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Biochemical Properties of Myofibrillar Protein and Gel Characteristics of Surimi from Rohu (*Labeo rohita*) as Affected by Frozen Storage

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ABSTRACT

The effects of frozen storage at -18ºC on the biochemical properties of myofibrillar protein (MF) extracted from rohu (*Labeo rohita*) surimi, and the gel characteristics of surimi were investigated. Biochemical changes were determined by the Ca^{2+}-ATPase activity, total and reactive sulfhydryl (SH) content, and SDS-PAGE patterns. The gel properties were determined by gel strength, water holding capacity (WHC), whiteness, and from observations of the SDS-PAGE patterns and microstructure. Extended frozen storage was found to affect myosin. This was reflected in Ca^{2+}-ATPase activity, reactive SH content, and myosin heavy chain intensity, which also decreased as the storage period was extended. This was confirmed by the decreasing of gel strength and WHC, the degradation of myosin heavy chain, and the formation of large protein clusters. The whiteness increased as the frozen storage time increased. This suggested that extended storage at -18ºC caused myosin denaturation, which in turn lead to a change of gel properties.

Keywords: Ca^{2+}-ATPase activity; Frozen storage; Myofibrillar protein; Rohu (*Labeo rohita*); Sulfhydryl groups; Surimi

1. Introduction

Rohu (*Labeo rohita*) is a freshwater fish species that is widely cultured in Thailand. In 2010, Thailand produced 1167 tons of rohu, with a market value of 1.3 million US dollars [1]. The decrease in marine fishery resources is affecting mince/surimi production. Many studies have attempted to utilize freshwater fish as an alternative resource including Nile tilapia

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(*Oreochromis niloticus*) [2], red tilapia (*Oreochromis niloticus* × *Oreochromis placidus*) [3], common carp (*Cyprinus carpio*), and small-scale mud carp (*Cirrhina microlepsis*) [4]. However, the use of Rohu in surimi had not yet been reported.

Surimi can be defined as a concentrated myofibrillar protein obtained from mechanically deboned fish flesh, which has been washed with water to remove water-soluble proteins, especially sarcoplasmic protein, blood, and enzymes [5]. Myofibrillar protein (MF), comprising 55-60% of total protein in muscle, plays an important role in the functional properties of muscle tissue, including gelation and water binding [6].

Freezing, an effective means of long-term preservation, is used to reduce the deterioration caused by microorganisms in surimi. However, MF deterioration has been reported during frozen storage. Changes in the intramolecular conformation and intermolecular aggregation of myosin occurs through the denaturation of MF, leading to the loss of functional properties such as gel-formation and water holding capacity (WHC) [7-9]. Frozen storage decreased the gel-forming ability of surimi from croaker (*Pennahai macrophthalmus*), lizardfish (*Sauruda micropectorails*), threadfin bream (*Nemipterus bleekeri*), bigeye snapper (*Priacanthus tayenus*) [10], and grass carp (*Ctenopharyngodon idellus*) [11], and MF from bighead carp surimi (*Aristichthys nobilis*) [12].

To explore the influence of frozen storage on myofibrillar protein and gel properties, in this study, biochemical properties of MF and gel characteristics of surimi from rohu kept at -18ºC for 6 months were investigated.

2. Materials and Methods

2.1 Materials

Rohu (*Labeo rohita*), weighing 1000±100 g each, were purchased from Ying Charoen Market (Bangkok, Thailand). Fish were packed in an ice box and transported to the laboratory within 90 min.

Trichloroacetic acid (TCA) was purchased from QRëC (New Zealand). Bovine serum albumin (BSA) and 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (USA). Maleic acid and calcium chloride (CaCl2) were purchased from Thermo Fisher Scientific Ltd. (Australia), Adenosine 5'-triphosphate sodium salt (ATP), Hexa-ammonium Heptamolybdate Tetrahydrate, and p-methylamino phenol sulfate (Elon solution) were purchased from Wako Pure Industries Ltd. (Japan). Reagents used for gel electrophoresis were purchased from Bio-Rad (USA).

