



# Solubilisation of myosin in a solution of low ionic strength L-histidine: Significance of the imidazole ring



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## ABSTRACT

Myosin, a major muscle protein, can be solubilised in a low ionic strength solution containing L-histidine (His). To elucidate which chemical constituents in His are responsible for this solubilisation, we investigated the effects of 5 mM His, imidazole (Imi), L- $\alpha$ -alanine (Ala), 1-methyl-L-histidine (M-his) and L-carnosine (Car) on particle properties of myosin suspensions and conformational characteristics of soluble myosin at low ionic strength (1 mM KCl, pH 7.5). His, Imi and Car, each containing an imidazole ring, were able to induce a myosin suspension, which had small particle size species and high absolute zeta potential, thus increasing the solubility of myosin. His, Imi and Car affected the tertiary structure and decreased the  $\alpha$ -helix content of soluble myosin. Therefore, the imidazole ring of His appeared to be the significant chemical constituent in solubilising myosin at low ionic strength solution, presumably by affecting its secondary structure.

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## 1. Introduction

Meat contains high-quality proteins that provide a wide range of peptides and amino acids for humans. Meat proteins, unlike those proteins from beans and whole wheat, contain all the essential amino acids, with none limiting, and are all highly digestible (Pereira & Vicente, 2013). However for component extraction, meat has not been utilised to the same extent as milk or soybean products because of their low solubility. Although myofibrillar proteins comprise approximately 50% of the total meat proteins, they are not readily soluble in low ionic strength solutions or water, and a relatively high concentration of salt (>0.3 M NaCl or KCl) is required for their solubilisation (Krishnamurthy et al., 1996). It is therefore of interest to determine whether, under certain conditions, the myofibrillar proteins can be solubilised at low ionic strength. Such a treatment might enable development of a liquid-meat protein diet for humans, which would be especially beneficial for the nutrition of elderly people and infants.

In order to accomplish the solubilisation of myofibrillar proteins in a low ionic strength solution, washing with a sodium chloride solution buffered with L-histidine (His) followed by dialysis against water is necessary. It has also been reported that more than 80% of chicken breast myofibrillar proteins were solubilised in a low ionic strength solution containing 5 mM His by washing and ultrasonication of muscle tissues (Ito, Tatsumi, Wakamatsu, Nishimura, & Hattori, 2003). The ultrasonication was essential for disruption of the highly-ordered structure of the myofibrils and their solubilisation.

The low solubility of myofibrillar proteins is largely the result of the spontaneous formation of myosin filaments that occurs *in vitro* or at low ionic strength. Recently, it was suggested that 5 mM His might affect the secondary structure of myosin (Guo, Peng, Zhang, Liu, & Cui, 2015) and cause elongation of light meromyosin (LMM), resulting in the inhibition of native myosin filament formation. This enabled solubilisation of 80% chicken breast myosin in a low ionic strength solution (1 mM KCl) (Hayakawa, Ito, Wakamatsu, Nishimura, & Hattori, 2009; Hayakawa, Ito, Wakamatsu, Nishimura, & Hattori, 2010). However, myosin was shown not to be soluble in a low ionic strength solution containing other amino

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acids, such as arginine or glycine (Takai, Yoshizawa, Ejima, Arakawa, & Shiraki, 2013). It therefore seems that His might have a specific role in the solubilisation of myosin in a low ionic strength solution, unlike other amino acids. Thus, we speculated that a specific chemical constituent of His might be responsible for the solubilisation effect on myosin in a low ionic strength solution (1 mM KCl). Identification of the critical chemical component in L-His may provide a new insight into the mechanism of enhanced myosin solubility by His in low ionic strength solutions.

His is a neutral amino acid with no net charge at the experimental pH of 7.5 (Takai et al., 2013), and its structure consists of an imidazole ring and an L- $\alpha$ -alanine (Fig. 1). To clarify the role of the chemical component in L-His that is responsible for the solubilisation of myosin at a low ionic strength, we investigated the effects of 5 mM L-His, imidazole, L- $\alpha$ -alanine, 1-methyl-L-histidine and L-carnosine on solubility, size distribution, zeta potential of myosin suspension and conformational characteristics of soluble myosin in a low ionic strength solution (1 mM KCl, pH 7.5).

## 2. Materials and methods

### 2.1. Materials

L-Histidine (His), L- $\alpha$ -alanine (Ala) and L-carnosine (Car) were purchased from Aladdin Industrial Corporation (Ontario, CA), imidazole (Imi) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and 1-methyl-L-histidine (1-M-his) was obtained from Sigma-Aldrich Corporation (St Louis, MO). All chemicals (Fig. 1) were of reagent grade.

### 2.2. Extraction of myosin

Myosin was extracted from chicken breast muscle (*musculus pectoralis major*) immediately after the animal was sacrificed by using a modified procedure originally reported by Hayakawa et al. (2009). All steps were performed at 0–4 °C in a cold chamber. Briefly, minced muscle was extracted with 3 volumes of modified Guba–Straub solution (0.3 M KCl, 100 mM  $\text{KH}_2\text{PO}_4$ , 50 mM  $\text{K}_2\text{HPO}_4$ , 5 mM  $\text{EDTA}\text{Na}_2$ , 4 mM sodium pyrophosphate, dissolved in Milli-Q water, pH 6.5) for 15 min and centrifuged at 1200g for 10 min. The supernatant was filtered through three layers of cheesecloth and diluted with 14 volumes of cold distilled water. After dilution it was stored for 4 h and then the floating material was removed via siphoning. The precipitated protein was then collected by centrifugation at 10,000g for 15 min. After centrifugation, the precipitate was solubilised in 0.3 M KCl, pH 7.0. The solution

was centrifuged at 20,000g for 30 min and the supernatant diluted with 10 volumes of cold distilled water. After centrifugation (10,000g, 15 min), the precipitate was dissolved in 0.6 M KCl, pH 7.5. The dissolved solution was then dialysed with a dialysis bag (diameter: 36 mm, MW: 3500) against 0.6 M KCl, pH 7.5 solution for 24 h. After centrifugation (20,000g, 20 min) of dialysed solution, the resulting supernatant was used as the stock myosin solution in 0.6 M KCl, pH 7.5. As previously reported, myosin was highly soluble in 0.6 M KCl (pH 7.5) solution and largely present in the monomer structure (Niederman & Pollard, 1975; Sinard, Stafford, & Pollard, 1989; Takai et al., 2013).

