit the formation of disulphide bonds.

Same phenomena were found in SDS-PAGE images. In Fig. 3(b) non-reducing mode, all six protein samples showed myosin aggregates at the top of the gels, indicating high level of –S–S– existence due to lack of β-MCE. In Fig. 3(c) reducing mode, both myosin aggregates and myosin heavy chain (shown in red frames) were observed, indicating a similar function to β-MCE. Most previous reports noted that myosin aggregates held by disulphide bonds are hard to solubilise (Chen et al., 2017; Chen, Zou et al., 2016), and the ability of proline preserving -SH bonds could be one of the reasons for the increased solubility of myosin with the presence of proline.

However, as shown in Fig. 3(b), proline could only have limited

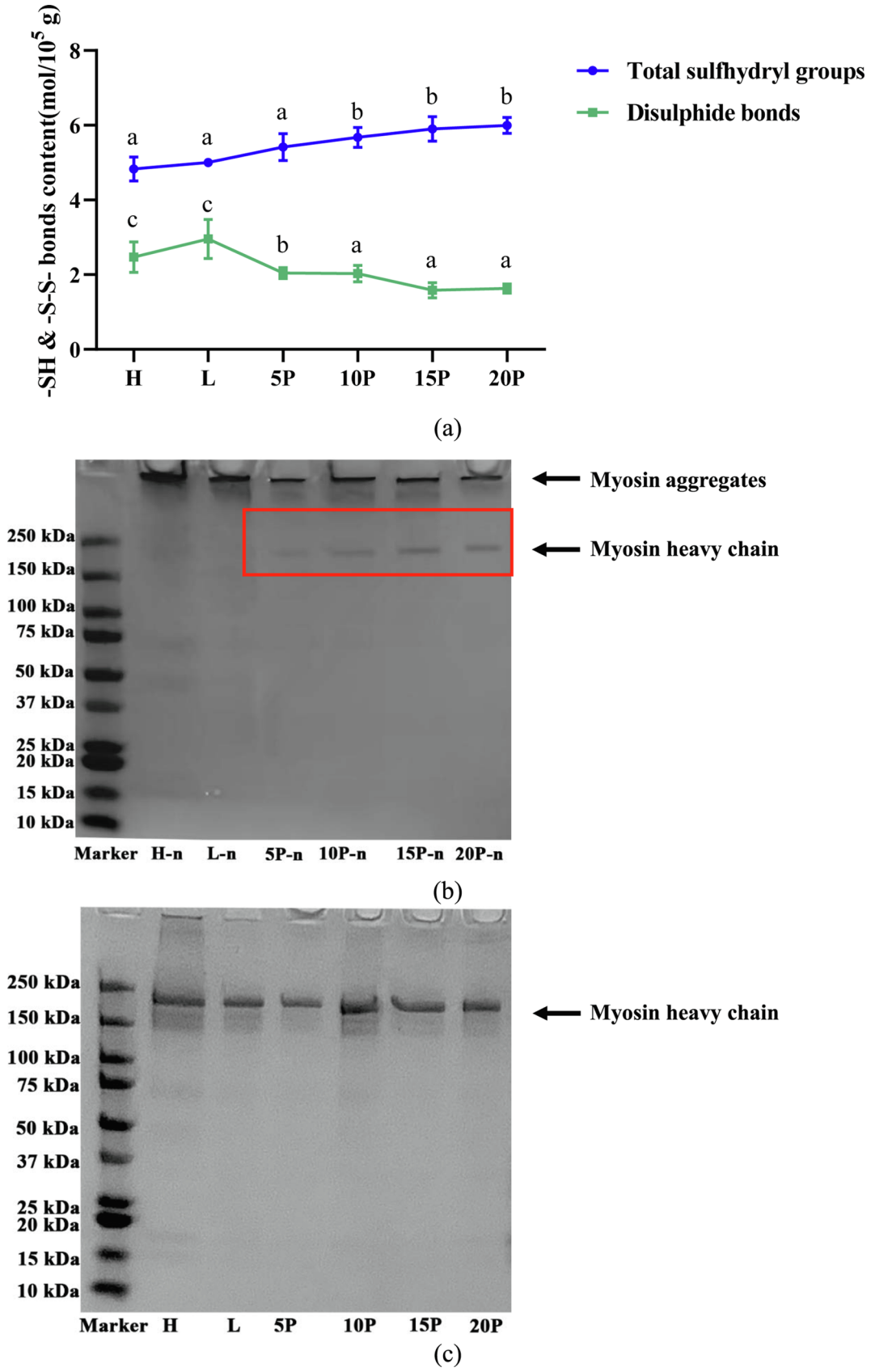


Fig. 3. (a) Total sulfhydryl (-SH) and disulphide bonds (–S–S–) contents; Protein patterns of myosin in (b) non-reducing mode and (c) reducing mode. H: myosin in 0.6 M NaCl solution; L: myosin in 0.1 M NaCl solution; 5P: L with 5 mM proline addition; 10P: L with 10 mM proline addition; 15P: L with 15 mM proline addition; 