2.2 Preparation of mince

Fish were gutted and washed with tap water. The fillet was minced using a meat grinder (2 mm hole diameter; Kenwood, AT950A, England). Mince was prepared following the method of Sutloet et al. [13].

2.3 Preparation of surimi

Sucrose (2.5%) (w/w), sorbitol (2.5%), sodium pyrophosphate (0.1%), and sodium tripolyphosphate (0.1%) were added to the mince during grinding. The mixture was then formed into a block (13.5 x 13.5 x 4 cm) with an approximate weight of 800 g. All surimi samples were rapidly cryogenically frozen in a cryogenic freezer (Minibatch Freezer, Thailand). Core temperatures were promptly reduced to below -23°C and maintained at -18±2°C for a six-month period.

2.4 Preparation of myofibrillar protein

The frozen surimi was thawed at 4±2ºC overnight and MF was prepared following the method of Sompongse et al. [14]. The protein concentration in the supernatant was determined following the method of Lowry et al. [15]. The MF was diluted to 2.5-4 mg/ml with 20 mM Tris HCl (pH 7.5) containing 3.0 M NaCl and 20 mM Tris-HCl (pH 7.5). Then, the mixture was

centrifuged at 10,000x for 30 min at 4ºC. The diluted MF was kept in an ice box for analysis. The initial value was taken from a sample kept at -18±2ºC for only 12 h. This is shown as month 0. Unfrozen control was prepared from unfrozen surimi and was held as control for analyzing the protein pattern.

2.5 Determination of biochemical properties of myofibrillar protein

2.5.1 Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity was determined following the method of Sompongse et al. [16]. The inorganic phosphate liberated in the filtrate was measured at 640 nm, following the method of Takashi et al. [17]. Specific activity was expressed as micromoles of inorganic phosphate released/mg protein/min. A blank solution was prepared by adding cold 15% (w/v) TCA prior to the addition of ATP. Measurements were made in triplicate.

2.5.2 Total and reactive SH content

Total and reactive SH content was determined using 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) following the method of Ellman [18] as modified by Sompongse et al. [14]. Briefly, 2 ml of MF solution, 18 ml of 0.2 M Tris-HCl buffer (pH 6.8) containing 8 M urea, 2% SDS, and 10 mM EDTA, were added. To 4 ml of the sample, 0.4 ml of 0.1% DTNB in 0.2 M Tris-HCl, urea-SDS-EDTA (pH 6.8) was added and the mixture was incubated at 40ºC for 25 min. The reactive SH groups were determined in the same manner using 0.2 M Tris-HCl (pH 6.8) containing 10 mM EDTA as buffer, and the reaction was incubated at 15ºC for 60 min. The sample was kept in the ice box throughout the determination. The total and reactive SH groups were determined at 412 nm, calculated using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}, and expressed as mol/10^5 g protein. Measurements were made in triplicate.

2.5.3 Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyze the protein pattern of the samples, following the method of Laemmli [19]. The protein concentration was measured by the method of Lowry et al. [15], using BSA as a standard protein. The MF extracted from frozen surimi was then mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 2% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) β-mercaptoethanol) and then heated at 95ºC for 5 min. The stacking and separating of gels and the analyzing of protein pattern was carried out following the method of Sutloet et al. [13].

2.6 Determination of gel characteristics

2.6.1 Textural properties

Gel samples were allowed to reach room temperature (approximately 30ºC). Five cylinder-shaped samples 25 mm in length and diameter were prepared from each gel. Textural properties were measured following the method of Sutloet et al. [13].

2.6.2 Expressible water content

The expressible water content was measured following the method of Sutloet et al. [20].

2.6.3 Whiteness

The whiteness of the samples was determined using a colorimeter (HunterLab, ColorFlex CX2687, USA) with D65 illuminant as the light source following the method of Sutloet et al. [20].