The protein composition of the extracted myosin was validated by SDS–PAGE with a gel imaging system (GT-800 F; Epson, Nagano, Japan). The purity of the myosin was greater than 90% (Supplementary Fig. 1) as determined by densitometry (Quantity One 1-D analysis software, Bio-Rad Co., Hercules, CA).

### 2.3. Preparation of myosin suspensions in low ionic strength solutions

Myosin suspensions were prepared in low ionic strength solutions according to the procedure of Hayakawa et al. (2009). Myosin (0.6 M KCl, pH 7.5) was dialysed against low ionic strength solutions (1 mM KCl, pH 7.5) containing 5 mM of different additives (His, Imi, Ala, 1-M-his or Car) over 24 h. After dialysis, the myosin suspensions were used for further treatment or measurements of solubility, particle size distribution, and zeta potential. Myosin dialysed against 1 mM KCl containing 5 mM Tris–HCl (pH 7.5) was regarded as the control.

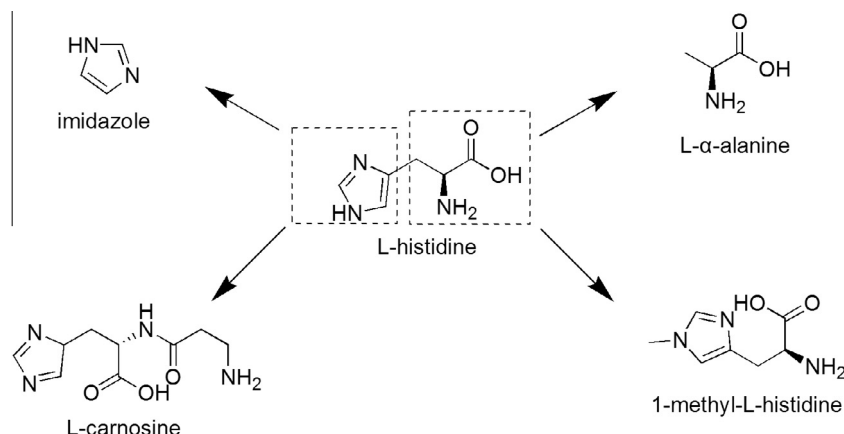
### 2.4. Myosin solubility

The dialysed myosin suspension was centrifuged at 20,000g for 20 min (Takai et al., 2013), and the obtained supernatant was defined as myosin solubilised in a low ionic strength solution containing the corresponding additive. The solubility was expressed as percent of protein concentration in the supernatant, with respect to that of dialysed myosin suspension before centrifugation, according to Hayakawa et al. (2010).

### 2.5. Particle properties of myosin suspensions

#### 2.5.1. Dynamic light scattering (DLS) measurement of particle size distribution (PSD)

DLS measurement was performed according to Shimada, Takai, Ejima, Arakawa, and Shiraki (2015) with a slight modification, by using a Zetasizer Nano ZS 90 (Malvern Instruments Ltd, Great Malvern, UK) equipped with a 4 mW He–Neon laser ( $\lambda = 633 \text{ nm}$ ). The



**Fig. 1.** Chemical structures of L-his, imidazole, L- $\alpha$ -alanine, 1-methyl-L-histidine and L-carnosine. L-His, imidazole, and L-carnosine contain an imidazole ring group whereas  $\alpha$ -alanine was the functional component in L- $\alpha$ -alanine and 1-methyl-L-histidine.

myosin suspensions (0.5 mg/mL) in low ionic strength solution containing additives (His, Imi, Ala, 1-M-his or Car) were placed in a 1-cm path-length quartz cuvette and subjected to DLS measurement with a detection angle of 90° at 25 ± 0.1 °C. Hydrodynamic diameters of myosin particles were estimated from the auto-correlation function, using the Cumulants method, based on a single exponential fit of the auto-correlation function to obtain the mean particle size (Z-average diameter). The distributions of scattering particle size based on the scattering intensity were monitored. The polydispersity index (PDI) value was determined as a measurement of the breadth of the size distribution.

### 2.5.2. Measurement of zeta potential

The zeta potential was measured by laser doppler electrophoresis using a Zetasizer Nano ZS 90 (Malvern Instruments Ltd) equipped with a 4 mW He-Ne laser with an output of 633 nm. The electrophoretic mobility was determined by measuring the direction and velocity of polymer movement in a well-defined electric field under the M3 Mode (Malvern Instruments Ltd). The zeta potential was calculated from the electrophoretic mobility using the Smoulokowski model.

## 2.6. Conformational characteristics of soluble myosin

The dialysed myosin suspensions in low ionic strength solution (1 mM KCl, pH 7.5) containing the corresponding additive (5 mM His, Imi or Car) were centrifuged at 20,000g for 20 min, and the obtained supernatants were defined as soluble myosin. The characteristics of its secondary and tertiary structures were investigated by comparing with that of native soluble myosin in 0.6 M KCl, pH 7.5.

### 2.6.1. Measurement of intrinsic tryptophan fluorescence

Changes in intrinsic tryptophan fluorescence were detected by recording the emission spectra from 320 to 420 nm of clarified soluble myosin (0.5 mg/mL) excited at 295 nm in a Spectramax microplate reader (Spectramax M2; Molecular Devices, Sunnyvale, CA).

