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Effects of calcium ion on gel properties and gelation of tilapia (*Oreochromis niloticus)* protein isolates processed with pH shift method

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ABSTRACT

The effect and mechanisms of calcium chloride and citric acid addition on the acid method of processing tilapia protein isolates and surimi gels were studied. The alkaline method shows better gel quality than the acid method with lower yield and high lipid content. The addition of calcium chloride increased the breaking force to 494.56 g and citric acid helped reducing the lipid content by 85.85%, resulting in a higher yield of 75.36%. Gels with calcium maintained low levels of expressible moisture (2.30%), indicating a well-organised gel matrix. The decrease in -SH content and increase in ionic bonds content suggested increased formation of disulphide bonds and ionic bonds. Oscillatory dynamic measurement indicated that more heat resistant bonds were formed. The morphology of myofibril proteins studied by atomic force microscopy (AFM) showed that the height of the proteins in the calcium-added gels decreased 79.4%, which suggested that the protein structure was “suppressed”.

1. Introduction

Surimi, a cooked paste or gel made from fish or other meat, is of great importance in aquatic products industry and has been developed rapidly. Conventionally, it is produced by constant washing of fish flesh with cool water to remove water-soluble proteins and unwanted substances, such as pigments, blood, lipids, or odorous substances which means the myofibril proteins are the only main concern. In making any surimi-based product, the crucial point is to increase the concentration of myofibril protein which improves gel network, thus helping to entrap moisture or to form a gel matrix (Kobayashi, Mayer, & Park, 2017).

One major problem associated with the washing method in surimi production is the low yield because of the multiple washing steps. By contrast, the pH shift method maximises the protein recovery of surimi products (Batista, Pires, & Nelhas, 2007; Freitas, Cortez-Vega, & Prentice, 2015; Undeland, Kelleher, & Hultin, 2002). With the help of the pH dependent properties of proteins, isolated proteins can be easily separated from unwanted substances, and the proteins can be re-precipitated with high concentration and purity (Matak, Tahergorabi, & Jaczynski, 2015). After pH shift treatment of fish mince, because of the distinct effects of acid and alkaline conditions on the myofibril proteins, the resultant isolated proteins and surimi gels showed different or even better functional characteristics (Marmon, Krona, Langton, & Undeland, 2012).

Tilapia (*Oreochromis niloticus*) is one of the most widely consumed fishery products in Southeast Asia, and it is usually further processed into surimi or fish ball products. One issue is the high content of triglycerides and membrane phospholipids, which are the main causes of lipid oxidation, off-odours, and quality deterioration. In the field of surimi food, the pH shift method and other numerous improvements have been made to extract proteins and enhance the quality (Kudre, Benjakul, & Kishimura, 2013; Lee, Yoon, & Park, 2017; Marmon et al., 2012). Previous research showed that calcium ions (Ca^{2+}) could cause phospholipoproteins from fat globule membranes to aggregate and precipitate. Citric acid, which contains several carboxylic groups, could interact with basic amino acid residues of cytoskeletal proteins and compete with the acidic phospholipids of membranes, thus decreasing the lipid component in surimi food effectively (Liang & Hultin, 2005). By reducing the content of lipids in fish products, the stability of the product is improved. In addition, the properties of the gel could be changed by the addition of external metal ions (Yin & Park, 2015), which could be of great help in aquatic food processing and could increase consumer satisfaction.

Although there are reports on using calcium chloride and citric acid in surimi processing, the mechanisms underlying the gelling properties of proteins remain to be explored. Textural properties are the determining factors of food quality and consumer acceptance; therefore, the current study aimed to determine the effects of calcium chloride and citric acid addition on functional and physicochemical properties of

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tilapia protein isolates and surimi gels. The present study increases our understanding of relationships among pH shift conditions, external additives, and fish myofibril proteins.

2. Materials and methods

2.1. Functional and physicochemical properties of tilapia protein isolates and surimi gels

Acid (A) and alkaline (B) methods of processing protein isolates were performed according to the method of Panpipat and Chaijan (2016) with slight modifications. Minced fish was homogenised with cold distilled water (4 °C) at a ratio of 1:6 (w/v) for 2 min. The pH of the homogenate was adjusted to 2 or 12 using 2 mol/L HCl or 2 mol/L NaOH. Homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was carefully collected, and the pH value was adjusted back to the isoelectric point (pI) pH 5.5. The precipitated fraction was centrifuged at 10,000 *g* for 10 min and collected as acid (A1) or alkaline (B1) method protein isolate.

Calcium-added protein isolates were prepared according to the method of Liang and Hultin (2005) with slight modifications. Fish mince was homogenised with 5 mmol/L citric acid containing 8 mmol/L calcium chloride at the ratio of 1:5 (w/v) for 2 min. The remaining steps were the same as the acid method to obtain protein isolates. The precipitated fraction was collected as the calcium method protein isolate (C1).

Conventional water washed mince was prepared according to the method of Chaijan, Panpipat, and Benjakul (2010) with slight modifications. Mince was stirred with cold distilled water (4 °C) at a ratio of 1:3 (w/w) for 10 min of three times, except that the third time was performed with cold 0.5% NaCl solution. The precipitated fraction was collected as the water washed protein isolate (W1).

After each processing method, the moisture contents of the protein isolates were adjusted back to 80% (the initial moisture content of tilapia muscle). Protein recovery after each processing condition was calculated as follows:

$$\text{Protein recovery}\,(\%) = \frac{\text{weight of recovered protien isolates}}{\text{weight of minced fish before processing}} \times 100$$

The lipid content in all protein isolates was determined using the method of Bligh and Dyer (1959). To every 1 g sample, homogenised with 8 mL of chloroform, methanol and water at the ratio of 1:2:1. The homogenate was treated with 2 mL of chloroform and homogenised again. After addition of 1 mL water and homogenisation, the mixture was centrifuged at 3000 *g* at 4 °C for 15 min. The chloroform phase was drained off into an Erlenmeyer flask containing about 1 g of anhydrous sodium sulfate, shaken very well, and decanted into a round-bottom flask through a filter paper. The solvent was evaporated at 40 °C and the lipid content in samples was calculated.

2.2. Functional and physicochemical properties of tilapia surimi gels

After processing, all protein isolates were chopped for 1 min followed by adding 2.5% NaCl and chopped for another 1 min at 4 °C, the paste was stuffed into a plastic casing with a diameter of 2.5 cm and both ends were tightly sealed. The pastes were incubated at 90 °C for 30 min and immediately cooled in iced water for 20 min. The surimi gels (A2, B2, C2, W2 as cooked surimi gels) obtained were stored at 4 °C overnight before further analysis.