2.6.4 Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS - PAGE)

SDS-PAGE was used to analyze the protein pattern of the gel samples using the method of Laemmli (1970). Preparation of solubilized sample, stacking and separating gels and analyzing of protein pattern was carried out following the method of Sutloet et al. [13].

2.6.5 Microstructure

The microstructure of the samples was examined using a scanning electron microscope (JEOL, JSM-IT300, Japan). The specimens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. The specimens were then rinsed twice with phosphate buffer and once with distilled water for 10 min. After that, they were dehydrated in ethanol with a serial concentration of 30%, 50%, 70, 95%, and 100% (v/v) for 10 min. The specimens were dried using a critical point dryer (Leica, EM CPD300, Austria), and were mounted and coated with gold (sputter coater, Bulzers, SCD 040, Germany).

2.6 Statistical analysis

The experiment used a randomized complete block design. Data were subjected to analysis of variance. Duncan's new multiple range test was used to determine the differences between sample means at $P \leq 0.05$. All experiments were done in triplicate.

3. Results and Discussion

3.1 Changes in biochemical properties of MF during frozen storage

3.1.1 Changes in Ca^{2+}-ATPase activity of MF during frozen storage

Fig. 1 shows the remaining Ca^{2+}-ATPase activity of MF. The activity slowly decreased throughout the six months of frozen storage. The activity in the samples remained at an average of 84.1% after three months. Ca^{2+} -ATPase activity provides a good index for the integrity of the myosin molecules [21], as the globular heads of the myosin are responsible for the activity. In the current study, the decline in activity across six months of frozen storage suggested the denaturation of the myosin had taken place mainly in the head region. The decrease in Ca^{2+} -ATPase activity has been reported to be caused by changes in the conformation of the myosin globular head, and the aggregation of this portion [22]. The change in the conformation is due to the generation of ice crystals. As a result, the ionic strength of the system is increased [23]. Rearrangement of the protein via protein-protein interactions is also presumed to contribute to the loss of Ca^{2+} -ATPase activity [23, 24]. The results of this study are in agreement with those of Pan et al. [11], who reported that the Ca^{2+} -ATPase activity of grass carp protein stored at -18ºC with or without cryoprotectants decreased as the storage time increased. The results in this study suggested that the myosin underwent slight denaturation during frozen storage, and demonstrated that the Ca^{2+}-ATPase activity at the sixth month of storage remained above 70% of the initial value after just 12 h of frozen storage.

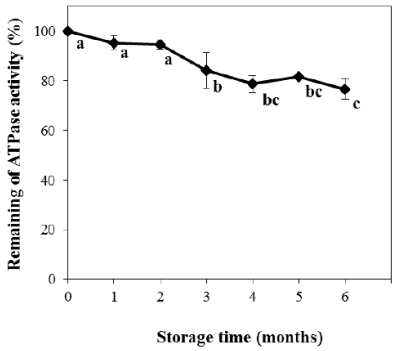

Fig. 1. Changes in Ca^{2+}-ATPase activity of MF extracted from surimi during frozen storage. Points represent the mean. Vertical lines represent the standard deviation. Different letters on each point indicate significant differences ($P \leq 0.05$).

3.1.2 Total and reactive SH content of MF

Fig. 2a shows the changes in total SH content of MF during frozen storage. The total SH content tended to decrease as the storage time increased. A decrease of 14.6% was observed in the first month of storage, after which the total SH content showed a small decrease. The decreases were in line with those reported by Benjakul et al. [21]. They reported decreases in total SH content of natural actomyosin after 24 weeks of frozen storage of 43.2% for threadfin bream (*Nemipterus bleekeri*), 47.8% for bigeye snapper (*Priacanthus tayenus*), 67.3% for lizardfish (*Sauruda micropectoralis*), and

29.6% for croaker (*Pennahai macrophthalmus*), compared with fresh sample. Cao et al. [9] reported that the total SH content of actomyosin from silver carp (*Hypophthalmichthys molitrix*) decreased significantly with extended frozen storage during 91 days of storage ($P \leq 0.05$).