### 2.6.2. Measurement of circular dichroism (CD) spectra and secondary structure calculation

The CD spectrum was measured using a Jasco J-715 spectropolarimeter (Jasco Co. Ltd., Tokyo, Japan). The soluble myosin (0.3 mg/mL) was transferred to a quartz cell with a 0.1 cm light-path length. Molecular ellipticity was measured in the range from 200 to 240 nm, the scan rate was 20 nm/min and the temperature was regulated with a control unit. The percentages of  $\alpha$ -helix structures were determined using the protein secondary structure estimation program (Yang's method) provided with a Jasco J-715 spectropolarimeter.

## 2.7. Statistical analysis

All data are presented as mean ± SD (standard deviation) values of three independent experiments. The analyses of variances, means and SDs were analysed with the Statistical Analysis System (SAS Institute Inc., Cary, NC). A  $p < 0.05$  significance level was used to determine the differences between the treatments.

## 3. Results and discussion

### 3.1. Effects of His, Imi, Ala, 1-M-his, and Car on solubility of myosin in low ionic strength solution

As shown in Fig. 2, the solubility of myosin was low (32%) when myosin was mixed in a low ionic strength solution containing

1 mM KCl at pH 7.5. In the presence of 5 mM His, more than 80% of myosin was solubilised in the low ionic strength solution (Fig. 2), which was consistent with the study by Hayakawa et al. (2009). Compared to the solubility of myosin in low ionic strength solution containing 1 mM KCl, it was interesting to observe that Imi and Car showed significant ( $p < 0.05$ ) effects in myosin solubility: Imi increased the solubility of myosin by more than 80% while Car increased its solubility to 60% (Fig. 2). However, the solubility of myosin in 1 mM KCl, pH 7.5 was not significantly changed ( $p > 0.05$ ) in the presence of Ala and 1-M-his, as compared with that of myosin in 1 mM KCl solution alone (Fig. 2). These results suggested that His, Imi and Car directly increased the solubility of myosin in low ionic strength solution whereas Ala and 1-M-his did not differ from the control. Overall, His and Imi were most effective in solubilisation of myosin at low ionic strength solution.

Previous works have established that myosin was practically insoluble in aqueous solutions at low ionic strengths as found here (Fig. 2) due to the formation of filamentous myosin aggregates *in vitro* (Guo et al., 2015; Hayakawa et al., 2009, 2010; Lin & Park, 1998; Niederman & Pollard, 1975; Sinard et al., 1989; Takai et al., 2013). Since then, Hayakawa et al. (2009, 2010) reported that more than 80% of myosin was solubilised in a low ionic strength solution with dialysis against a solution containing 1 mM KCl and 5 mM His at pH 7.5, as supported by our study (Fig. 2). However, the mechanism for the effect of these compounds remains unclear, as discussed in the Introduction. Since His is an amino acid that contains two major chemical constituents: Imi and Ala (Fig. 1), our immediate interest was in testing the two chemicals separately. Results showed that a similar solubilisation of myosin occurred with His and with Imi (Fig. 2). It appears that the imidazole ring is the functional group contributing to the solubilisation effect of His. The significance of the imidazole ring was further demonstrated by our methylation experiments. Methylation at the N-1 position of imidazole, 1-M-his (Fig. 1) reduced myosin solubilisation at low ionic solution (Fig. 2) compared with the non-methylated form. Next we examined whether other amino acids containing the imidazole ring had a similar effect to His. We found that Car, which, like His, had an imidazole ring (Fig. 1), exhibited an improved solubilisation effect (60%, Fig. 2) when compared with the control (1 mM KCl, pH 7.5). It was probably due to the larger molecular structure of Car when compared with His and Imi,

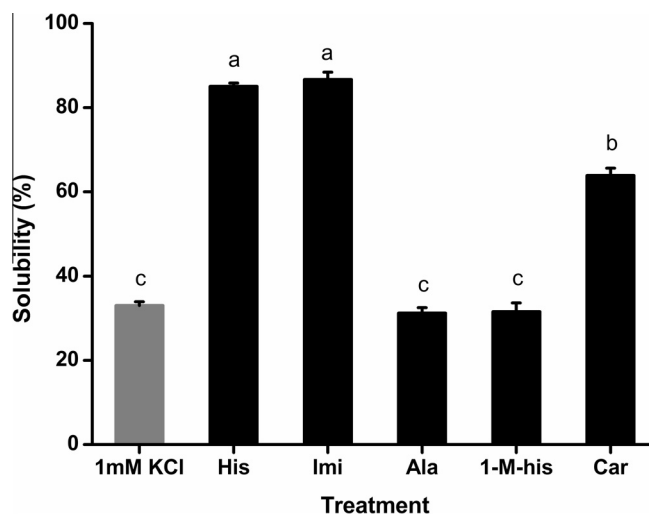


Fig. 2. The solubility of myosin in 1 mM KCl solutions (pH 7.5) containing 5 mM different additives: His, Imi, Ala, 1-M-his and Car. Values are means ± SD ( $n = 3$ ), a–c indicates that the different letters are significantly different ( $p < 0.05$ ).