20P: L with 20 mM proline addition; n: non-reducing mode protein patterns. Different letters in (a) indicate significant differences ($P < 0.05$) within each of the two groups.

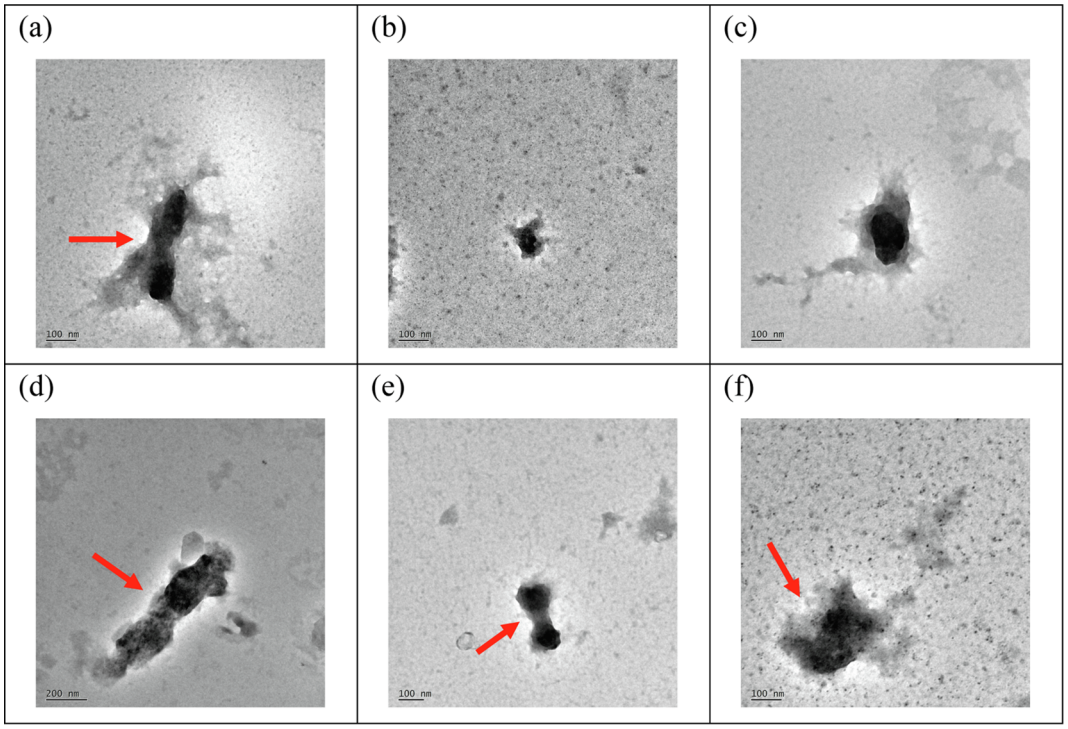


Fig. 4. Transmission electron microscope images of myosin after different treatments. (a): H, myosin in 0.6 M NaCl solution; (b): L, myosin in 0.1 M NaCl solution; (c): 5P, L with 5 mM proline addition; (d): 10P, L with 10 mM proline addition; (e): 15P, L with 15 mM proline addition; (f): 20P, L with 20 mM proline addition.

ability in increasing the band intensity of MHC. For L and 10P, both the slight decrease of –S–S– from 2.96 to 2.03 mol/10^5 g proteins and the slight increase of –SH from 5.00 to 5.68 mol/10^5 g proteins confirmed that, most of the proteins still remained as aggregates and stayed at the top of the SDS gel, while in Fig. 3(c) with β-MCE added, there was only trace of myosin aggregates at the top. The observations again confirmed our theory that the solubilising effects of proline was multifactorial, and the reduction-like function of proline was only one of the factors.