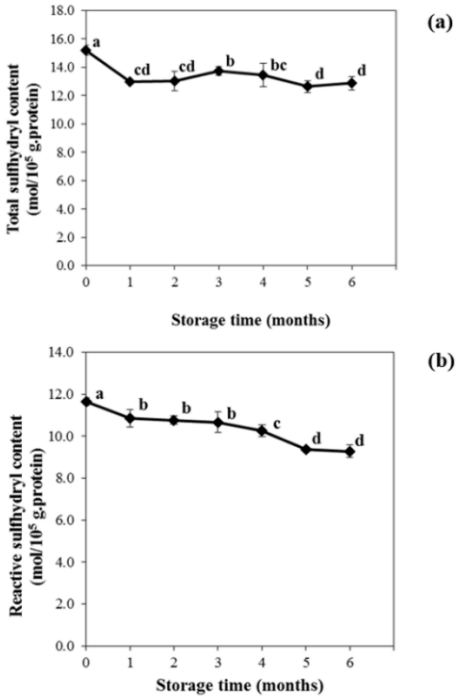

Fig. 2. Changes in SH content of MF extracted from surimi during frozen storage. (a) total SH content and (b) reactive SH content. Points represent the mean. Vertical lines represent the standard deviation. Different letters on each point indicate significant differences ($P \leq 0.05$).

Fig. 2b presents the changes in reactive SH content of MF during frozen storage. The reactive SH content decreased as the frozen storage time increased. The content decreased significantly by the end of the first month of storage (6.8%, $P \leq 0.05$). This trend continued throughout the six months of storage.

SH groups are the most reactive functional group in actomyosin [21, 25]. The decrease in total and reactive SH content has been attributed to the formation of disulfide bonds via oxidation or disulfide interchanges [9, 21]. The continuous decrease of reactive SH groups in samples across the frozen storage period agreed with the findings of Benjakul and Bauer [23], who studied the physicochemical and enzymatic changes in cod muscle protein when subjected to different freeze-thaw cycles. They reported that the changes in tertiary structure became more intense as frozen storage time increased. As a result, the reactive SH groups were more exposed to and oxidized by disulfides, thus decreasing the reactive SH content. The results suggest that frozen storage exposes SH groups, which are then further oxidized to form disulfide bonds [26]. Furthermore, a decrease in SH content has been associated with a decrease in Ca^{2+}-ATPase activity. Buttkus [27] reported that myosin from a freshly killed fish contains 42-43 SH groups per 5 x 10^5 g of protein. SH_1 and SH_2, located in the head portion of the myosin molecule, have been reported to play an important role in ATPase activity [28]. This would suggest that the decrease in Ca^{2+}-ATPase activity was caused by oxidation of the SH groups, especially the groups in the head region. In this experiment, by the sixth month, the total SH content of the MF had declined by approximately 15%. The reactive SH content had reduced by approximately 20%.

3.1.3 Protein pattern of MF

Fig. 3 shows the protein patterns of the MF in the course of frozen storage. The myosin heavy chain (MHC) intensity decreased as the frozen storage time increased. The MHC intensity declined dramatically in the first month (lane 4), then steadily across the remaining five months of frozen storage. The intensity of actin showed no marked change over the course of the frozen storage. The clear decrease in the MHC intensity at the end of the first month was consistent with the significant decrease in total and reactive SH group content (Figs. 2a and b). Benjakul and Sutthipan [29]

reported MHC crosslinking by disulfide and nondisulfide covalent bonds during frozen storage and the formation of high molecular weight polymers and aggregates. The results from this study suggested the reduction in MHC intensity was associated with the total and reactive SH group contents.