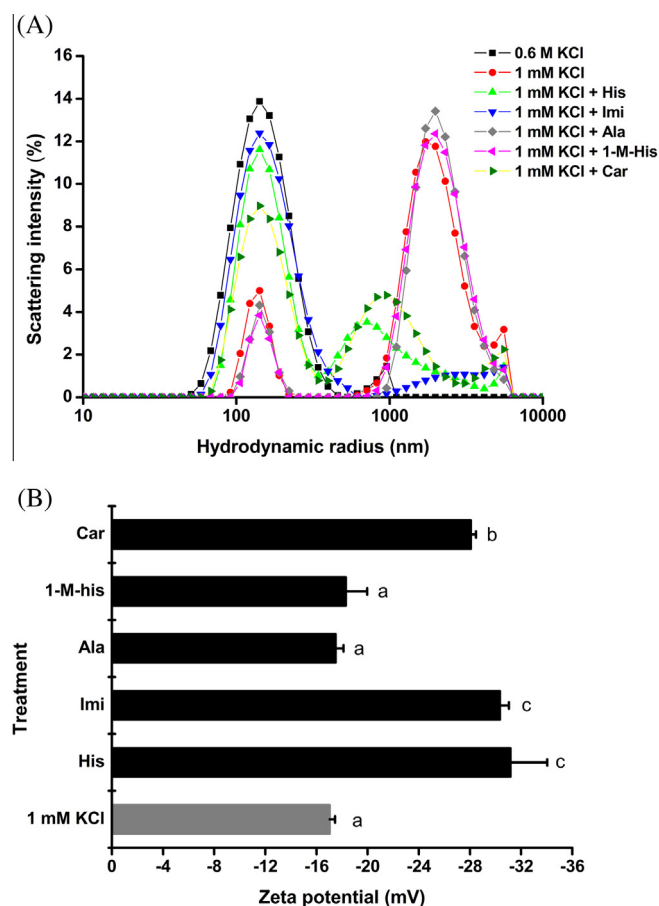
making it more difficult to interact with myosin during the dialysis process; only 60% of myosin (lower than that of His and Imi) was soluble in low ionic solution (1 mM KCl, pH 7.5) in the presence of Car (Fig. 2). In addition, the pH values of myosin solution after dialysis for 24 h were also measured, and no significant difference among each treatment was observed (data not shown). Thus, it was unlikely that the solubilisation effect was due to pH. It can be concluded that the imidazole ring of His played an important role for the solubilisation of myosin in a low ionic strength solution.

### 3.2. Effects of His, Imi, Ala, 1-M-his, and Car on particle properties of myosin suspensions

#### 3.2.1. Particle size distribution (PSD)

Monomer myosin forms filamentous structures at low salt concentrations, which make it relatively insoluble in water or in dilute salt solutions (Niederman & Pollard, 1975). The effects of the various test solvents used on myosin solubility resulted from their effects on the monomer-filament equilibrium (Shimada et al., 2015; Sinard et al., 1989; Takai et al., 2013; Tsunashima & Akutagawa, 2004). DLS is a useful technology used to monitor myosin filament dissociation or monomer aggregation through measuring the hydrodynamic radius of particles in the solution (Shimada et al., 2015; Tsunashima & Akutagawa, 2004). In the present study, the particle size distribution, Z-average hydrodynamic diameter (Z-average), hydrodynamic diameter (nm) of the more intense peak ( $D_H$ ) and the polydispersity index (PDI) were investigated by DLS (Fig. 3A, Table 1).

The experiments were performed by dialysing soluble myosin in 0.6 M KCl against low ionic strength solution (1 mM KCl) containing the individual test additives. The myosin in the high ionic strength solution (0.6 M KCl) was largely monomeric (Sinard et al., 1989; Tsunashima & Akutagawa, 2004) which we have confirmed (Fig. 3A and Table 1). One major peak at 142 nm with a Z-average of 147 nm was observed for myosin solubilised in 0.6 M KCl (Fig. 3A). The value of PDI for soluble myosin in 0.6 M KCl was 0.26 (Table 1), lower than 0.3, indicating a homogenous and mono-dispersed population in this solution (Barros, Carvalho, Alves, Carvalho, & Tabak, 2015; Di Marzio, Marianecci, Petrone, Rinaldi, & Carafa, 2011). Although it is not possible to assign the myosin structure based on DLS experiments, the single mono-dispersed peak in the high ionic strength solution was suggested as myosin monomers (Niederman & Pollard, 1975; Shimada et al., 2015; Sinard et al., 1989). After dialysis, the ionic strength of the myosin dialysate decreased from 0.6 to 0.001. At a low ionic strength of 1 mM KCl, myosin polymerised to form filaments through rod-rod electrostatic interaction (Nakasawa et al., 2005; Sohn et al., 1997), so the intensity distribution curves of myosin suspension presented two peaks with a  $D_H$  of 1809 nm and a Z-average of 1008 nm, which were significantly higher than those of soluble myosin in 0.6 M KCl (Fig. 3A, Table 1). The first small contribution around 141.77 nm was assigned to the monomer species and the second one at 1809 nm was probably due to the contribution of myosin filaments. More than 80% of myosin molecules displayed an assembled filament state in low ionic strength solution, as observed by electron micrographs (Hayakawa et al., 2010; Sinard et al., 1989). It is believed that the filaments were the predominant species at 1 mM ionic strength solution in this study (Fig. 3A). This dialysis process from 0.6 M to 1 mM produced particles with broader scattering intensity distribution, as the PDI was significantly ( $p < 0.05$ ) increased to 0.71 (Fig. 3A, Table 1). Therefore, our DLS data strongly support the suggestion that monomer myosin assembled to filament species with larger particle size during the dialysis process from 0.6 M to 1 mM.



**Fig. 3.** Particle properties of myosin suspension: (A) size distributions in 0.6 M KCl, 1 mM KCl, 1 mM KCl + His, 1 mM KCl + Imi, 1 mM KCl + Ala, 1 mM KCl + 1-M-his, and 1 mM KCl + Car solutions (pH 7.5), respectively and (B) zeta potential in 1 mM KCl, 1 mM KCl + His, 1 mM KCl + Imi, 1 mM KCl + Ala, 1 mM KCl + 1-M-his, and 1 mM KCl + Car solutions (pH 7.5), respectively, monitored by DLS at 25 °C. Values are means  $\pm$  SD ( $n = 3$ ), a–c in (B) indicates that the different letters are significantly different ( $p < 0.05$ ).