Expressible moisture was determined using the method of Panpipat and Chaijan (2016). Samples were weighed and placed between filter papers (one on top and two below). A 5 kg weight was then placed on top of the sample for 2 min and the sample was weighed again. The weight differences were defined as expressible moisture.

Protein solubility was determined as follows: to every 1 g protein isolates, 15 mL cold water was added and homogenised. After centrifugation at 10,000 *g* for 10 min at 4 °C, supernatant was separated and saved while cold 0.3% NaCl solution was added to the pellet and mixed. The mixture was centrifuged at the same condition and the supernatant was saved and combined together as solution of water-soluble proteins. The pellet was added with 15 mL cold 0.6 M NaCl (20 mM Tris–maleate, pH 7.0), homogenised, and kept at 4 °C with occasional vortex for 1 h. The homogenate was salt-soluble proteins. Concentration of water and salt soluble proteins was determined using the bicinchoninic acid assay (BCA method) with bovine serum albumin as standard.

The texture of the surimi gels was measured using a texture analyser (TA-XT2i, Stable Micro System, Surrey, UK) with a spherical plunger (5-mm diameter). The testing parameters were set as pre-test speed of 5 mm/s, a test speed of 1 mm/s, and a penetration distance of 15 mm. Gels were equilibrated at room temperature for at least 2 h before analysis. Cylindrical samples of 2 cm in length were cut. Breaking force (gel strength) and deformation (elasticity) were determined.

2.3. Determination of -SH groups ionic bonds content

Total -SH groups, reactive -SH groups, and ionic bonds in all samples were examined according to previously published methods (Zhang, Li, Shi, Zhu, & Luo, 2018). For -SH groups, 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. Surface reactive -SH groups were measured using the same system except buffer A was made in the absence of urea. The concentration of -SH bonds content was calculated according to the Ellman method (Ellman, 1959).

For concentration of ionic bonds, 1 g sample was homogenised with 10 mL 0.05 M NaCl, and another 1 g sample was homogenised with 0.6 M NaCl. The concentration of ionic bonds was determined by the differences of the protein concentration in each of the solution, with the help of Bradford method (Bradford, 1976) using bovine serum albumin as standard.

2.4. Ca^{2+}-ATPase activity of myofibrillar proteins

Myofibril proteins of all samples were extracted according to previous method with modifications (Zhang et al., 2018). To every 1 g of sample, 5 mL of solution 1 (0.05 M NaCl, 0.02 M Tris–maleate buffer at pH 7.0 and 0.05 mmol/L phenylmethylsulfonyl fluoride) was added and homogenised. The pellet was collected by centrifugation at 4 °C, 10,000 *g* for 10 min. After this step was repeated once, the pellet was homogenised with solution 2 (0.6 M NaCl, 0.02 M Tris – maleate buffer at pH 7.0), kept at 4 °C for 1 h. Then the pellet was centrifuged at 10,000 *g* for 10 min at 4 °C, added with 30 mL chilled deionised water (DI water) and recentrifuged again at 10,000 *g* for 10 min at 4 °C. The pellet was then collected and dissolved in 5 mL 0.6 M NaCl. After centrifugation at the same condition, the supernatant was collected as myofibril protein solution.

The concentration of protein solution was adjusted to 1 mg/mL. To every 1 mL of myofibril protein solution, 0.5 mL of 0.5 M Tris–maleate (pH 7.0), 0.5 mL of 0.1 M $CaCl_2$, 7.5 mL water were added and mixed well. Then, 0.5 mL of 20 mM ATP (pH 7.0) was added to all the solutions to initiate the reaction. After incubation at room temperature for 5 min, 0.8 mL of 15% trichloroacetic acid (TCA) was added to terminate the reaction. The mixture was centrifuged at 10,000 *g* for 2 min and supernatant was collected. Blank was prepared by adding TCA prior to ATP solution. The reactivity of Ca^{2+}-ATPase was expressed as the inorganic phosphate released by every 1 mg protein per minute. The amount of inorganic phosphate was measured by the method of Fiske and Subbarow (1925).

2.5. Protein patterns, microstructure, and secondary structure of myofibrillar proteins

The protein patterns of fresh fish (F) without any processing conditions, protein isolates (A1 – W1), and surimi gels (A2 – W2) were

Table 1
Effects of processing conditions on the properties of tilapia protein isolates and surimi gels.

Processing condition	Protein recovery (%)	Lipid reduction (%)	Expressible moisture (%)	Breaking force (g)	Deformation (mm)	Water-soluble protein concentration ($\times 10^{-2}$ mg/mL)	Salt-soluble protein concentration ($\times 10^{-2}$ mg/mL)
Fresh/F	–	–	–	–	–	119.8 ± 5.6^{c}	67.7 ± 3.9^{a}
Acid/A	81.60 ± 1.79^{c}	72.22 ± 3.18^{b}	3.62 ± 0.46^{b}	360.22 ± 24.14^{b}	12.51 ± 2.29^{a}	3.4 ± 1.2^{a}	216.6 ± 0.5^{b}
Alkaline/B	67.44 ± 5.02^{b}	70.22 ± 6.03^{b}	3.64 ± 1.80^{b}	595.92 ± 95.73^{c}	10.760 ± 0.72^{a}	1.6 ± 0.2^{a}	298.5 ± 1.6^{d}
Calcium ion/C	75.36 ± 3.91^{c}	85.85 ± 4.05^{c}	2.30 ± 1.14^{a}	494.56 ± 30.14^{c}	10.83 ± 2.29^{a}	3.9 ± 0.9^{a}	221.1 ± 1.3^{c}
Water Washing/ W	40.69 ± 5.81^{a}	51.52 ± 5.34^{a}	2.59 ± 1.67^{a}	141.05 ± 31.77^{a}	9.52 ± 0.86^{a}	19.2 ± 7.5^{b}	70.6 ± 2.6^{a}

Within the same column, different letters indicates a significant difference ($P < 0.05$).

determined using SDS-PAGE according to the method of Laemmli (1970). After getting the images of protein patterns, ImageJ v1.47 software was used to analyse the bands intensity.

