Changes in Biochemical Properties and Microstructure of Scallop (*Patinopecten yessoensis*) Striated Adductor Muscle during Freeze-thawing, Freeze-drying and Rehydration Process

（凍結解凍、凍結乾燥および復水プロセスによるホタテガイ（*Patinopecten yessoensis*）閉殻横紋筋の生化学的特性と微細構造の変化）

A dissertation

Presented to the United Graduated School of Agricultural Sciences

Iwate University

In partial fulfillment of

The Requirements for the Degree of Doctor of Philosophy (Bioresources Science)

By

Huamao Wei

September 2020

Acknowledgement

Like every research project requiring years of research and thinking, this study owes a great deal to several individuals, without which completion of this dissertation could not have been possible. I would like to express my gratitude to all those who helped me during my Ph.D course study.

First of all, I would like to express my greatest gratitude, deepest sense of appreciation, best regards and profound indebtedness to my respected academic supervisor, Dr. Chunhong Yuan associated professor, Faculty of Agriculture, Iwate University, Japan, for her incisive guidance, supervision and encouragement and particularly for the valuable time that she has devoted to all the periods of my study in Japan, over three years. It has been an honor to be her Ph.D student. I have benefited in numerous ways from her close supervision, constant encouragement and untiring help throughout the research work and during the writing up of this dissertation.

Secondly, I would like to express my heart felt gratitude to Prof. Tetsuro Yamashita, Prof. Hayato Maeda, and Dr. Hideharu Tsukagoshi, for their encouragement, guidance and support for my Ph.D study. Moreover, thanks to Prof. Takao Ojima of Hokkaido University and Takeshi Nagai of Yamagata University, for their advises for my Ph.D research. Their deep thoughts and suggestions have been beneficial in reorganizing and revising the final draft and putting it in better form.

Thirdly, my sincere gratitude goes to Prof. Koichi Takaki, Dr. Kefeng Yu, Prof. Yuanyong Tian and Dr. Arifa Rahman. Without their helping and suggestions, I could not conduct my study smoothly in these three years.

Besides, I am extremely grateful for Jielin Ran, Yabin Niu, Seika Ogasawara, Akira Muto, Yumeng Lin, Shiliang Dong and Riko Sato in our lab, for their active help in research work and their

friendship. Thank United Graduate School of Agricultural Science, Iwate University, for providing me such excellent studying conditions and experimental conditions.

Finally, I am extremely grateful to my parents for their love, care, sacrifice and understanding throughout my life.

Huamao Wei

Table of Contents

List of Table

List of Figures

List of Abbreviations

ATP: adenosine triphosphate

ADP: adenosine diphosphate

AMP: adenosine monophosphate

IMP: inosine monophosphate

AdR: adenosine

HxR: inosine

Hx: hypoxanthine

EDTA: ethylene Diamine Tetraacetic Acid

EGTA: ethylene glycol-bis (β-aminoethyl ether)-N, N, N, N -tetraacetic acid

CP: chloramphenicol

kDa: kilodalton

PMSF: phenylmethylsulfonyl fluoride

Mf: myofibril

MHC: myosin heavy chain

Rod: myosin rod

S-1: myosin subfragment-1

SDS: sodium dodecyl sulfate

Tris: tris (hydroxymethyl) aminomethane

General Introduction

Japanese scallop (*Patinopecten yessoensis*), a kind of cold-water bivalve, also known as Yesso scallop, Giant Ezo scallop, Ezo giant scallop in the family *Pectinidae*. The scallop is usually aquacultured in the northern Japan and also can be found around the far eastern Asian coast, from China, Korea, Japan and Sakhalin, and possibly as far north as the Kamchatka Peninsula and the Aleutian Islands (Nam, Lee, Moon, & Huh, 2012).

In Japan, scallop is a very important economic species as seafood. According to the report of Ministry of Agriculture, Forestry and Fisheries (MAFF) (https://www.maff.go.jp/), the productions of scallop in 2018 was 388 kilotons, which is 106 kilotons (38%) higher than last year. Moreover, the export of scallop was 84 kilotons (up 76.6% than last year) and the export amount was 47.7 billion yen (up 3.1% than last year) in 2018. The main edible and processing part of scallop is adductor muscle, which has high nutritive value and is traditionally regarded as delicacy.