**Table 1**  
Hydrodynamic properties of myosin suspensions in various solutions obtained from DLS data.

Treatment	Hydrodynamic properties		
	Z-average (nm) <sup>a</sup>	$D_H$ (nm) <sup>b</sup>	PDI <sup>c</sup>
0.6 M KCl	147 $\pm$ 5 d	142 $\pm$ 0 b	0.26 $\pm$ 0.01 d
1 mM KCl	1008 $\pm$ 51 a	1809 $\pm$ 157 a	0.71 $\pm$ 0.04 a
1 mM KCl + His	204 $\pm$ 5 b	157 $\pm$ 13 b	0.44 $\pm$ 0.02 b
1 mM KCl + Imi	166 $\pm$ 4 c	142 $\pm$ 0 b	0.34 $\pm$ 0.01 c
1 mM KCl + Ala	965 $\pm$ 102 a	2095 $\pm$ 182 a	0.64 $\pm$ 0.07 a
1 mM KCl + 1-M-his	866 $\pm$ 91 a	1990 $\pm$ 0 a	0.84 $\pm$ 0.14 a
1 mM KCl + Car	231 $\pm$ 17 b	142 $\pm$ 0 b	0.63 $\pm$ 0.08 a

Note: values were mean of triplicate values  $\pm$  S.D.

<sup>a–d</sup> Different letters in the same column indicate significant differences at  $p < 0.05$ .

<sup>a</sup> Z-average hydrodynamic diameter obtained by DLS.

<sup>b</sup> Hydrodynamic diameter (nm) of the more intense peak shown in Fig. 4.

<sup>c</sup> PDI is the polydispersity index.

In the presence of His, the PSD of myosin suspensions at 1 mM ionic strength displayed a two-peak curve with a  $D_H$  of 157 nm and a Z-average of 204 nm (Fig. 3A, Table 1). This likely resulted from the formation of mini-filaments (Craig & Woodhead, 2006; Sinard et al., 1989). Myosin suspensions in low ionic strength solutions in the presence of His contained a small amount of larger particle size species (Fig. 3A). However, the smaller size distribution at



around 157 nm for myosin suspension became dominant when compared with that of myosin suspensions at 1 mM KCl solution in the absence of His. This indicated that the contribution of monomer or small size myosin mini-filament was more pronounced in 1 mM KCl solution containing His. This result is in good agreement with the study using transmission electron microscopy, which reported that His could participate in the depolymerisation of the myosin filament (Hayakawa et al., 2010). It has also been verified that the depolymerisation effect was not caused by the dissociation or degradation of any polypeptides of monomer myosin (Hayakawa et al., 2009), which we have also confirmed by SDS-PAGE (data not shown). Hence we confirmed that His can affect the myosin monomer-filament transition during dialysis from high ionic (0.6 M) to low ionic (1 mM) solution by dissociation of myosin filament.

Next we examined the effects of the functional groups in His (Imi, Ala, 1-M-his and Car) on the monomer-filament transition in myosin suspensions during dialysis from high ionic strength to low ionic strength solutions. After dialysis to low ionic strength, Imi induced a PSD curve of myosin suspensions profiled with a major peak at 142 nm ( $D_H$  in Fig. 3A and Table 1) and a small broad peak near 2000 nm (Fig. 3A). The PSD of myosin suspensions in Imi solution at low ionic strength was similar to that in 0.6 M KCl solution (Fig. 3A). The PDI at this condition was 0.34 (Table 1), indicating a nearly mono-dispersed contribution of particle size at around 142 nm. It appears that Imi can retain most of the monomers by affecting the monomer-filament equilibrium in the myosin suspensions during dialysis. However, the presence of Ala and 1-M-his there appeared to be little influence on the monomer-filament transition in the myosin suspension during dialysis, since we observed a similar particle size distribution with 1 mM KCl solution without additions (Fig. 3A). There were no significant differences in the values of Z-average,  $D_H$  or PDI among 1 mM KCl, 1 mM KCl + Ala and 1 mM KCl + 1-M-his treatment (Table 1). These results suggested that the imidazole group in His might be responsible for the disruption of the mono-filament equilibrium in myosin suspensions during dialysis from high to low ionic strength solution.

Thus we tested the PSD of myosin suspensions at low ionic solution containing Car by DLS. Two populations were observed: one centred at 142 nm ( $D_H$ ) and the other at 1000 nm (Fig. 3A, Table 1). With the addition of Car, the Z-average was significantly decreased to 231 nm as compared to the 1 mM KCl solution (Table 1), indicating that Car, which also contains an imidazole ring, can inhibit the mono-filament transition during dialysis; probably by dissociation of myosin filaments as discussed above. The inhibitory ability was weaker than that of His and Imi, as the major peak area of Car was centred at near 141 nm, which represented the monomer species of myosin in suspensions and was lower than that of both His and Imi (Fig. 3A). Therefore, it is proposed that the imidazole ring in His might be indispensable for the dissociation of myosin filament, thus affecting the mono-filament equilibrium in myosin suspensions during dialysis from 0.6 M KCl to 1 mM KCl solutions, resulting in the formation of small particle size species in myosin suspensions at low ionic strength solution.

The well documented properties of myosin solubility suggest that it is the formation of filaments that determines myosin solubility (Sinard et al., 1989; Sohn et al., 1997; Tsunashima & Akutagawa, 2004). It had been assumed that the monomeric myosin form was soluble at both low and high salt concentrations (Takai et al., 2013). Monomeric myosin formed filamentous structures and became insoluble at low ionic strength (Lowey, Slayter, Weeds, & Baker, 1969). Thus, the low solubility of myosin at 1 mM KCl (Fig. 2) was attributed to the myosin suspension comprising wholly of filaments and not the monomers (Fig. 3A, Table 1). When adding 5 mM His, Imi or Car to 1 mM KCl solution, myosin

suspensions favoured the small particle size species (most likely monomeric myosin as shown in Fig. 3A and Table 1), leading to a increasing effect on the solubility of myosin (Fig. 2). The solubilisation effects of His and Imi were more apparent than that of Car (Fig. 2) as the quantity of the monomer species in myosin suspensions of His and Imi was higher than with Car (Fig. 3A). However, the PSD of myosin suspensions in the presence of either Ala or 1-M-his consisted predominantly of the filament species (Fig. 3A, Table 1), similar to that of 1 mM KCl, resulting in the low solubility of myosin (Fig. 2).