Prior to protein morphology study using atomic force microscopy (AFM), each myofibrillar protein solution was diluted 20 times to avoid protein overlap and 20 μL of diluted solution was pipetted onto a freshly peeled mica sheet attached to a magnetic specimen disc. Each sample disc was air dried at room temperature overnight before scanning. Scanning was performed using a TT-AFM atomic force microscope (AFM Workshop, Signal Hill, CA, USA) equipped with a Sensaprobe TM190-A-15 tip (Applied Nanostructures, Mountain View, CA, USA) with resonance frequencies of 145–230 KHz, force constants of 25–95 N/m, and a Z scanner around 0.2–0.4 Hz; the mode was selected as vibration mode. The morphology of the myofibrillar proteins was analysed using the Gwyddion software (Sow, Chong, Liao, & Yang, 2018).

Changes occurring in the protein secondary structure were examined by Fourier transform infrared spectroscopy (FTIR). Spectra between 450 and 4500 cm^{-1}, with a resolution of 4 cm^{-1} and scan number of 32, were recorded using a Spectrum One FTIR spectrometer (PerkinElmer, Waltham, MA, USA). Samples were free dried and grinded with KBr with at least five repetitions performed in scanning. The spectra in the amide I region (1600–1700 cm^{-1}) was deconvoluted by software Origin Pro 9 (OriginLab, Northampton, MA, USA) using Gaussian curve fitting function. The final content of each protein structure component was measured by the area of the component peak divided by the overall area of the amide I region after deconvolution.

2.6. Rheological behaviour

About 0.5 g of protein isolate sample was placed under a 25-mm-diameter parallel plate on an Anton Paar MCR-102 (Anton Paar, Ashland, VA, USA) stress controlled rheometer. A temperature sweep was performed from 5 to 90 °C with a 2 °C/min heating rate, 1% strain, and 0.1 Hz frequency. Prior to heating, the exposed composites of the rheometer sample plate were sealed with silicone oil to prevent contact of sample and the surroundings. The gap between the plate and sample holder was fixed at 0.5 mm. The storage modulus (G′) and loss modulus (G″) were recorded.

2.7. Statistical analysis

At least three samples were prepared for each experiment, and the experiment was conducted in triplicate. Data were statistically analysed by 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) to assess the effects of different treatments on tilapia muscle protein isolates and surimi gels. Additionally, differences with a P value ≤ 0.05 were considered significant.

3. Results and discussion

3.1. Effects of processing conditions on biochemical properties of tilapia surimi gels

As shown in Table 1, the pH shift method generally resulted in a higher protein yield than the conventional washing method. Myofibril proteins are pH sensitive and could be precipitated at the isoelectrical point (pI), therefore, more proteins were collected, thus increasing the concentration of myofibril proteins (Marmon et al., 2012). According to previous studies (Panpipat & Chaijan, 2016; Yongsawatdigul & Park, 2004), the acid method usually shows a higher yield than the alkaline method. In the present study, the protein yield of C1 was 75.36%. Lipid reduction was 85.85% which was the highest among all the gels. This high lipid reduction efficiency could be attributed to the polycarboxylic groups from citric acid, which could interact with the membrane phospholipids and help to remove them from the protein isolates during the centrifugation steps (Liang & Hultin, 2005).

3.2. Effects of calcium chloride and citric acid addition on functional and physicochemical properties of tilapia surimi gels

In addition to the high yield and lipid reduction, the highest water holding capacity (WHC) was obtained by sample C2, as presented in Table 1. The lower the expressible moisture, the higher the WHC. These factors are usually used to evaluate protein-water interactions and the gel matrix structure (Zhang et al., 2018). The expressible moisture of C2 was 2.30%, which suggested a more organised gel matrix and more water entrapped. Surimi gels with better ordered networking could restrict water movement while less well-ordered gels could not (Buamard, Benjakul, & Konno, 2017). WHC is commonly deemed as an essential parameter to evaluate surimi gels mouthfeel; therefore, calcium ions enhanced the quality of the gels.

In the case of breaking force of gels, Galvez-Rongel et al. (2014) reasoned that there might be a better protein–protein interaction within the sol–gel transition during alkaline processing. Comparing with alkaline method processed gels, more conformational changes occurred during the acid method process, and myosin were possibly dissociated in a greater extend from the original compact structure of myofibril proteins, which could also lower the breaking force of the acid method-processed gels (Chomnawang & Yongsawatdigul, 2013), thus alkaline method-processed gels usually produce surimi gels with better functional and physicochemical qualities. However, the significant increment in breaking force from 360.22 g in A2 to 494.56 g in C2 demonstrated the importance of calcium ions in mediating the gel formation procedure to yield gels with enhanced quality.

According to Table 1, all pH shift extracted protein isolates were found to have low water-soluble protein and high salt-soluble protein concentration. Since only the pH sensitive proteins precipitated, the remaining water-soluble proteins were discarded during the pH shift procedure. Among all water or salt-soluble proteins, mainly myofibril

Table 2
Concentration changes of -SH groups and ionic bonds in protein isolates and surimi gels under different processing conditions.

	Conc. of -SH groups (10^{-5} mol/g)	Conc. of surface reactive -SH groups (10^{-5} mol/g)	Decrease in conc. of -SH groups/ reactive -SH groups (10^{-5} mol/g)	Conc. of ionic bonds (mg/mL)	Ca^{2+}-ATPase activity ($\times 10^{-1}$ μmol/min/mg)
F	1.05 ± 0.12^{e}	0.86 ± 0.08$^{c, d}$	–	1.34 ± 0.26^{b}	4.40 ± 0.25^{d}
A1	0.82 ± 0.05^{d}	0.77 ± 0.03^{c}	–	1.66 ± 0.52^{b}	3.80 ± 0.07^{c}
B1	1.03 ± 0.10^{e}	0.83 ± 0.15$^{c, d}$	–	0.92 ± 0.42$^{a, b}$	2.89 ± 0.47^{b}
C1	1.02 ± 0.07^{e}	0.93 ± 0.12^{d}	–	2.82 ± 0.68^{c}	1.96 ± 0.08^{a}
W1	0.55 ± 0.07^{c}	0.48 ± 0.03^{b}	–	5.45 ± 0.87^{d}	2.96 ± 0.10^{b}
A2	0.45 ± 0.02$^{b, c}$	0.41 ± 0.05$^{a, b}$	0.37/0.36	0.55 ± 0.20$^{a, b}$	–
B2	0.39 ± 0.04$^{a, b}$	0.32 ± 0.04^{a}	0.71/0.51	0.28 ± 0.04^{a}	–
C2	0.32 ± 0.04^{a}	0.27 ± 0.06^{a}	0.70/0.66	0.57 ± 0.08$^{a, b}$	–
W2	0.27 ± 0.04^{a}	0.26 ± 0.09^{a}	0.28/0.22	0.73 ± 0.15$^{a, b}$	–

Within the same column, different letters indicates a significant difference ($P < 0.05$).
F: fresh fish muscle; A1-W1: protein isolates before the heating process; A2-W2: surimi gels.

proteins are precipitated at pH 5.5, and it's commonly agreed that myofibril proteins are salt-soluble proteins (Yongsawatdigul & Park, 2004), thus they show low solubility in water and high solubility in high salt solution. In the case of water washing protein isolates, most of the water-soluble proteins and materials were also discarded during the washing procedure, the remaining portion was mainly constituted of lipids, myofibril proteins and myostromin. Since both lipid and myostromin were neither water nor salt soluble, and myofibril proteins were enriched, therefore the outcome was lower water-soluble protein concentration and higher salt-soluble protein concentration compared with fresh fish meat.