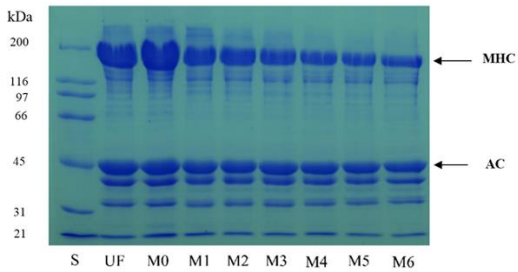

Fig. 3. SDS-PAGE patterns of MF extracted from surimi during frozen storage. MHC: myosin heavy chain; AC: actin. S: standard protein; UF: unfrozen mince control; M0: initial value (after 12 h of frozen storage), M1-M6: Frozen surimi at months 1-6.

3.2 Changes in gel characteristics of surimi during frozen storage

3.2.1 Changes in textural properties of surimi gel during frozen storage

Fig. 4a shows the change in textural properties of the surimi gels throughout frozen storage. The gel strength gradually decreased as the storage period extended. Significant changes were found at the end of the fifth month, with a decrease of 7.3% from the initial value. A continuous decrease in gel strength was observed by the end of the sixth month, with a total decrease of 10.3%. Freezing reduced the gel strength, though no significant change was observed until the end of the fourth month. This suggested that surimi kept at -18ºC maintained its gel strength for four months.

Gel-forming ability is an indicator of fish protein quality [3]. MF, especially myosin, plays an important role in gel formation. Denaturation of protein has an adverse effect on gel formation, inhibiting the development of a three-dimensional network [7, 11]. In this study, the gel strength was found to decrease with frozen storage time. The decline increased as the storage time increased. This suggested that denaturation of the myosin molecule took place, resulting in the formation of an inferior gel network and a decrease in gel strength. Lu et al. [12] argued that the denaturation of protein during frozen storage is due to protein aggregation via disulfide bonding and hydrophobic interactions. Their results supported those of Benjakul et al. [10]. They reported that the breaking force and deformation of surimi from croaker (*Pennahai macrophthalmus*), lizardfish (*Sauruda micropectorails*), threadfin bream (*Nemipterus bleekeri*), and bigeye snapper (*Priacanthus tayenus*) decreased over 24 weeks of frozen storage. Pan et al. [11] reported that the breaking force and deformation of surimi from grass carp (*Ctenopharyngodon idellus*) with or without cryoprotectants decreased as the frozen storage time increased. The present findings suggest that the gel-forming ability of surimi was decreased by frozen storage.

3.2.2 Changes in expressible water content of surimi gel during frozen storage

Fig. 4b shows the change in expressible water content of surimi gel during six months of frozen storage. The expressible water content increased linearly as the frozen storage time increased. By the end of the third month, the content had increased by 20.2%, a significant change ($P \leq 0.05$). No further significant changes were observed over the final three months of storage. Freezing, therefore, caused a significant decrease in the WHC of surimi within the first three months of storage, but no further significant decrease over the remaining months of storage. These expressible water results suggested that gel prepared from the first three months of storage was able to retain more water within its structure than gel prepared from the final three months. The WHC, as shown by the

expressible water content, represents the amount of water retained within the structure of a protein gel network [12]. The increase in expressible water content confirmed the decrease in textural properties. Lu et al. [12] reported that the WHC of MF gels from bighead carp decreased during frozen storage, and suggested that some of the immobile water in the MF gel had become free water. Generally, the changes that take place in fish muscle protein during frozen storage include denaturation, ice crystallization, and intermolecular conformation changes [30-32]. Denaturation of myosin reduces the strength of the formed gel network. A weakened gel network is less able to retain water within its structure. Ice crystal formation causes tissue damage and the leakage of organelles [21]. These current findings confirmed a decrease in the WHC during frozen storage.