### 3.2.2. Zeta potential

Zeta potential is related to the charge residing on the surface or near-surface of a suspended particle. The effective surface charge of particles is an important factor for determining their dispersion and aggregation (Malhotra & Coupland, 2004; Teng, Luo, & Wang, 2012). In the present study, we studied the zeta potential of myosin suspensions so as to investigate the myosin dispersion properties in low ionic strength solutions containing different additives as shown in Fig. 3B. Compared to the 1 mM KCl without test additives, myosin suspensions with the addition of His, Imi or Car possessed significantly higher ( $p < 0.05$ ) absolute zeta potential (approximately 30 mV; Fig. 3B). In the presence of His and Imi the myosin suspensions had a significantly higher ( $p < 0.05$ ) absolute zeta potential than with Car (Fig. 3B). Nevertheless there was no significant difference ( $p > 0.05$ ) in the zeta potential values among the 1 mM KCl, Ala and 1-M-his (Fig. 3B).

It was well known that increasing the surface charge (increasing the absolute zeta potential) of colloidal particles can strengthen their inter-particle electrostatic repulsion and disrupt existing protein aggregates as well as discourage further aggregate formation (Song, Zhou, Fu, Chen, & Wu, 2013; Teng et al., 2012). It had also been reported that a physical stable nano-suspension solely stabilised by electrostatic repulsion, would have a minimum absolute zeta potential of 30 mV (Di Marzio et al., 2011). The high negative zeta potential (around  $-30$  mV) in myosin suspensions containing His, Imi or Car indicated very stable myosin dispersions. As a result, the solubility of myosin suspensions in His, Imi, or Car would increase (Fig. 2). However, the addition of either Ala or 1-M-his gave a lower absolute zeta potential compared with that of His, Imi or Car, indicating that the electrostatic repulsion among myosin particles was weaker. This might easily promote formation of particle aggregates and prevent their dissociation (Li et al., 2007). Then, as expected, the solubility of myosin suspension in Ala or 1-M-his solution was low (Fig. 2).

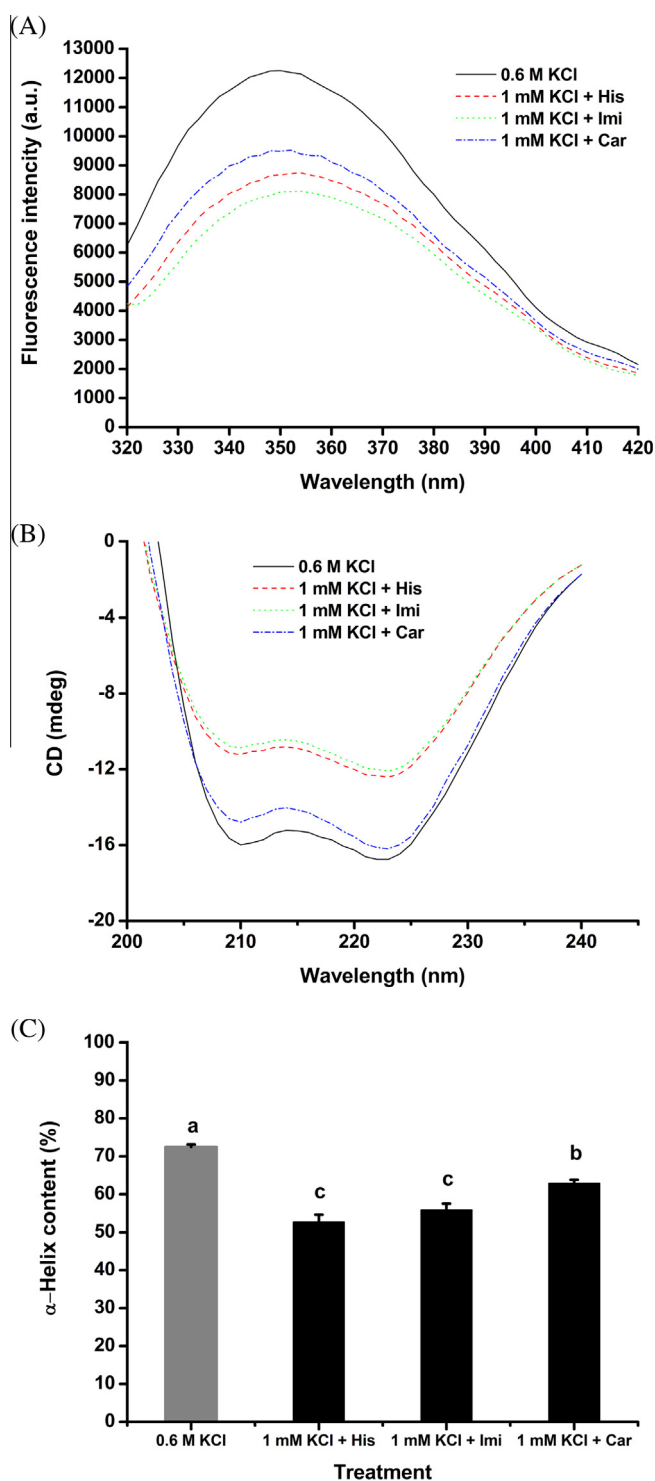
The results on particle properties of myosin suspensions suggest that the imidazole ring in His might be the significant functional group affecting the mono-filament equilibrium in myosin suspensions during dialysis from 0.6 M KCl to 1 mM KCl solutions. It can induce a myosin suspension to form small particle size species (most likely monomeric myosin) with a high absolute zeta potential, thus increasing its solubility.

### 3.3. Effects of His, Imi and Car on the conformational characteristics of soluble myosin

Generally, the native monomer myosin in 0.6 M KCl solution can form insoluble filamentous structures after dialysis into low ionic strength solutions. As discussed above, more than 60% of myosin (most of them were monomer myosin) was soluble in low ionic strength solution containing His, Imi or Car (all containing the imidazole ring as shown in Fig. 1). By comparison with the structure of native soluble myosin in 0.6 M KCl solution, our intent was to determine the mechanisms for the solubilising effect of the imidazole ring.