As shown in Table 2, after heat treatment, the concentration of total -SH in C1 to C2 decreased from 1.02×10^{-5} to 0.32×10^{-5} mol/g. The declining trend of both total and surface reactive -SH concentration in group C was quite similar to that in group B. After the heating process, the decrease in the surface reactive -SH concentration in group C was 0.66×10^{-5} mol/g, which was the largest changes among all the groups indicating the most abundant consumption of -SH groups. The decrease in sulfhydryl concentration is believed to be caused by the formation of disulphide bonds, because of oxidation of two cysteine molecules or disulphide bond interchanges (Vate & Benjakul, 2016). The formation of disulphide bonds makes a large contribution to the texture properties (Yin & Park, 2014); however, disulphide bonds are limited in acid conditions (Yongsawatdigul & Park, 2004), thus the relatively small decrease in the concentration of -SH groups shown in the A groups suggested reduced formation of disulphide bonds, resulting in lower gel strength.

However, the concentration of surface reactive -SH groups was higher in C1 than in A1, indicating the effect of calcium ions in altering the protein structures to a greater extent, to expose more buried -SH groups. In addition, the degree of hydrolysis was the highest, as shown in the protein patterns revealed by SDS-PAGE (Fig. S1), indicating that more -SH groups could be exposed to form disulphide bonds with the help of Ca^{2+}.

Another interesting phenomenon observed in C1 was the relatively high concentration of ionic bonds. As shown in Table 2, C1 showed an ionic bond concentration of 2.28 mg/mL, which was almost twice that of the fresh fish muscles, and was also higher than that for A1 (1.66 mg/mL). Presumably, bonds appeared among Ca^{2+} and other functional groups before heat treatment. One possible bonding was between Ca^{2+} and carboxylic groups residing at the end of the myofibril proteins. After adjusting the pH value back to pI, the overall net charge of the proteins/amino acids should be 0, i.e., the charge state of the two major functional groups in proteins/amino acids should be $-COO^{-}$ and $-NH_4{}^{+}$. Therefore, Ca^{2+} would be able to bind to the negatively charged $-COO^{-}$ and contribute to the increased ionic bonds content. However, the loss of ionic bonds occurred after the heat treatment. The bond energy of ionic bonds is much weaker than that of disulphide bonds, which are covalent bonds. Notably, the concentration of ionic bonds in A2 was quite the same as that in C2, while no external positively charged ions were added to A1. Therefore, we postulated that the ionic bonds in the C group formed by Ca^{2+} and $-COO^{-}$ were weaker than those in the A group gels, because the diameter of Ca^{2+} was much larger than any other cations present in the A group gels, while some of the ionic bonding in A1 could be safely preserved through the heating process.

By contrast, the highest concentration of ionic bonds was actually found in W1 and W2. During the processing procedure of water washing, proteins were not purified or concentrated, and numerous impurities remained in the mixture; thus, it was reasonable that these samples would contain the highest ionic bond content.

After pH shifting, the Ca^{2+}-ATPase activity pH shift processed protein was relatively lowered than that of proteins from fresh fish and water washed protein. It indicated the loss of protein integrity and functionality but proteins were still able to form gels after processing. Even though the content of -SH groups can be an indication of protein unfolding, Ca^{2+}-ATPase is also useful in examining the integrity of protein. The decrease in Ca^{2+}-ATPase activity usually indicates the destruction of myosin S-1 structure (Vate & Benjakul, 2016). According to Vate and Benjakul (2016), all Ca^{2+}-ATPase activity was lost at high temperature, thus only protein isolates were tested. The highest reactivity was found to be 0.440 μmol/min/mg in the raw fish meat and the lowest was 0.196 μmol/min/mg in C1. It was typical that processed samples showed lower activity than the original samples due to the external turbulence caused by the processing method (Panpipat & Chaijan, 2016). Ishizaki, Tanaka, Takai, and Taguchi (1995) had shown that the unfolding of myosin subfragment-1 (S-1) exposed hydrophobic groups, with a concomitant loss of solubility. Similarly, Riebroy, Benjakul, Visessanguan, Erikson, and Rustad (2008) found that a low Ca^{2+}-ATPase activity might lead to protein aggregation and decreased solubility. Our results showed that the solubility of C1 water-soluble protein was significantly lowered, and the highest amount of surface reactive -SH groups was found in C1 all agreed with the low Ca^{2+}-ATPase activity of C1 and the fact that combination of pH shift and addition of Ca^{2+} affected the myofibril protein structure in a greater manner.

3.3. Patterns, nanostructure and morphology of proteins

The protein patterns of the protein isolates are shown in Fig. S1(a) and surimi gels are shown in Fig. S1(b). The band intensity on SDS-PAGE gels are shown in Table S2. The most intense bands were myosin heavy chain bands and actin bands, which are the two major proteins in myofibrillar proteins. A comparison of the washed minced flesh and the protein isolates showed that the band intensities of the myosin heavy chain and actin were markedly reduced in the protein isolates, which indicated degradation or hydrolysis after treatment. Yongsawatdigul and Park (2004) reported similar results for rockfish muscle proteins and pacific whiting muscle proteins. When comparing the three pH shift treatments, C1 showed the lowest myosin heavy chain band intensity