One of the reasons for the export of Japanese scallops to the world is its excellent processing technology. In Japan, large amount of scallop products are consumed as fresh, frozen, dried, smoked or canned products, some surimi products are also sold in supermarket (Kawashima & Yamanaka, 1995). However, one of the challenges in market distribution is to maintain the freshness of adductor muscle, because it is easy to become hardening and blackening without good storage condition, so that loses its market value (Kawashima & Yamanaka, 1992; Kimura, Narita, Nomata, Kaneko, & Yamanaka, 1997).

With the improvement of living standard, more and more people choose to consume fresh scallop

or frozen scallop (Wei, Tian, Yamashita, et al., 2020). Recently, frozen scallops become increasingly popular due to their long-time shelf life. Before retail distribution and consumption of frozen scallop, thawing is necessary process. There are many thawing methods reported, such as high pressure (Rouille, Lebail, Ramaswamy, & Leclerc, 2002), microwave (Boonsumrej, Chaiwanichsiri, Tantratian, Suzuki, & Takai, 2007) or infrared radiation (Hong, Shim, Choi, & Min, 2009), but the usual thawing methods are running water (Ogata, Iwane, & Kimura, 2018), or in still air (Abe, Osako, Watanabe, & Suzuki, 2009). The purpose of thawing is to keep the quality attributes as close as possible to those of fresh, unfrozen meat (Wang, Luo, Shi, & Shen, 2015). However, meat and fish may undergo quality losses such as drip, cell damage, color deterioration and softening during unsuitable thawing (Li & Sun, 2002; Ersoy, Aksan, & Ozeren, 2008). Although many studies have examined the effects of thawing on post-mortem biochemical changes in meat and fish, few basic information regarding the effect of thawing process on the post-mortem biochemical changes of pre-rigor scallop adductor muscle is available. Moreover, in some of studies, samples were placed in refrigerator for 12 h or more time to thaw, which indicated that there is no strict distinguish between thawing and post thawing refrigeration (Yoneda, Kasai, Hatae, Hirota, & Nakamura, 2006; Yoneda, Awazuhara, & Hatae, 2008; Makri, 2009).

In addition, among many scallop products, dehydrated scallops are also very popular, especially in China. Drying technology is also a good storage method for reduction in weight and volume, so that resulting in lower costs of package storage and transportation (Mayor & Sereno, 2004; Taheri-Garavand, Rafiee, & Keyhani, 2011; Zhang, et al., 2017). Many high-quality drying methods were reported in vegetable, fruit and meat fields, such as air impingement drying (Lujan-Acosta, Moreira,

& Seyed-Yagoobi, 1997), low-pressure superheated steam drying (Nimmol, Devahastin, Swasdisevi, & Soponronnarit, 2007), freeze drying (Babić, Cantalejo, & Arroqui, 2009; Nawirska, Figiel, Kucharska, Sokół-Łętowska, & Biesiada, 2009), et al. Different drying methods cause different modifications on the biochemical structural properties of foods (Deng, et al., 2014). Freeze-drying is a modern drying technology that provides dried products with little or no shrinkage and structure change, good rehydration capacity, and minimal loss in physicochemical and nutritional qualities (Crapo, Oliveira, Nguyen, Bechtel, & Fong, 2010). In addition, rehydration process is also an important step before freeze-dried scallop utilization. Unsuitable rehydration method results in loss of nutritional and functional properties and changes in structure. Arai, Kobayashi and Saito (1968) indicated the ATP-related compounds of freeze-dried scallop and abalone muscle did not change during dry storage, while ATP decreased rapidly during rehydration. However, few studies focus on changes in biochemical properties and microstructure during freeze-drying and rehydration process.