### 3.3.1. Changes in tertiary structure

The intrinsic tryptophan fluorescence spectra of soluble myosin in low ionic strength solution in the presence of different test additives (His, Imi and Car) is shown in Fig. 4A, and was used to characterise the tertiary structural changes. The intensity of the intrinsic fluorescence spectrum of myosin obviously decreased



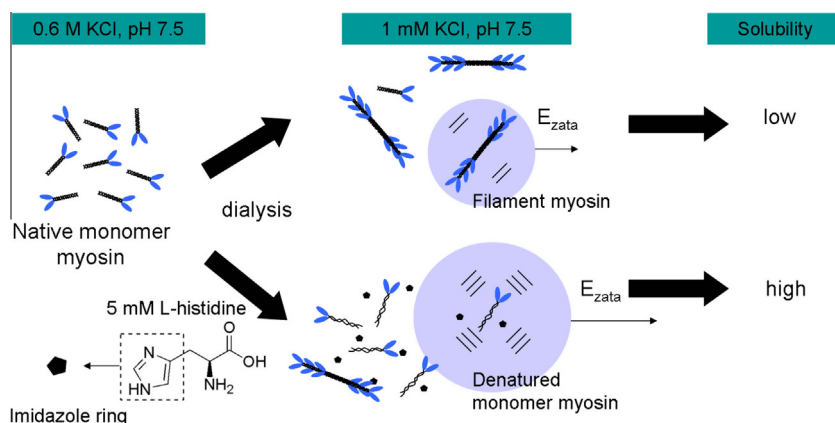
**Fig. 4.** Conformational changes of soluble myosin: (A) intrinsic tryptophan fluorescence spectra, (B) CD spectra and (C)  $\alpha$ -helix structure content of soluble myosin in 0.6 M KCl, 1 mM KCl + His, 1 mM KCl + Imi and 1 mM KCl + Car solutions (pH 7.5), respectively. Values are means  $\pm$  SD ( $n = 3$ ), a–c in (C) indicates that the different letters are significantly different ( $p < 0.05$ ).

with the addition of His, Imi or Car, as compared to the native myosin in 0.6 M KCl (Fig. 4A). The maximum intensity of myosin treated by His was lower than that of Car, and Imi gave the lowest intrinsic intensity of myosin among the test solvents (Fig. 4A). The maximum fluorescence of native myosin in 0.6 M KCl was observed at 350 nm, while it shifted to 352 nm with Car addition (Fig. 4A). A further red shift in maximum fluorescence ( $\sim 354$  nm) was observed in soluble myosin treated with His or Imi (Fig. 4A).

The decline in the intrinsic fluorescence intensity indicated that the environment of the peripheral tyrosine and tryptophan residues was more polar due to the presence of the test additives inducing changes in the tertiary structures of myosin (Kristinsson & Hultin, 2003). The results of red shifting fluorescence suggested that, in native myosin, the tryptophan residues were partially buried, and, in denatured myosin, were exposed to a polar environment (Iwasaki & Yamamoto, 2003). A notable quenching of fluorescence and red shifting fluorescence (Fig. 4A) suggested possible tertiary structural changes of myosin induced by His, Imi or Car. The red shift of myosin observed with His or Imi was only 4 nm, suggesting that the structural changes of myosin differed from those induced by denaturants such as guanidine hydrochloride (Gu-HCl). The fully unfolded myosin molecule in 6 M Gu-HCl exhibited more quenching of tryptophan fluorescence and was significantly red shifted (Kristinsson & Hultin, 2003). The red shift at the maximum fluorescence wavelength of myosin S1 was 14 nm with 5 M urea (Nozais, Bechet, & Houadjeto, 1992). Tertiary structure changes of myosin induced by His had been investigated through surface hydrophobicity and sulfhydryl content measurement (Guo et al., 2015). They suggested that His was likely to bind to the charged residues of myosin via an electrostatic effect, thereby disrupting the intra- and inter-molecular ionic linkages and alter the protein structure. However, at pH 7.5, as used in the present study, His was neutral in net charge; thus the electrostatic interactions were unexpected. Moreover, from SDS-PAGE analysis, these structural changes of myosin induced by His were unlikely to have been caused by the degradation of any polypeptides of myosin (Hayakawa et al., 2009). The morphological profile of myosin solubilised in His solution was similar to that of native myosin in 0.6 M KCl, except that the rod of myosin was lengthened in the presence of His as observed by TEM (Hayakawa et al., 2009, 2010). It has been concluded that tryptophan fluorescence is a particularly sensitive and informative structural probe for the myosin rod (Kato & Konno, 1993). A decrease in tryptophan fluorescence intensity is thought to be attributed to the partial unfolding of the helical-helix structure consisting of two peptides in the myosin rod of chicken skeletal muscle (Iwasaki & Yamamoto, 2003). However, some studies have suggested that changes in the tertiary structure actually represented changes in the head region of myosin, since the rod region only contributed approximately 27% of the total fluorescence of the entire myosin molecule (King & Lehrer, 1989; Raghavan & Kristinsson, 2008). Therefore, it was difficult to determine which part of myosin (head or rod region) was influenced by the additives (His, Imi and Car) through the tryptophan fluorescence intensity measurement. However, we can speculate that the imidazole ring in His might be responsible for the tertiary structural changes of soluble myosin.