3.7. Protein morphology

It is generally believed that myosin has a rod-like structure (Eakins, Al-Khayat, Kensler, Morris, & Squire, 2002) and a similar morphology was observed in this study. Fig. 4 shows the morphology of myosin with or without different treatments, as imaged using TEM. All myosin samples showed a rod-like morphology, with different features caused by the solubilisation environment. Myosin solubilised in high (Fig. 4a) and low (Fig. 4b) ionic strength solutions showed similar morphologies to those observed Hayakawa et al. (2010). The main differences between Fig. 4(a) and (b) were the lengths of the myosin rods and the connections between them. Myosin in the high ionic strength solution had longer rods, which corresponded with results for the particle size, in which H had larger particles than L. In addition, single myosin proteins were observed more easily in L than in H. Myosin proteins tend to have “connections” between single proteins and same phenomena applied to 10P (d), 15P (e) and 20P (f) (indicated by arrows). However, fewer connections were found in 5P (c), which suggested that the more proline was needed to effectively solubilise myosin, and 5 mM proline was obviously insufficient.

As discussed above, proline was confirmed to have shielding effects on proteins (Schobert & Tschesche, 1978) and could reduce disulphide bonds in SDS-PAGE gels, thus the single proteins observed in 5P could be the result of proline breaking the disulphide bonds formed between proteins to increase the solubility. With the addition of more proline, more disulphide bonds were broken thus more proteins could be solubilised in a low ionic strength solution. However, we also observed that when more proline was added, significantly more hydrophobic interactions occurred, which in turn suggested that proline promoted myosin to form oligomer aggregates by shielding around myosin protein particles and stabilising the oligomers via hydrophobic interactions instead of forming multimer aggregates, which might be the case in H.

3.8. Solubilisation mechanism

A proposed solubilisation mechanism is shown in Fig. 5. According to previous studies, myosin has a mostly rod-like structure (Kristinsson & Hultin, 2003; Xue et al., 2017), it contains the myosin heavy chain (MHC) and myosin light chain (MLC) at the “head” region and a tail structure at the end region. The tail structure is a highly compact helix structure with few sheets or turn structures.

It was widely accepted that tryptophan and tyrosine residues tend to be on the surface of proteins, and this was confirmed by results from TFE shown in Fig. 1, indicating that proline tends to interact with tryptophan and tyrosine. Hence, these two residues were chosen as representatives to be shown in the myosin protein structure when interactions were illustrated. As shown in Fig. 5, when myosin is in a high ionic strength solution, some hydrophobic sites, including Tyr, Trp, and disulphide bonds, would be exposed. As major bonds in the myosin protein, disulphide bonds are hydrophobic, and protein surface hydrophobicity showed that under high ionic strength conditions, BPB binding in H was the highest among all the groups (18.25 mg/g). Hydrophobic site exposure usually indicates structural changes in both the head and tail regions, and it would allow more interactions between myosin and its surroundings (Chen, Xu et al., 2016). In addition, at pH 7.0, myosin is negatively charged (Zhou & Yang, 2019). The thorough unfolding of myosin caused by high concentration of Na^+ resulted in the protein aggregates in H showing good stability (zeta potential value −13.53 mV), and most of the negative charges could be neutralised by Na^+.

Under low NaCl conditions, little unfolding of myosin occurred, and the negatively charged myosin could not be completely neutralised because of insufficient amounts of Na^+. The surface hydrophobicity