To evaluate the fresh and quality of aquatic products, ATP-related compounds and K value is a very important index which is widely accepted by many scholars (Iwamoto, Yamanaka, Watabe, & Hashimoto, 1991; H. Hong, Regenstein, & Luo, 2017; Ogata, et al., 2018). Nucleotide degradation in most aquatic muscle proceeds as follows: ATP → ADP → AMP → IMP → HxR → Hx or ATP → ADP → AMP → AdR → HxR → Hx (Yokoyama, Sakaguchi, Kawai, & Kanamori, 1992; Kawashima, et al., 1995; Vidode Mattio, Paredi, & Crupkin, 2001). Many studies reported that these two kinds of pathway are co-existed in some marine invertebrates, such as scallop (Pacheco-Aguilar, et al., 2008), abalone (Watanabe, Yamanaka, & Yamakawa, 1992), prawn (Matsumoto & Yamanaka, 1990), et al. However, the process of IMP generation in scallops is controversial (Lazou, 1989). There have been

no studies focused on the products of AMP and AdR decomposition determined directly by high-performance liquid chromatography (HPLC). Moreover, few studies have focused on investigating the effect of different ions on AMP decomposition pathway.

In addition, proteins are basic component (15-20%) of fish and shellfish, and they can be divided into water soluble sarcoplasm proteins, salt soluble myofibrillar proteins and insoluble matrix proteins (Blafsdbttir, et al., 1997). Myofibrillar proteins are important component of muscle protein and their biochemical properties can be also used as an index for quality evaluation. Myosin and actin are the important parts of myofibrillar protein. Benjakul, Visessanguan, Hongkaew and Tanaka (2003) indicated that Ca^{2+}-ATPase activity can be used to evaluate the integrity of myosin molecules for its globular heads which are responsible for Ca^{2+}-ATPase activity. The salt solubility of myofibrils can also be a good response to the interaction of myosin and actin (Azuma & Konno, 1998), which can be used to infer whether the protein is denatured. Changes in protein can be also examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Martinez, Slizyte, & Dauksas, 2007; Godiksen, Morzel, Hyldig, & Flemming, 2009). Moreover, the quality of a scallop muscle also depends on its taste and texture. Texture is influenced by microstructure (Dunajski, 1980; Crapo, Himelbloom, Pfutzenreuter, & Lee, 1999). Changes in the microstructure during freeze-thawing, freeze-drying and rehydration process can be used as an index to evaluate the quality of scallop.

Therefore, to investigate the biochemical properties and ultra-structure of scallop adductor muscle during freeze-thawing, freeze-drying and rehydration process, and to study the pathway of AMP decomposition in scallop, This study was divided into four parts as follows: In chapter 1, four different thawing methods were compared to explore the quality of adductor muscle by comparing

the changes in the biochemical properties including pH, ATP-related compounds and the microstructure of scallop adductor muscles after thawing and post cold storage. In Chapter 2, IMP, AMP, and AdR as respective substrates were used to study the enzyme activity related to the breakdown of major adenine nucleotides in scallop, and effects of heat treatment and EDTA on AMP and AdR decomposition were also investigated. In chapter 3, AMP decomposition pathway was studied in scallop crude enzyme with EDTA, K^+, Ca^{2+} and Mg^{2+} addition. Moreover, adenosine deaminase was detected by LC-MS/MS directly. In chapter 4, quality of scallop adductor muscle after freeze-drying and rehydration process was evaluated by examining ATP-related compounds, biochemical properties of myofibrillar protein and microstructure during freeze-drying and rehydration process.