### 3.3.2. Changes in secondary structure

The CD spectrum of native monomer myosin in 0.6 M KCl solution exhibited two negative bands near 208 and 222 nm (Fig. 4B), implying that the predominant form present was due to the myosin tail having a supercoiled  $\alpha$ -helix structure (Cao & Xiong, 2015). When subjecting myosin to solubilisation in 1 mM KCl solution containing His, Imi or Car, the distinct helical pattern underwent significant negative attenuation at 208 and 222 nm of the band region (Fig. 4B), denoting significant losses of  $\alpha$ -helix structure



**Fig. 5.** Proposed mechanism of solubilisation of myosin at low ionic solution (1 mM KCl, pH 7.5) in the presence of 5 mM His: native monomer myosin generally polymerised to form filaments consisting of large particle sizes with relatively low absolute zeta-potential in low ionic strength solution (1 mM KCl, pH 7.5), resulting in low solubility; with the addition of 5 mM His, the loss of  $\alpha$ -helix structure and elongation in the myosin rod might occur. Thus, the filament assembly process was impeded, resulting in a myosin suspension with small particle size species and high absolute zeta-potential. Therefore, the solubility of myosin in 1 mM KCl, pH 7.5 was increased. The imidazolium moiety of His was the representative structure for solubilisation of myosin in low ionic strength solution (1 mM KCl, pH 7.5).

(Fig. 4C) of light meromyosin (Cao & Xiong, 2015). Compared to the native myosin in 0.6 M KCl, the  $\alpha$ -helix content of soluble myosin at 1 mM KCl in the presence of Car significantly decreased ( $p < 0.05$ ) from 72.47% to 62.97% (Fig. 4C). Further marked loss ( $p < 0.05$ ) of  $\alpha$ -helix content (decreased to around 55.00%) was observed for soluble myosin in 1 mM KCl solution with the addition of His or Imi (Fig. 4C). This result confirmed a previous finding that His decreased the  $\alpha$ -helix content of myosin in 1 mM ionic strength solutions (Guo et al., 2015). The effects of other amino acids on myosin conformation have been investigated by others, with the result being contradictory. L-Arginine or L-lysine caused no secondary structural changes in the presence of 200 or 300 mM NaCl (Takai et al., 2013), suggesting a different solubilisation mechanism for L-arginine or L-lysine compared to His. The denaturing effect of His was also different from Gu-HCl, which led to full unfolding and complete loss of the secondary structure spectrum of myosin (Kristinsson & Hultin, 2003). It can be concluded that His, Imi and Car, each containing imidazole rings, decreased the  $\alpha$ -helix structure of native myosin. As the  $\alpha$ -helix structure of myosin was mainly stabilised by hydrogen bonds between the carbonyl oxygen ( $-\text{CO}$ ) and amino hydrogen ( $\text{NH}-$ ) of the polypeptide chain (Cao & Xiong, 2015; Liu, Zhao, Xiong, Xie, & Qin, 2008), the presence of nucleophilic centres in the imidazole ring may have disturbed these hydrogen bonds, hence affecting its structure.

Myosin mainly consisted of two globular head regions and a rod-like tail portion consisting of a coiled-coil  $\alpha$ -helix (Harrington & Rodgers, 1984). The 100% helical rod has been found to represent the great majority of the secondary structure spectra of myosin (King & Lehrer, 1989; Kristinsson & Hultin, 2003). Therefore, the  $\alpha$ -helix losses of myosin induced by His, Imi and Car (Fig. 4B and C) might be attributed to the conformational changes of the rod region. An interesting phenomenon had been reported that His can induce lengthening of the LMM region of the myosin rod and increase the periodicity of LMM para-crystals (Hayakawa et al., 2009, 2010), probably resulting from changes in the secondary structures in the myosin rod induced by His (Fig. 4B and C). As discussed above, it is the dynamic myosin filament assembly process occurring during dialysis that determines the myosin solubility. The positive and negative charge clusters in the rod region of myosin are essential for filament formation (Nakasawa et al., 2005). By the interaction between charge clusters in the rod region, native monomer myosin can assemble to form filaments during dialysis from 0.6 M KCl to 1 mM KCl (Craig &

Woodhead, 2006; Nakasawa et al., 2005; Sohn et al., 1997). Loss of  $\alpha$ -helix structure in the myosin rod region might cause a change in the interaction between the molecules, thus disrupting the filament assembly process as discussed for Fig. 3A. Therefore the solubility of myosin in 1 mM KCl containing 5 mM His, Imi or Car was increased as shown in Fig. 2.

Based on our results and previous studies, a proposed mechanism for solubilisation of myosin at low ionic solution (1 mM KCl, pH 7.5) in the presence of 5 mM His is depicted in Fig. 5. After dialysis into a low ionic strength solution (1 mM KCl, pH 7.5), native monomer myosin generally polymerised to form filaments consisting of large particle sizes (Fig. 3A, Table 1) and relatively low absolute zeta-potential (Fig. 3B); thereby, its solubility was low (Fig. 2). With the addition of 5 mM His in low ionic strength solution (1 mM KCl, pH 7.5), the transformation of native myosin conformation occurred (Fig. 4). Probably because of the loss of  $\alpha$ -helix structure and the elongation in the myosin rod, the filament assembly process was impeded, resulting in a myosin suspension with small particle size species and high absolute zeta-potential (Fig. 3A, Table 1 and Fig. 3B). Subsequently, the solubility of myosin in low ionic strength solution was increased in the presence of 5 mM His (Fig. 2). We firstly confirmed that the imidazolium moiety of His was the representative structure for solubilisation of myosin in low ionic strength solution. It would be of great interest to test the significance of the imidazole ring in His to see if it can be used beneficially for other food additives in the development of liquid dietary meat products containing myosin, based on its ability to increase the solubility of myosin in solutions of low ionic strength.

#### 4. Conclusions

In 1 mM ionic strength solutions (pH 7.5), the solubility of myosin can be increased by addition of His, Imi or Car but not with Ala or 1-M-his. Myosin suspensions having small particle size species (most likely monomeric myosin) with high absolute zeta potential, were induced by addition of His, Imi or Car but not with Ala or 1-M-his. His, Imi and Car effected tertiary structural changes, whereby the  $\alpha$ -helix content of soluble myosin was decreased. The conformational changes in the rod region of myosin might result in increased solubility. The imidazolium moiety of His was the critical structure for solubilisation of myosin in low ionic strength solution.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.09.039>.

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