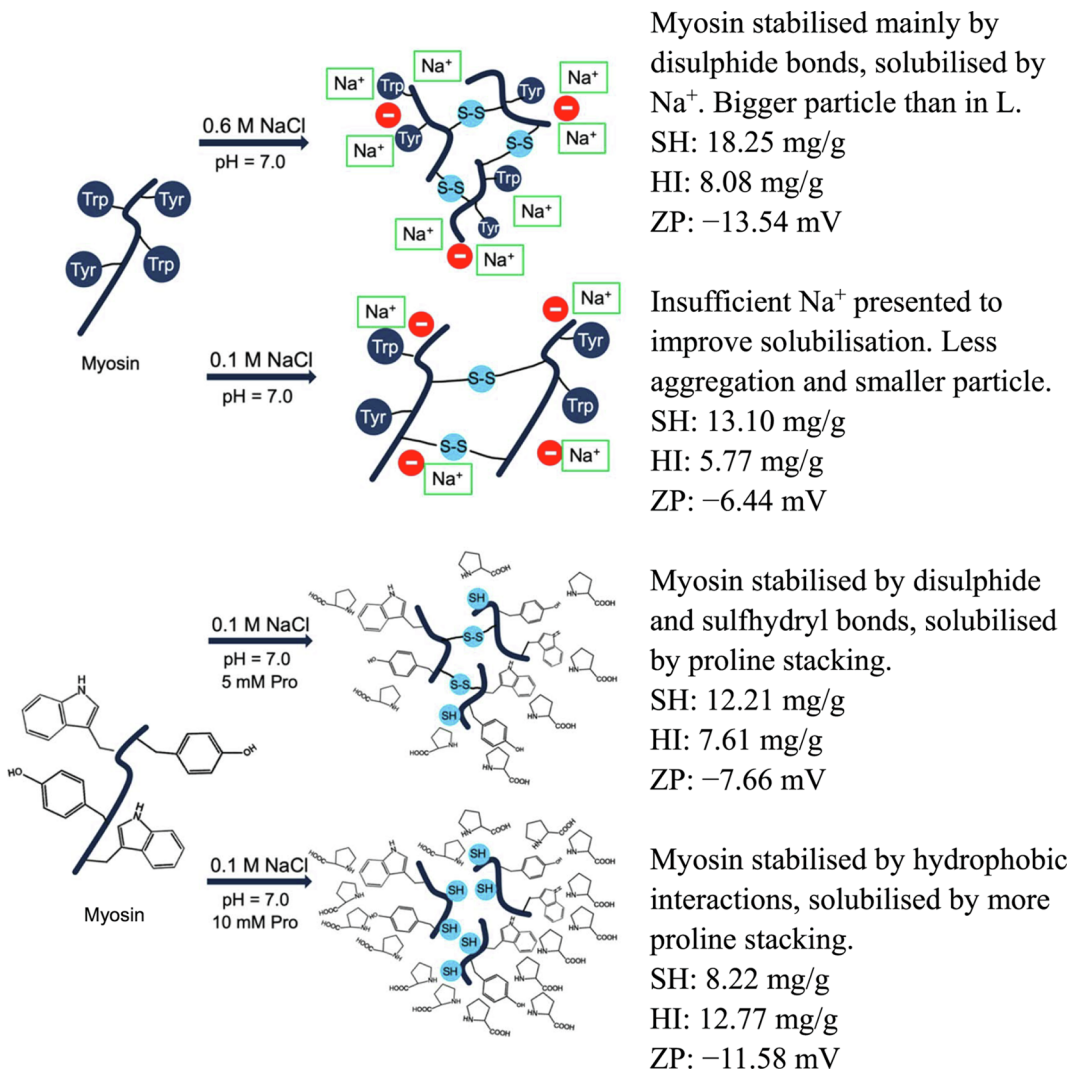


Fig. 5. Proposed mechanism of proline addition in solubilisation of myosin in low ionic strength solution. SH: surface hydrophobicity; HI: hydrophobic interactions; ZP: zeta potential.

was lower than that in H, but was still relatively high, which could be detrimental to solubilisation; all of these factors led to low solubility of myosin.

When 5 mM proline was added to the low ionic strength solution, fewer hydrophobic sites were exposed. One possible reason was that the low concentration of salt was unable to unfold the protein. Another reason was the shielding effects of proline, as mentioned before (Schobert & Tschesche, 1978). Although solubilisation effects were not obvious, proline still had certain effects on myosin. Proline addition increased the particle stability, making myosin less prone to aggregation. Thus, 5 mM proline caused not only the shielding effects, but also affected aggregation. Furthermore, based on the SDS-PAGE pattern, some of the aggregates were disassociated in the presence of proline, thus fewer disulphide bonds and more sulfhydryl bonds were present.