Materials and Methods

Sample preparation

Live scallops were purchased from Kamaishi Aquaculture company in Iwate, Japan, and transported to the laboratory within 4 h. The scallops were shucked and the striated muscles (weight: 30±3.2 g) of the scallops were taken out, washed by filtered seawater and cut into 8 fan-shaped parts, each part 3-4 g. Then, all samples were frozen in a freezer at -30 ℃. The unfrozen samples were used as a control (C). Unfrozen samples and frozen-thawed scallops were used for the experiments. Four thawing methods were used: running water (18 ℃) thawing (R); ice-water (0 ℃) thawing; air (4 ℃) thawing (A); and ice-saltwater (-2 ℃) thawing (S). The freezing and thawing curves recorded by a Thermo Recorder TR-52i (T&D, Ltd, Japan) and thawing time of R, I, A and S group were 8, 56, 80 and 74 min when the center temperature of R, I and A group reached 0℃ and S group reached -2 ℃. After thawing, the samples were placed in a refrigerator at 4℃ for 24 h, and all analytical measurements were taken at 0, 6, and 24 h during storage. The freezing curve and thawing curves of pre-rigor scallop adductor muscle was shown in Figure 1.

Measurement of muscle pH

The pH determination method was described by Jeacocke (1977). One gram meat with 10 mL 20 mM sodium iodoacetate was homogenized, and the pH was determined by a pH meter.

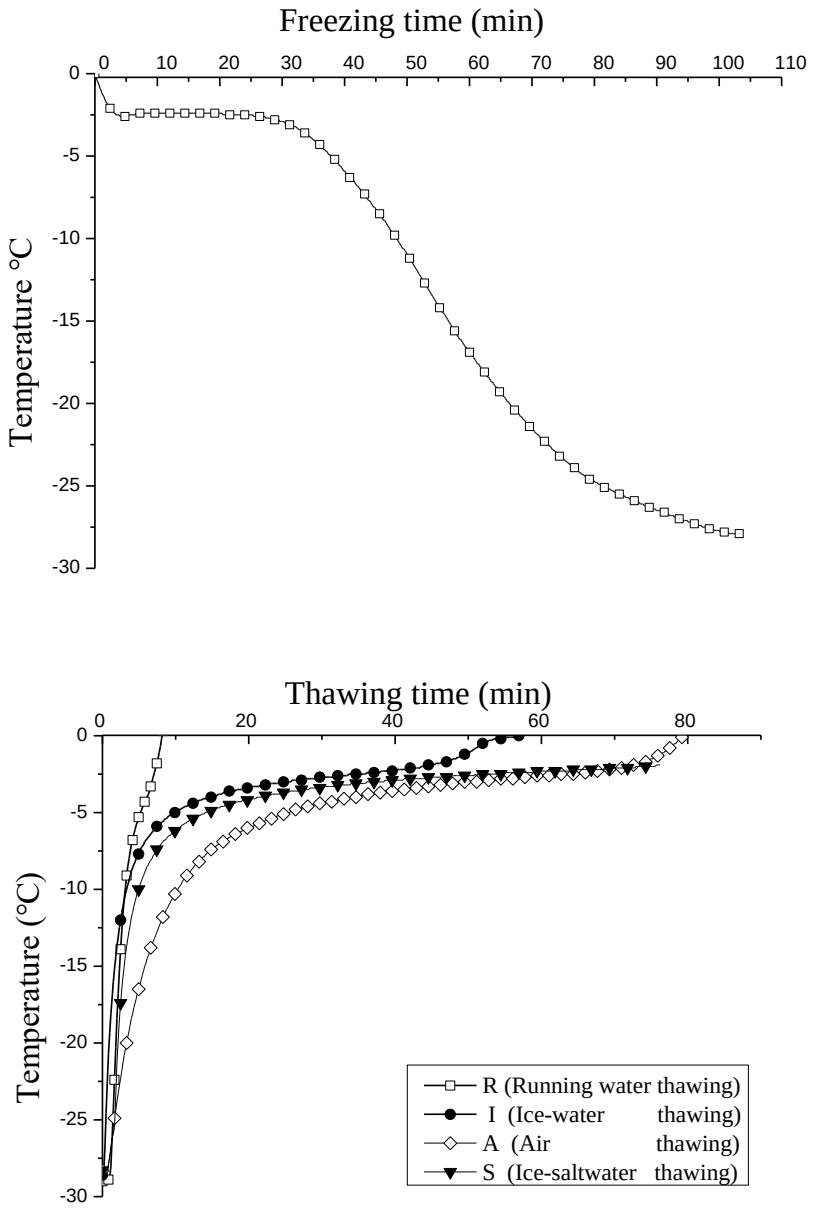

Figure 1 Freezing curve (left) and thawing curves (right) of pre-rigor scallop adductor muscle.