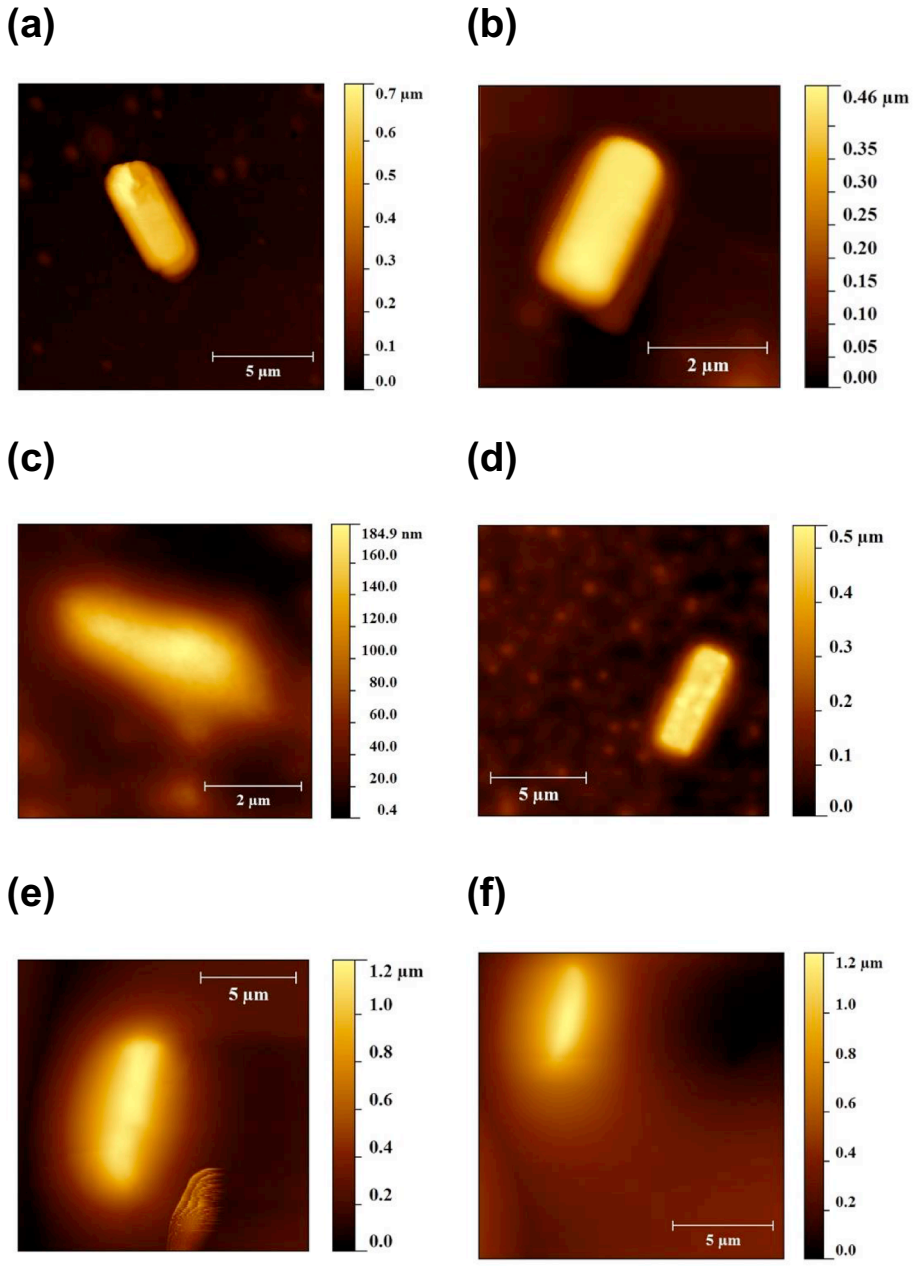

Fig. 1. Microstructures of myofibrillar proteins extracted from protein isolates and surimi gel samples. (a)–(f) represent the top view of proteins from F, A1, B1, C1, W1 and A2; (g), (i) and (k) represent the top view of proteins from B2, C2 and W2, (h), (j) and (l) represent the 3D view of proteins from B2, C2 and W2. F: raw tilapia muscle protein before any treatment or heating process; A1: acid-treated protein isolate; B1: alkaline-protein isolate; C1: calcium ion mediated acid-treated protein isolate; W1: conventional water washing-treated protein isolate; A2: acid -treated surimi gel; B2: alkaline-surimi gel; C2: calcium ion mediated acid-treated surimi gel; W2: conventional water washing-treated surimi gel.

(8.99%) followed by A1 (9.56%), indicating higher degradation or hydrolysis caused by calcium ions. The least degradation was found in the water washing method. Buamard et al. (2017) observed that at in acid conditions, cathepsin L showed higher activity, which could lead to increased degradation degree using the two acid methods.

After heat treatment, all proteins showed high molecular weight protein (HMWP) bands at the top of the gel. The formation of HMWPs indicates gel matrix or aggregates formation. Therefore, with the lowest HMWP band intensity, A2 should contain the lowest content of matrix or aggregates, which was confirmed by the AFM images.

The most concentrated proteins aggregated in the surimi gel matrices are myofibril proteins, therefore, the morphology of the myofibril protein microstructure plays a crucial part in affecting the macro attributes. To clarify these macro changes, AFM was introduced as a state-of-the-art tool to study the morphology of the myofibrillar proteins. AFM has been widely applied to image various biomolecules, such as polysaccharides or proteins (Feng, Bansal, & Yang, 2016). After obtaining more than 5 images for all protein samples, the clearest examples of scanning images of each sample via AFM are shown in Fig. 1. Image (a)–(i) are the top view of proteins and (j)–(r) represent the 3D view of the proteins. Quantitative analysis, including the length, width, and height of the myofibrillar proteins is shown in Table S1.

Fresh tilapia fish muscle proteins as shown in Fig. 1(a) and (j), without any processing procedure, exhibited a rod-like structure, which was in consistent with previous findings (Feng, Zhu, Liu, Lai, & Yang, 2017). The length, width, and height of the F group proteins (Table S1)