When 10 mM proline added, more proline could interact with the hydrophobic sites, which lowered the surface hydrophobicity and zeta potential. Compared with 5P, fewer disulphide bonds formed, which led directly to more hydrophobic interactions inside the myosin aggregates. The whole system was markedly stabilised by the low hydrophobicity on the surface and the strong hydrophobic interactions inside, leading to high solubility and stability.

It was worth mentioning that, when higher concentrations of proline were added to the low ionic strength system (15 and 20 mM proline), no significant increase of solubility of myosin was found. Based on our theory, the addition of proline would stack around the myosin proteins and decrease the surface hydrophobicity and stabilise the protein aggregates, therefore increase the protein-water interactions and increase the solubility. However, in most studies, the increase of protein size would decrease the solubility (Chen, Xu et al., 2016; Chen, Zou et al., 2016). By referring to existed results and our findings, it was suggested that there was a balance among the increase of protein size, decrease of protein surface hydrophobicity and protein solubility. When the concentration of proline was too low (5P) or no proline addition at all, the surface hydrophobicity was too high to increase the solubility, and protein-protein interactions played a leading role. When more proline was added (10P), there was an equilibrium among the three parameters mentions above, and at this proline concentration, myosin reached the highest solubility. According to the results from transfer free energy in Fig. 1(a), interactions spots for proline and proteins were limited, therefore when even more proline was added (15 and 20P), little interactions could take place to decrease the surface hydrophobicity, thus no significant increase in solubility was observed. It also could be observed from results of FTIR (Fig. 2) that when 15 and 20 mM proline were added, no significant increase or decrease of the secondary structure was found, indicating that no or very little proline-protein interactions took place at these two concentrations.

4. Conclusions

The results of present study confirmed the hypothesis that proline would increase the solubility of myosin under low salt conditions. The addition of 10 mM proline to myosin in 0.1 M NaCl increased the solubility from 30.48% to 83.36%. The results for TFE and myosin total amino acid contents confirmed that the interactions between myosin and proline were favourable and aided solubilisation. The addition of 10 mM proline resulted in myosin with low surface hydrophobicity and stable protein particles. More β-sheet structures formed at the expense of α-helices, indicating that 10 mM proline could unfold the myosin secondary structure. Protein patterns showed that proline could partially break disulphide bonds in myosin. Proteins visualised by TEM showed that sufficient proline addition helped myosin to form oligomer aggregates by shielding around myosin protein particles. Overall, the results demonstrated that the addition of 10 mM proline could greatly enhance the solubility of myosin, and would contribute to meeting the high demand for healthier fish products in the future.

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.

Acknowledgements

This work was supported by Singapore Ministry of Education Academic Research Fund Tier 1 (R-160-000-A40-114). The authors also thank the financial support from industry projects from Fujian Putian Sea-100 Food Co., Ltd (R-143-000-633-597) and Polymer Science (Shenzhen) Ke Ju New Material Co., Ltd (R-143-000-672-597).

Credit author information

Y. Z. performed all the experimental work and drafted the manuscript. H. Y. secured funding, reviewed and edited the manuscript and supervised the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126665.

References

An, Y., Liu, Q., Xie, Y., Xiong, S., Yin, T., & Liu, R. (2018). Aggregation and conformational changes of silver carp myosin as affected by the ultrasound–calcium combination system. *Journal of the Science of Food and Agriculture, 98*(14), 5335–5343.

Arakawa, T., Ejima, D., Tsumoto, K., Obeyama, N., Tanaka, Y., Kita, Y., & Timasheff, S. N. (2007). Suppression of protein interactions by arginine: A proposed mechanism of the arginine effects. *Biophysical Chemistry, 127*(1), 1–8.

Biedermannova, L. E., Riley, K., Berka, K., Hobza, P., & Vondrasek, J. (2008). Another role of proline: Stabilization interactions in proteins and protein complexes concerning proline and tryptophane. *Physical Chemistry Chemical Physics, 10*(42), 6350–6359.

Borzova, V. A., Markossian, K. A., Kara, D. A., & Kurganov, B. (2015). Kinetic regime of dithiothreitol-induced aggregation of bovine serum albumin. *International Journal of Biological Macromolecules, 80*, 130–138.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254.