Determination of ATP-related compounds and K values

The contents of ATP and its related compounds in scallop adductor muscle were determined using HPLC according to the method of Hu, Zhang, Ebitani and Konno (2013) with slight modifications. Two grams sample was mixed with 20 mL of 5% perchloric acid (PCA), homogenized and then centrifuged at 3,000 x g for 3 min at 4 ℃. The supernatant was collected and adjusted to pH 3 by KOH. After centrifugation at 3,000 x g for 3 min at 4 ℃, the supernatants were filtered through a 0.22 μm filter membrane, and the filtrates were subjected to analysis using HPLC (Nanospace, Shiseido Ltd. Osaka, Japan) with a column (Shodex GS-320 HQ, Showa Denko K.K., Tokyo, Japan). The conditions were as follows: mobile phase: 0.2 M phosphate buffer (pH 3.7); flow rate: 0.6 mL/min; temperature: 30 °C. A UV detector (Nanospace 3002, Shiseido Ltd. Osaka, Japan) was used, and the absorbance at 254 nm was monitored. The ATP-related compounds standard curves were shown in Figure 2.

$$\text{K value (\%)} = [(\text{HxR}+\text{Hx})/(\text{ATP}+\text{ADP}+\text{AMP}+\text{IMP}+\text{HxR}+\text{Hx})] * 100 \quad (1)$$

Preparation of myofibril

Myofibrils were prepared from the scallop adductor muscle using the method described by Yuan, Wang, Chen, Qu and Konno (2011). The scallop adductor muscle was washed in 0.1 M NaCl 20 mM Tris-HCl (pH 7.5) two times, homogenized, and rewashed in the same buffer. The myofibril suspension was filtered through two layers of gauze, and the filtrate was used as the myofibrils in subsequent analyses.

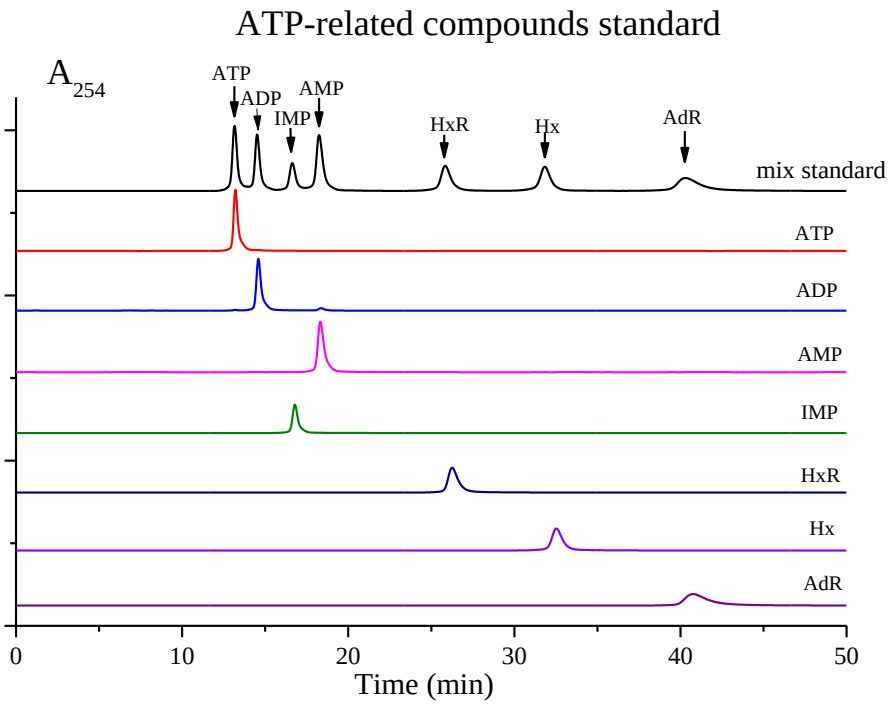

Figure 2. Chromatogram of ATP-related compounds.