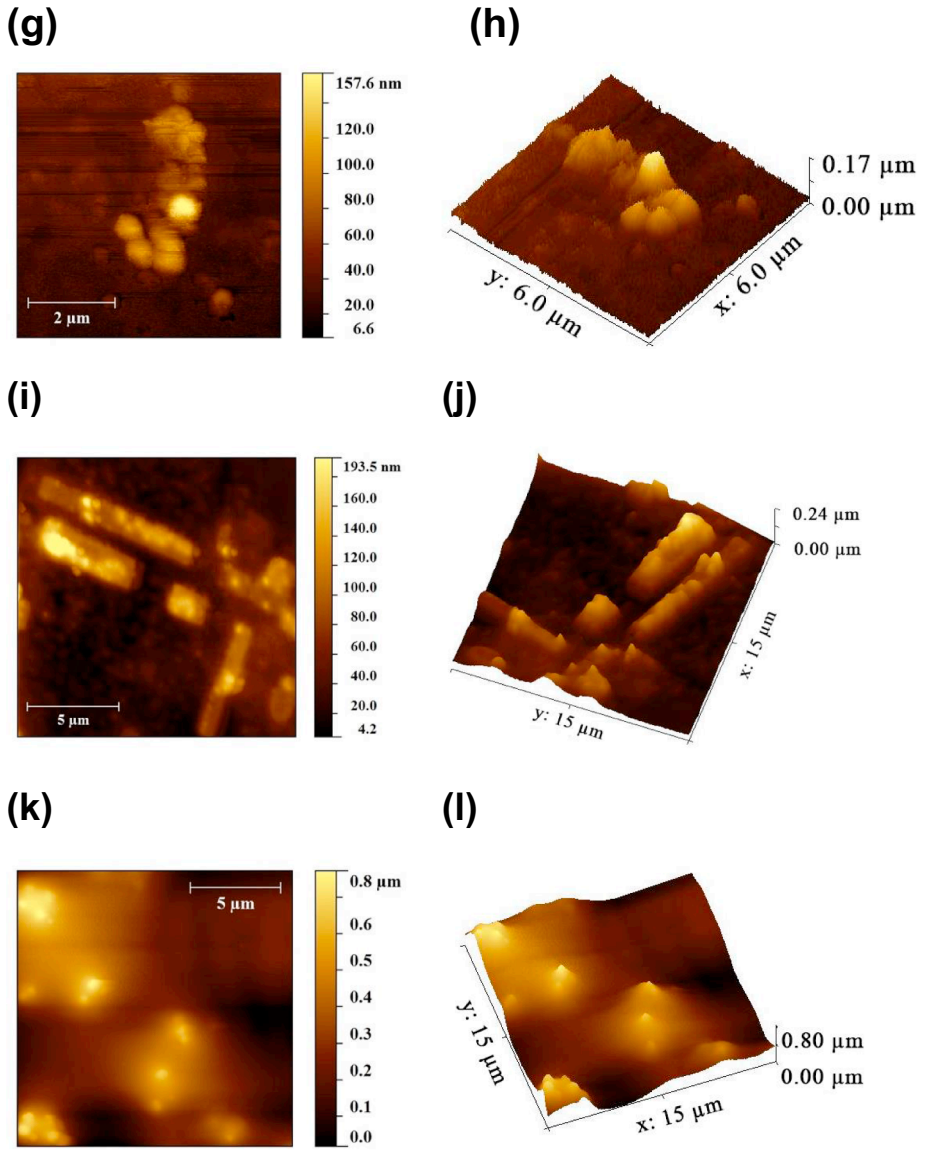

Fig. 1. (*continued*)

Table 3
The effects of different treatment on content of secondary structures in protein isolates and surimi gels.

	α- helix (%)	β- sheet (%)	β- turn (%)	Disordered coil (%)
F	57.29 ± 3.65$^{b, c}$	32.95 ± 3.87$^{a, b}$	8.99 ± 1.35^{b}	0.77 ± 1.37^{a}
A1	53.57 ± 2.44$^{b, c}$	29.33 ± 1.53^{a}	8.53 ± 1.92^{b}	8.24 ± 1.02$^{b, c, d, e}$
B1	55.62 ± 4.58$^{b, c}$	29.12 ± 0.58^{a}	5.87 ± 0.18$^{a, b}$	9.40 ± 4.99$^{c, d, e}$
C1	58.71 ± 2.14$^{b, c}$	30.99 ± 4.00$^{a, b}$	4.57 ± 0.56^{a}	5.74 ± 1.30$^{a, b, c, d}$
W1	56.13 ± 2.23$^{b, c}$	32.60 ± 3.71$^{a, b}$	7.05 ± 0.55$^{a, b}$	4.23 ± 2.04$^{a, b, c}$
A2	51.26 ± 0.13^{b}	31.23 ± 6.28$^{a, b}$	7.79 ± 0.96$^{a, b}$	9.72 ± 5.44$^{d, e}$
B2	52.65 ± 2.83$^{b, c}$	37.45 ± 4.31^{b}	4.50 ± 1.29^{a}	3.92 ± 2.30$^{a, b}$
C2	54.96 ± 2.55$^{b, c}$	29.84 ± 1.04^{a}	6.50 ± 2.34$^{a, b}$	8.41 ± 2.81^{d}
W2	44.80 ± 2.75^{a}	36.18 ± 1.63^{b}	7.76 ± 2.17$^{a, b}$	11.26 ± 0.87^{e}

Within the same column, different letters indicates a significant difference ($P < 0.05$).
F: fresh fish muscle; A1-W1: protein isolates before the heating process; A2-W2: surimi gels.

were 6.29 μm, 3.14 μm, and 602.07 nm, respectively. Compared with the control, the length, width, and height of the A1 and B1 proteins were lower. For C1 proteins, the length and width were greater, but the height was lower. For the W1 proteins, the increase in length and height was significant. The addition of calcium flattened the height to 441.70 nm and enlarged the protein structure at the same time (the length increased to 7.99 μm, and the width increased to 4.16 μm), while A1 proteins were not only flattened, but also shortened to 3.47 μm, and narrowed down to 2.24 μm. These findings suggested that the protein structures were more disturbed or disrupted by the addition of calcium chloride, and even though the pH value was adjusted back to the pI, it was harder, or even impossible, for the proteins to re-fold back to the original state.

Another interesting finding was that after heat treatment, B2 and W2 lost all their rod-like structure, only matrices/aggregates and spherical structures were found. In addition, there were gel matrices found in B2 and C2, and granulation found in W2. The morphology of A2 and C2 remained rod-like structure; however, the proteins in C2 were more randomly distributed in all three dimensions. Although no matrix was found in A2, there was also no aggregation, indicating that there were some heat resistant interactions within the A2 gels.