Cando, D., Herranz, B., Borderías, A. J., & Moreno, H. M. (2015). Effect of high pressure on reduced sodium chloride surimi gels. *Food Hydrocolloids, 51*, 176–187.

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

Chen, X., Xu, X., Liu, D., Zhou, G., Han, M., & Wang, P. (2018). Rheological behavior, conformational changes and interactions of water-soluble myofibrillar protein during heating. *Food Hydrocolloids, 77*, 524–533.

Chen, X., Zou, Y., Han, M., Pan, L., Xing, T., Xu, X., & Zhou, G. (2016). Solubilisation of myosin in a solution of low ionic strength L-histidine: Significance of the imidazole ring. *Food Chemistry, 196*, 42–49.

Duan, R., Zhang, J., Xing, F., Konno, K., & Xu, B. (2011). Study on the properties of gelatins from skin of carp (*Cyprinus carpio*) caught in winter and summer season. *Food Hydrocolloids, 25*(3), 368–373.

Eakins, F., Al-Khayat, H. A., Kensler, R. W., Morris, E. P., & Squire, J. M. (2002). 3D structure of fish muscle myosin filaments. *Journal of Structural Biology, 137*(1), 154–163.

Feng, X., Hang, S., Zhou, Y., Liu, Q., & Yang, H. (2018). Bromelain kinetics and mechanism on myofibril from golden pomfret (*Trachinotus blochii*). *Journal of Food Science, 83*(8), 2148–2158.

Fu, Y., Zheng, Y., Lei, Z., Xu, P., & Zhou, C. (2017). Gelling properties of myosin as affected by L-lysine and L-arginine by changing the main molecular forces and microstructure. *International Journal of Food Properties, 20*(sup1), S884–S898.

Guo, X., Peng, Z., Zhang, Y., Liu, B., & Cui, Y. (2015). The solubility and conformational characteristics of porcine myosin as affected by the presence of L-lysine and L-histidine. *Food Chemistry, 170*, 212–217.

Hayakawa, T., Ito, T., Wakamatsu, J., Nishimura, T., & Hattori, A. (2010). Myosin filament depolymerizes in a low ionic strength solution containing L-histidine. *Meat Science, 84*(4), 742–746.

Hayakawa, T., Yoshida, Y., Yasui, M., Ito, T., Iwasaki, T., Wakamatsu, J., ... Nishimura, T. (2012). Heat-induced gelation of myosin in a low ionic strength solution containing L-histidine. *Meat Science, 90*(1), 77–80.

Kristinsson, H. G., & Hultin, H. O. (2003). Changes in conformation and subunit assembly of cod myosin at low and high pH and after subsequent refolding. *Journal of Agricultural and Food Chemistry, 51*(24), 7187–7196.

Li, K., Fu, L., Zhao, Y.-Y., Xue, S.-W., Wang, P., Xu, X.-L., & Bai, Y.-H. (2020). Use of high-intensity ultrasound to improve emulsifying properties of chicken myofibrillar protein and enhance the rheological properties and stability of the emulsion. *Food Hydrocolloids, 98*, 105275.

Li, S., Zheng, Y., Xu, P., Zhu, X., & Zhou, C. (2018). L-lysine and L-arginine inhibit myosin aggregation and interact with acidic amino acid residues of myosin: The role in increasing myosin solubility. *Food Chemistry, 242*, 22–28.

Liu, R., Zhao, S., Liu, Y., Yang, H., Xiong, S., Xie, B., & Qin, L. (2010). Effect of pH on the gel properties and secondary structure of fish myosin. *Food Chemistry, 121*(1), 196–202.

Mrozek, A., Karolak-Wojciechowska, J., & Kieć-Kononowicz, K. (2003). Five-membered heterocycles. Part III. Aromaticity of 1, 3-imidazole in 5+ n hetero-bicyclic molecules. *Journal of Molecular Structure, 655*(3), 397–403.

Nieuwenhuizen, W. F., Weenen, H., Rigby, P., & Hetherington, M. M. (2010). Older adults and patients in need of nutritional support: Review of current treatment options and factors influencing nutritional intake. *Clinical Nutrition, 29*(2), 160–169.