Ca^{2+}-ATPase activity assay

The Ca^{2+}-ATPase activity of myofibrils (0.8 mg/mL) was assayed in a medium of 0.5 M KCl, 20 mM Tris-maleate (pH 7.0), 5 mM $CaCl_2$ and 1 mM ATP at 25 ℃ (Zheng, et al., 2012). The concentration of inorganic phosphate (Pi) was determined to analyze ATP hydrolysis. The Ca^{2+}-ATPase activity of myofibrils was measured in 0.1 M and 0.5 M NaCl solutions. The results of the Ca^{2+}-ATPase activity were expressed in μmol Pi/min/mg of protein. The protein content was measured by the biuret method (Gornall, Bardawill, & David, 1949).

Salt solubility

The solubility of myofibrils was measured by the following procedures. Myofibrils were dissolved in different concentrations of NaCl together with 20 mM Tris-HCl (pH 7.5), and then the solution was centrifuged at 10,000 x g for 15 min. The same solution was added with 5 mM Mg-ATP to dissociate actomyosin into myosin and actin and then measured after centrifugation (Kimura, Takahashi, Nagahisa, & Fujita, 1982).

The protein content before and after centrifugation was measured by the biuret method (Gornall, Bardawill, & David, 1949). The content of the sample before centrifugation (C_0) and the supernatant protein content of the sample after centrifugation (C_t) were determined. The solubility was calculated using the following equation:

$$\text{Solubility (\%)} = (C_t/C_0)*100 \quad (2)$$

Transmission electron microscopy (TEM)

The morphology of the fresh scallop adductor muscle and thawed meat was observed using TEM according to the method of Ando, Oishi, Mochizuki, Tsukamasa and Makinodan (2002) with slight modifications. The samples were cut into 5 blocks (1 mm x 1 mm x 5 mm) and fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h and then washed three times with 0.1 M phosphate buffer (pH 7.4); the specimens were then post-fixed with 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). Dehydration and substitution with ethyl alcohol and propylene oxide were performed before embedding in resin. Then, the specimens were ultra-sectioned, stained and observed using a transmission electron microscope (JEM-2100, JEOL LTD., JAPAN).

Effect of antibiotic treatment and storage temperature on IMP accumulation

Fresh scallop adductor muscles were divided into 2 portions. One portion was washed with sterilized seawater, and the other portion was washed with sterilized seawater with 150 ppm of antibiotics (chloramphenicol). Then, all samples were frozen in a freezer at -30°C and thawed in ice-water before treatment. The frozen-thawed scallops were stored at 4°C and 20°C for 1 day. The AMP and IMP contents of the samples were measured by HPLC.

Comparison of the decomposition rates of AMP, IMP and AdR assay

A modified experimental method was applied based on that of Fujisawa and Yoshino (1987).

Crude enzyme from scallop striated adductor muscles was prepared by homogenization with 10 mM sodium phosphate buffer (pH 7.0), in which a part of the homogeneous solution was separated into the supernatant and precipitate by centrifugation (10,000 × g for 10 min at 4°C). The scallop muscle tissue solution (suspended solution), myofibrillar proteins (precipitate) and sarcoplasmic proteins (supernatant) were mixed with 10 mM sodium phosphate buffer (pH 7.0) to a final volume of 25 mL. The prepared solution was used to determine scallop muscle AMP, IMP and AdR decomposition using 4 mM AMP, IMP and AdR as the respective substrates at 25°C and pH 7.0. The ATP-related compounds in the samples were measured by HPLC.

Comparison of the AdR decomposition rate in scallop muscle extracellular solution

To elucidate whether the scallop AdR deaminase is intracellular or extracellular, scallop adductor muscles were soaked in 4 volumes of 10 mM sodium phosphate buffer (pH 7.0) at 4°C for 1.5 h and 24 h. The decomposition ability of the soaking solution using AdR as a substrate was determined at 4 °C and pH 7.0. The ATP-related compounds in the samples were measured by the same method as above. The protein concentration of the soaking solution was examined by the biuret method (Gornall, Bardawill, & David, 1949).