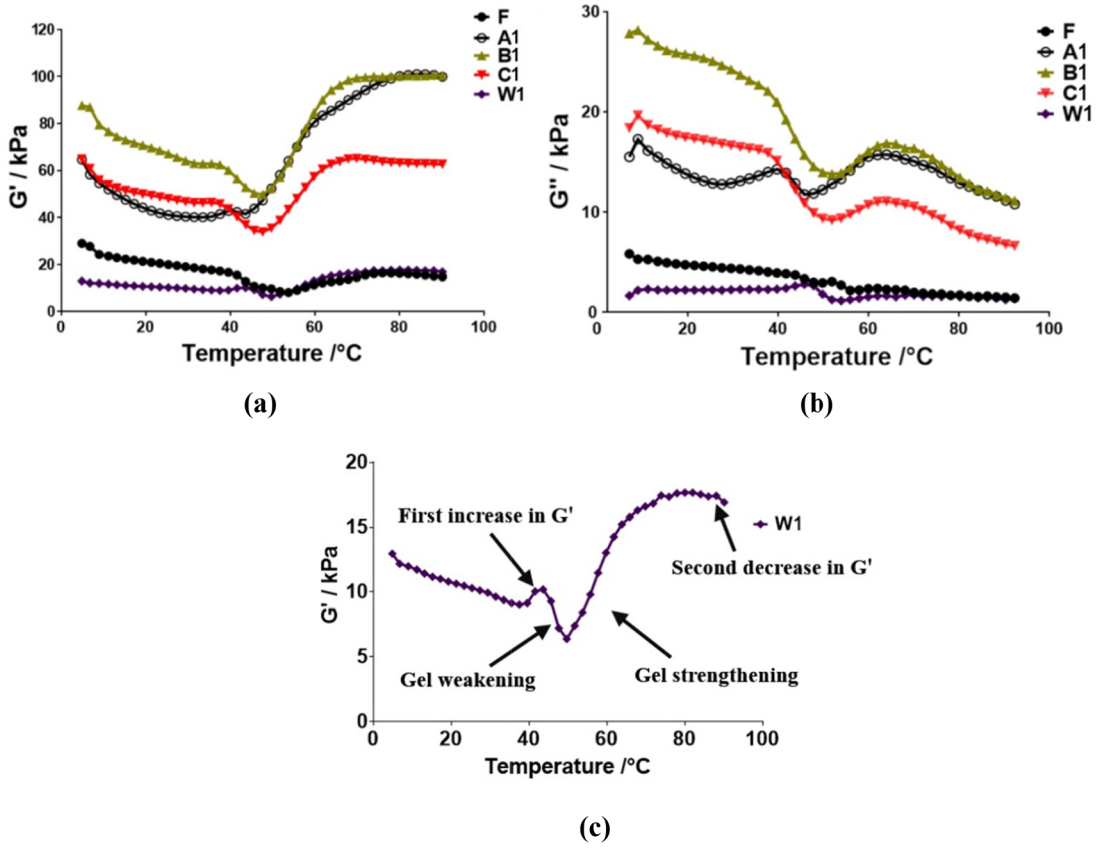

Fig. 2. Rheological analysis of fresh muscles and protein isolates. (a) and (b): Rheological behaviour measurements of thermal gelation profiles from 5 to 90 °C for fresh fish muscle (F) and protein isolates prior to heating process (A1 – W1). (c): Rheological behaviour of W1 under the same experimental conditions.

Similarly, one could speculate that the acid processing conditions preserved the morphology of the proteins well, because both the A2 and C2 (Fig. 1(f) and (h)) group maintained a rod-like structure after the heating process, whereas B2 and W2 (Fig. 1(g) and (i)) didn't. Protein aggregation after heating was also found in a study by Vate and Benjakul (2016). Another possible explanation for these phenomena found in B2 was the existence of the 'molten globule' state as a protein configuration at low or high pH. Upon pH shifting, the conformation of proteins could be greatly altered. By dissolving and reprecipitation, proteins would be unfolded and refolded. In most cases, the refolding could not recover the proteins back to the initial state, and the alkaline method usually resulted in lower recovery (Kristinsson & Hultin, 2003).

As the basic "building blocks" of the gel matrix, the morphological changes of proteins are closely related to the macro structural changes. As shown in Table 1, the highest gel strength was 595.92 g obtained by B group gels, followed by C group gels as 494.56 g. For granules or spherical structure proteins with small width and height, the network could be compact and stable, resulted in higher gel strength. In addition, the formation of disulphide bonds was found mostly in B2 gels (0.71 mol/10^5 g) followed by C2 gels (0.70 mol/10^5 g). With their lower heights and narrower width, the arrangement of the proteins could be compact enough to resist the external breaking force. There have been reports concerning whey proteins and tofu making, in which the addition of calcium ions could induce coagulation via the cross-links between anionic groups originating from protein molecules and calcium ions, thus enhancing the gel strength (Arii & Takenaka, 2014).

3.4. Changes in the secondary structure of proteins

As a robust, non-destructive analytical approach, FTIR has been employed extensively to examine protein secondary structure changes (Cando, Herranz, Borderías, & Moreno, 2016; Kobayashi et al., 2017). Amide I region (1600–1700 cm^{-1}) is mainly examined for secondary structure estimation, based on the assumption that only structures including α-helices, β-sheets, β-turns, and random coils are considered as a linear sum of the protein structure (Cando, Moreno, Tovar, Herranz, & Borderias, 2014). The four structures mentioned above were assigned according to various literature reports (Cando, Herranz, Borderías, & Moreno, 2015; Kobayashi et al., 2017; Sow et al., 2017) and presented in Table 3.

Compared with the original protein secondary structure (F), only C1 showed a higher helix content and most samples showed lower sheet content. The washing method, as a mild procedure, does not affect the protein secondary structure thus W1 showed no significant differences compared with the F. As mentioned before, alkaline conditions usually lead to a lower recovery of original protein structures (Kristinsson & Hultin, 2003); indeed, B1 proteins showed a much higher disordered coil content while the morphology was less rectangular-shaped compared with the other proteins. C1 formed more α-helices at the expense of β-sheets; however, C1 showed lower β-turn content (4.54%) than A1 (8.53%). We postulated that the addition of calcium caused the refolding of proteins to a greater extend and less helices unwound into β-turn structure.

Heat treatment lowered the α-helix content in all surimi gels. Generally, a higher sheet content results in the formation of a more organised gel matrix (Cando et al., 2014); thus, B2 showed the highest gel strength with its highest sheet content. However, A2 and C2 treatment showed no significant differences in the content of both helices or sheets structures, while C2 showed a better WHC and gel strength. Apart from secondary structure, disulphide bonds contribute most to the tertiary structure. In addition, the morphology has crucial influence on the macro attributes. More disulphide bonds were formed in the C2 gels, and the "building blocks" were smaller than those in the A2 gels; therefore, an organised gel could still be formed to better express the WHC and gel strength.