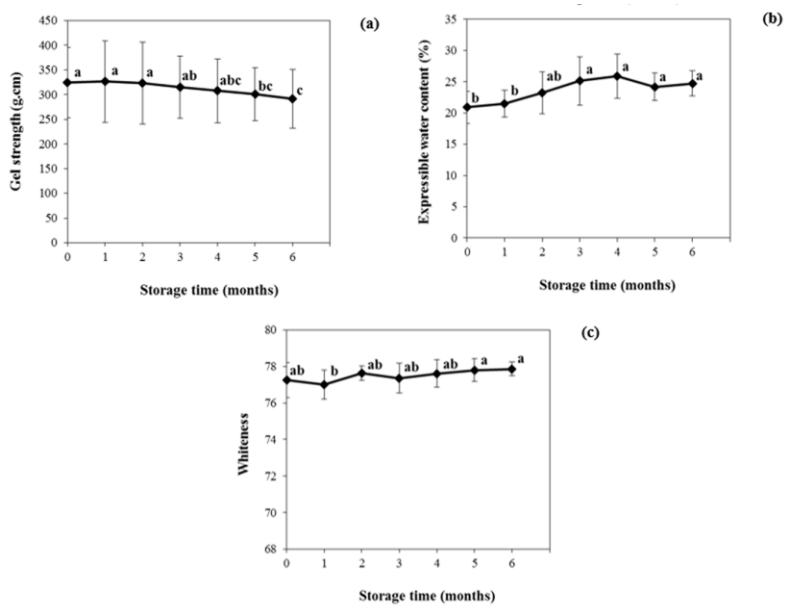

Fig. 4. Changes in gel characteristics of surimi during frozen storage. (a) gel strength (b) expressible water content and (c) whiteness. Lines represent the mean and standard deviation. Different letters on each line indicate significant differences ($P \leq 0.05$).

3.2.3 Changes in whiteness of surimi gel during frozen storage

Fig. 4c shows the change in whiteness during frozen storage. The whiteness of gel increased as the frozen storage time increased. After six months of storage, whiteness had increased by 0.8%. The increase in whiteness was associated with the L* value, and this increased as the frozen storage time increased (data not shown). This may have been due to denaturation of MF that had an adverse effect on gel formation. Therefore, no well-structured three-dimensional protein network emerged. Benjakul et al. [10] reported that gels from frozen fish comprised a coarser network with a large void, compared with fresh fish. In this study, fine-stranded gels from frozen surimi did not form from an ordered association of protein molecules. Instead, the protein network exhibited larger protein clusters than the unfrozen control (Fig. 6). As a result, less light was able to pass through this structure and the L* value increased. This accounted for the increase in whiteness of surimi gel as the storage time increased.

This may have been due to reduced water retention by the weak gel structure of

the denatured protein, and the release of free water to the outside structure. As a result, more light was reflected and less passed through the structure.

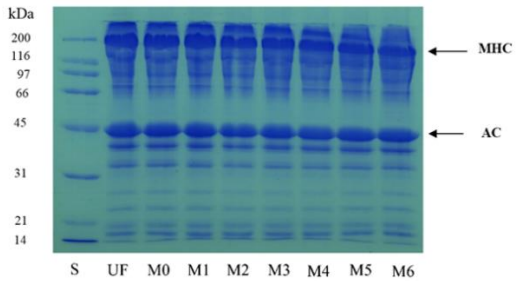

Fig. 5. SDS-PAGE patterns of surimi gels at different points of frozen storage. MHC: myosin heavy chain; AC: actin. S: standard protein; UF: unfrozen mince control; M0: initial value, M1-M6: frozen storage time at months 1-6.