Effect of EDTA addition and short-term heating on the AMP and AdR decomposition rates

Crude enzyme (soluble fraction) was prepared from scallop striated adductor muscles by homogenization with 10 mM sodium phosphate buffer (pH 7.0). The supernatant was divided into

the following 4 groups: one mixed with 5 mM EDTA, one heated in a 100°C water bath for 1 min (solution temperature reached 45-50°C) or 5 min and one without any treatment (control). The crude enzyme was used to determine AMP and AdR decomposition using AMP and AdR as the respective substrates at pH 7.0 and at 25 and 4°C. The ATP-related compounds from the samples under the treatment different conditions were measured by HPLC.

Effect of EGTA addition on AMP decomposition

The crude enzyme solution was divided into two groups: one mixed with 5 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, N, N -tetraacetic acid (EGTA) and one without any treatment (control). The crude enzyme was used to determine the AMP decomposition ability using 3 mM AMP as substrates at pH 7.0 and 25℃. The ATP-related compounds of the samples under the treatment different conditions were measured by HPLC.

Effect of buffer with different ions and microbial activity on AMP decomposition

The phosphate buffer (pH 7.0) which has 10 mM sodium or potassium ions was used for crude enzyme solution dialysis. Each of crude enzyme solution was dialyzed against 10 times volume of phosphate buffer (pH 7.0) for 8 h, and then dialyze against new 10 times volume of the same buffer (pH 7.0) for 16 h to make sure all ions were removed. All the dialysis experiments were performed at 5 ℃.

Then, each dialyzed crude enzyme solutions were divided into two groups: one with 0.1%

chloramphenicol (Na^+-CP or K^+-CP) and one without any treatments (Na^+-C or K^+-C). These four group of crude enzyme solutions were used to determine AMP decomposition using 3 mM AMP as substrate at 25 ℃ for 0, 8 and 24 h by HPLC.

Moreover, the whole solution, supernatant and precipitate were also measured during dialysis and incubation process. Supernatant from crude enzyme solutions were obtain after 10,000 x g for 15 min centrifugation at 4 ℃. The precipitate was added buffer constant volume to initial volume. Protein content of crude enzyme after dialysis and during incubation was detected by biuret method (Nowotny, 1979). The protein compositions were measured by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Effect of $CaCl_2$ and $MgCl_2$ on AMP decomposition

The crude enzyme solution which dialyzed with 10 mM sodium phosphate buffer (pH 7.0) was divided into 3 groups: one mixed with 1 mM $CaCl_2$ (Ca group), one mixed with 6 mM $MgCl_2$ (Mg group), and one mixed with 1 mM $CaCl_2$ and 6 mM $MgCl_2$ (Mix group). All groups in this part were added 0.1% chloramphenicol to remove the influence of microbial activity. The crude enzyme was used to determine the AMP decomposition ability using 3 mM AMP as substrates at pH 7.0 and at 25 and 4℃. The ATP-related compounds of the samples under the treatment different conditions were also measured by HPLC.

Determination of adenosine deaminase by LC-MS/MS

Adjust the concentration of sodium phosphate buffer (pH 7.0) of crude enzyme solution to 0.5 M and added 2 mM Mg-ATP and 40% ammonium sulfate (A.S) to remove the actin as much as possible. The supernatant was obtained after 15,000 x g for 15 min centrifugation at 4 ℃. Then, enzyme solution was fractionated by added ammonium sulfate in the 60% saturation range, and the precipitate collected by centrifugation was dissolved by 10 mM sodium phosphate buffer (pH 7.0) for SDS-PAGE analysis.