Panpipat, W., & Chaijan, M. (2017). Functional properties of pH-shifted protein isolates from bigeye snapper (*Priacanthus tayenus*) head by-product. *International Journal of Food Properties, 20*(3), 596–610.

Park, J., Yongsawatdigul, J., Choi, Y., & Park, J. (2008). Biochemical and conformational changes of myosin purified from pacific sardine at various pHs. *Journal of Food Science, 73*(3), C191–C197.

Schiffman, S. S., & Dackis, C. (1975). Taste of nutrients: Amino acids, vitamins, and fatty acids. *Perception & Psychophysics, 17*(2), 140–146.

Schobert, B., & Tschesche, H. (1978). Unusual solution properties of proline and its interaction with proteins. *Biochimica et Biophysica Acta (BBA) - General Subjects, 541*(2), 270–277.

Sow, L. C., Tan, S. J., & Yang, H. (2019). Rheological properties and structure modification in liquid and gel of tilapia skin gelatin by the addition of low acyl gellan. *Food Hydrocolloids, 90*, 9–18.

Sow, L. C., Toh, N. Z. Y., Wong, C. W., & Yang, H. (2019). Combination of sodium alginate with tilapia fish gelatin for improved texture properties and nanostructure modification. *Food Hydrocolloids, 94*, 459–467.

Tahergorabi, R., & Jaczynski, J. (2012). Physicochemical changes in surimi with salt substitute. *Food Chemistry, 132*(3), 1281–1286.

Takai, E., Yoshizawa, S., Ejima, D., Arakawa, T., & Shiraki, K. (2013). Synergistic solubilization of porcine myosin in physiological salt solution by arginine. *International Journal of Biological Macromolecules, 62*, 647–651.

Tokifuji, A., Matsushima, Y., Hachisuka, K., & Yoshioka, K. (2013). Texture, sensory and swallowing characteristics of high-pressure-heat-treated pork meat gel as a dysphagia diet. *Meat Science, 93*(4), 843–848.

Troitzsch, R. Z., Tulip, P. R., Crain, J., & Martyna, G. J. (2008). A simplified model of local structure in aqueous proline amino acid revealed by first-principles molecular dynamics simulations. *Biophysical Journal, 95*(11), 5014–5020.

Wang, G., Liu, M., Cao, L., Yongsawatdigul, J., Xiong, S., & Liu, R. (2018). Effects of different NaCl concentrations on self-assembly of silver carp myosin. *Food Bioscience, 24*, 1–8.

Xue, S., Yang, H., Wang, H., Tendu, A. A., Bai, Y., Xu, X., ... Zhou, G. (2017). High-pressure effects on the molecular aggregation and physicochemical properties of myosin in relation to heat gelation. *Food Research International, 99*, 413–418.

You, J., Pan, J., Shen, H., & Luo, Y. (2012). Changes in physicochemical properties of bighead carp (*Aristichthys mobilis*) actomyosin by thermal treatment. *International Journal of Food Properties, 15*(6), 1276–1285.

Yu, X., Li, Z., Zhao, M., Lau, S. C. S., Tan, H. R., Teh, W. J., ... Zhang, Y. (2019). Quantification of aflatoxin B1 in vegetable oils using low temperature clean-up followed by immunomagnetic solid phase extraction. *Food Chemistry, 275*, 390–396.

Zhao, X., Zhou, Y., Zhao, L., Chen, L., He, Y., & Yang, H. (2019). Vacuum impregnation of fish gelatin combined with grape seed extract inhibits protein oxidation and degradation of chilled tilapia fillets. *Food Chemistry, 294*, 316–325.

Zhou, C., Li, J., & Tan, S. (2014). Effect of L-lysine on the physicochemical properties of pork sausage. *Food Science and Biotechnology, 23*(3), 775–780.

Zhou, Y., & Yang, H. (2019). Effects of calcium ion on gel properties and gelation of tilapia (*Oreochromis niloticus*) protein isolates processed with pH shift method. *Food Chemistry, 277*, 327–335.

Zhuang, X., Jiang, X., Zhou, H., Chen, Y., Zhao, Y., Yang, H., & Zhou, G. (2020). Insight into the mechanism of physicochemical influence by three polysaccharides on myofibrillar protein gelation. *Carbohydrate Polymers, 229*, 115449.