3.2.4 Changes in protein patterns of surimi gel during frozen storage

Fig. 5 shows the protein patterns of the surimi gels after different periods of frozen storage. A slight decrease in MHC intensity was observed at the end of the second month of storage (Lane 5). A polymer band was observed at the top of the separating gel after 12 h of frozen storage (Lane 3), suggesting that polymerization occurred during setting. This may be due to the denaturation temperature of protein being lower in surimi as affected by frozen storage. When MF, extracted from mince, is heated at the setting temperature (40ºC), almost complete unfolding of the protein may produce inter-molecular aggregation through polymerization. This would explain the presence of a polymer band before freezing (Lane 2). However, the intensity of both polymer and MHC bands decreased as the storage time increased, possibly due to protein deterioration or degradation. As a result, an inferior gel network from the denatured protein molecule interaction was formed during setting. These results agreed with those from textural property analysis, which found a decrease in gel strength as the storage time increased. Moreover, the degraded protein with molecular weights of 97-116 kDa and actin showed no visible change.

Myosin is considered the MF most susceptible to freeze denaturation [30, 32]; on the other hand, actin is more stable than myosin during frozen storage [30]. In this study, the decrease in MHC intensity of surimi suggested that myosin molecules degraded during frozen storage, as shown by the intensity of high molecular weight polymers or degraded protein. The results were in agreement with those for biochemical property changes, gel strength and expressible water content, and suggested that the denaturation of myosin had an adverse effect on the gel network, reducing water retention within the structure.

3.2.5 Changes in microstructure of surimi gel during frozen storage

Fig. 6 shows the microstructure of surimi gel at each point of the frozen storage. No visible difference was observed between the gels prepared at the beginning of storage and those prepared in the third month, but larger protein clusters appeared in the gel prepared in the sixth month. The results confirmed that the weaker networks of gels prepared from frozen surimi reduced gel-forming ability and the WHC.

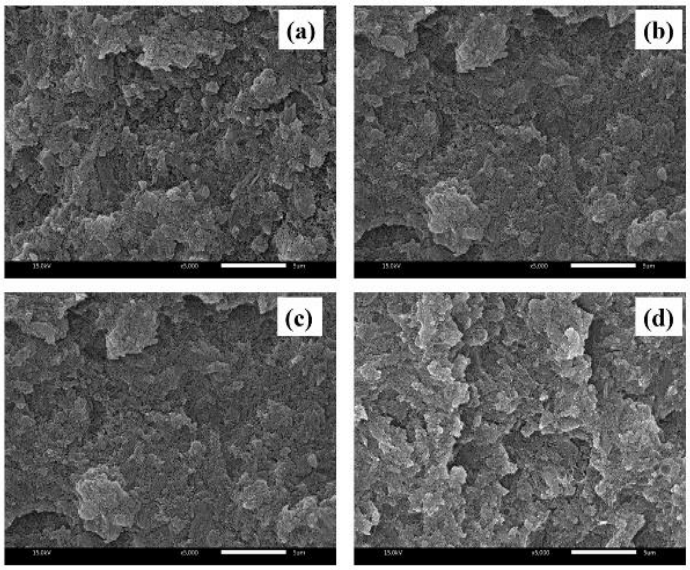

Fig. 6. Microstructure of surimi gels at different frozen storage times (Magnification: 10,000x). (a) control, (b), (c), and (d) frozen surimi at 0, 3, and 6 months, respectively.

4. Conclusion

Denaturation of myosin occurred when surimi was frozen and stored at -18ºC. This produced biochemical changes in the MF. After six months of storage, Ca^{2+}-ATPase activity had reduced by approximately 20%, total SH by 15%, and reactive SH by approximately 20%. These reductions were supported by the reduced intensity of the MHC and the increased intensity of degraded protein. Biochemical changes led to a reduction in gel strength and WHC. At the end of the sixth month, the gel strength decreased by approximately 10% and the WHC by approximately 18%. This was associated with the degradation of myosin, reflected in the SDS-PAGE patterns, and the aggregation of protein clusters in the microstructures. These findings confirmed that the frozen storage of surimi adversely affected its gel properties, mainly attributable to the partial denaturation of the MF; however, the gel prepared from fish frozen for six months can be used as raw material for surimi-based products.

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