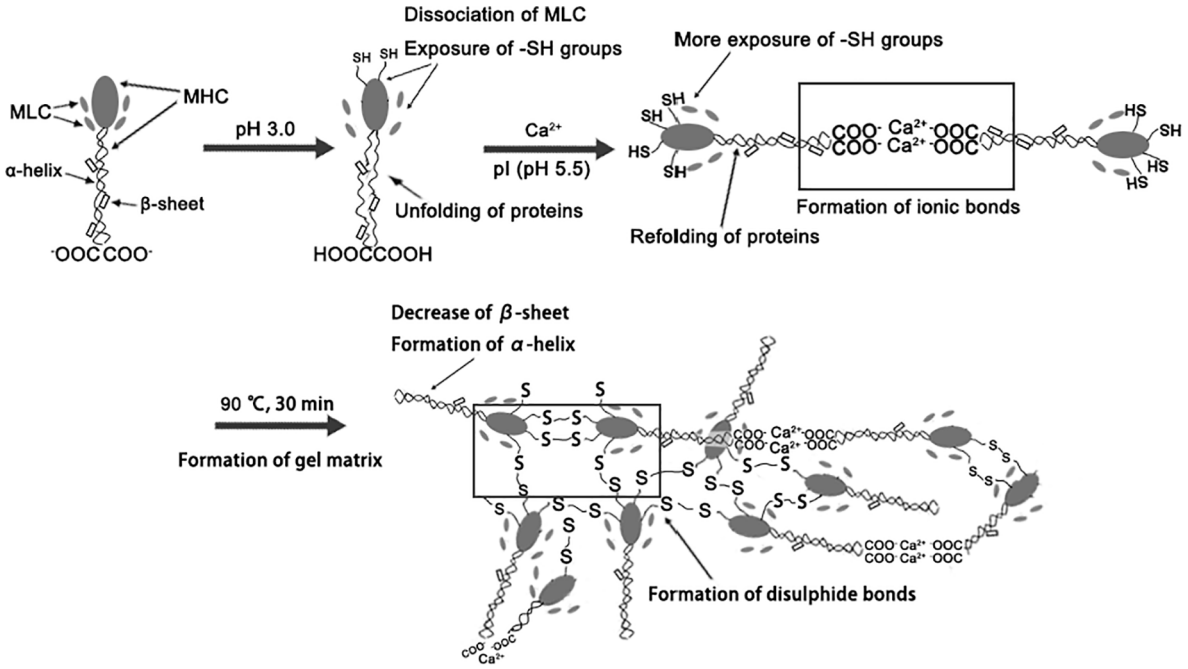

Fig. 3. Schematic mechanism of the protein gelation in surimi with added calcium ions.

3.5. Rheological behaviour of proteins

The rheological behaviour of all protein isolates before heating treatment (A1–W1), and fresh fish muscle as a control (F), are shown in Fig. 2(a) and (b), with both storage modulus (G′) and loss modulus (G″) recorded. With a view to providing more detailed behaviour information, Fig. 2(c) showed only the rheological curve of W1. In general, G′ represents the energy stored as mechanical energy after applying a deforming force (mostly the elastic portion), while G″ measures the energy dissipated as heat, mostly representing the viscous portion (Zhang, Yang, Zhou, Zhang, & Wang, 2017). The more pronounced change in G′ and G″ in proteins that underwent the pH shift process could be translated as a more rigid gel system.

According to previous studies (Cando et al., 2016; Chen and Huang, 2008; Yin and Park, 2015), the gelling process of surimi gels usually undergoes four stages: (1) an increase in between 20 and 36 °C; (2) a decrease in G′ around 40–45 °C as the "gel weakening" state; (3) another increase in G′ as the "gel strengthening" state between 45 and around 70 °C; (4) a second decrease in G′ after 70 °C. However, these four stages were fully observed in W1, while the other three pH shift methods only showed the final three stages clearly, with much higher G′ and G″ values. Yongsawatdigul and Park (2004) reported the same trend in rockfish muscle treated with the pH shift method: the first stage was indistinct while the other three stages were clearer. In the early phases, A1 proteins showed that increments of G′ and G″ happened at a lower temperature compared with those in B1 and C1, indicting the aggregation of proteins. A less stable myofibril protein structure caused by the denaturation of proteins might have occurred during acidic conditioning process and caused aggregation of proteins at low temperature (Yongsawatdigul & Park, 2004). Aggregation rather than matrix formation via intermolecular bonding is detrimental to the properties of gels, which was proved by the low gel strength of A2. Although the final G′ and G″ values were the same as the B1 proteins, the protein network was poorly organised. Interestingly, the trends of B1 and C1 were similar, indicating the role of the calcium ions in mediating the gel matrix formation. They both showed the gel weakening and strengthening starting at 39 °C and 54 °C, which was higher than that for the A1 proteins. It allowed sufficient time for the proteins to network in a more organised manner rather than simply aggregation because of the temperature elevation. Notably, C1 showed higher G' and G" values than A1 between 5 and 35 °C, which could be an evidence for the formation of intermolecular "salt bridges" by Ca^{2+} between partially dissolved and unfolded proteins (Yin & Park, 2014). For the fresh fish muscle, no sharp peak, or obvious increment or decline were observed, indicating the low concentration of myofibril proteins in the fresh fish muscle and bad gelling behaviour.

3.6. Schematic mechanism of protein gelation with calcium ions

The schematic model is shown in Fig. 3. The original protein structure is mainly composed of myosin heavy chain (MHC) and myosin light chain (MLC) (Kristinsson & Hultin, 2003). The helices and sheets structure mainly reside in MHC. After adjusting the pH to 3.0, proteins were positively charged, thus $-COOH$ and $-NH_4^+$ will exist simultaneously. In addition, low pH could lead to the dissociation of MLC and exposure of -SH groups. The added calcium chloride would then hydrolyse the proteins further, exposing more -SH groups. After adjusting the pH to isoelectric point (5.5), the proteins refolded and would be neutrally charged. Ionic bond formation would then occur because a net charge zero requires both the existence of $-COO^-$ and $-NH_4^+$, and ionic bonds formed between Ca^{2+} and $-COO^-$, which agrees with the observation of Sow et al. (2017). The subsequent heat treatment induces the gelation of proteins and the formation of matrices. Disulphide bonds formed, while some ionic bonds were broken. During this step, the number of α-helices increases at the expense of β-sheets. The final gel matrix is held together mainly by disulphide bonds, whilst retaining certain ionic bonds content.

4. Conclusion

pH shift methods in protein isolation and surimi gel preparation have advantages, including higher yields, better lipid removal, and better gel strength compared with the conventional washing method. Introducing calcium ions and citric acid into the processing procedure resulted in smaller proteins (as shown by AFM), a better lipid reduction rate (85.85%), better WHC (2.30%), and higher gel strength (494.56 g). Smaller protein structure and the formation of disulphide bonds and ionic bonds are main factors that affect the macro attributes of gels.

Overall, the results demonstrated that the two additives could greatly enhance the processing of surimi gels, and would contribute to meeting the higher demand for aquatic products in Southeast Asia.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.10.110.

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