The protein fraction near 40 kDa in SDS-PAGE gel was used to LC-MS/MS analysis. The LC-MS/MS methods was performed as described by Hynek, Svensson, Jensen, Barkholt and Finnie, (2006) with slight modification. The bands of interest were excised from the SDS-PAGE gel and cut into 1 mm^3 cubes. And then washed in 1:1(v/v) 50 mM NH_4HCO_3 in acetonitrile. Cys residues were reduced with DTT and alkylated with iodoacetamide. Digestion was carried out with 0.01% Protease MAX solution which contain 4 ng/μL trypsin at 37℃ for 2-3 h. The 1% TFA was used to stop the digestion. The supernatant was obtained after 1,5000 x g for 15 min centrifugation and stored in -20℃ freezer until LC-MS/MS analysis.

Digested peptides were applied onto a L-colomn2-ODS (0.1mm x 150 mm, CERI, Tokyo, Japan) in an Advance UHPLC system (Michrom Bioresources, Auburn, CA, USA) equilibrated with 0.1% formic acid in acetonitrile, and eluted using a linear gradient from 5% to 45% acetonitrile at a flow rate of 500 nL/min. The mass spectrometer used was an LTQ Orbitrap XL (Thermo Scientific) operated with the Xcalibur software (version 2.0.7, Thermo Scientific). Peptides were identified using an in-house Mascot server (MS/MS ion search, Mascot version 2.5, Matrix Science Inc.) using the SwissProt database.

Digestion of myofibrillar protein

The methods of chymotryptic digestion of scallop myofibrils were performed as described by Zheng, et al. (2012) with slight modification. Myofibrils digestion was performed to cleave the myosin as S-1/rod fragment in a medium containing 0.1 NaCl, 20 mM Tris-HCl (pH 7.5) and 5 mM EDTA, using 1/1,000 N HCl treated α-chymotrypsin to myofibril ratios of 1/400 (w/w) at 25℃ to study the temperature-dependent flexibility of myosin conformation.

In addition. Chymotryptic digestion was also carried out for heated myofibrils to elucidate the structural changes of myosin upon heating. Thermal denaturation profiles of fresh scallop, frozen-thawed scallop and freeze-dried scallop myofibrillar protein were studied by the amounts of chymotryptic fragments produced when digested at S1/rod junctions for 0, 5 and 20 min at 40℃.

Chapter 1 Effects of Thawing Methods on the Biochemical Properties and Microstructure of Pre-rigor Frozen Scallop Striated Adductor Muscle

Scallop (*Patinopecten yessoensis*), one of the most valuable commercial aquatic species in Japan, is sold fresh or processed into frozen, dried, canned, or boiled forms (Kawashima, et al., 1995). In recent year, frozen scallop has been increased remarkably due to the improved technology of aquaculture and freezing (Kawashima, et al., 1995). Thawing of the frozen scallop is required before retail distribution and consumption. Many studies have examined about the effects of thawing on post-mortem biochemical changes in meat and fish (Kawashima, et al., 1995; Abe, et al., 2009). Rapid thawing by running water is recommended due to less protein denaturation and drip loss (Abe, et al., 2009). On the other hand, slow thawing with salt iced water (-1 and -2℃) can supply high quality pre-rigor freeze-thawed olive flounder for sashimi and the properties of myofibrillar protein were largely maintained (Ogata, et al., 2018). For high fresh whale, thawing with pretreatment at -3℃ for 3-7 days was suitable for the inhibition of thaw rigor (Murata, et al., 2008). The above different conclusions might be due to the initial freshness before freezing and the intrinsic characteristics of different species. Moreover, in some of studies, samples were placed in refrigerator for 12 h or more time to thaw, which indicated that there is no strict distinguish between thawing and post thawing refrigeration (Yoneda, et al., 2006; Yoneda, et al., 2008; Makri, 2009).

It is well-known that fish which are frozen in pre-rigor have a better quality than that of in rigor or post-rigor stage (Ogata, Shindo, & Kimura, 2012; Ogata, et al., 2018). Post-mortem biochemical changes in frozen-thawed fish are closely related to the eating quality of the fish meat (Vaughan, 1979). However, few basic information regarding the effect of thawing process on the post-mortem

biochemical changes of pre-rigor scallop adductor muscle is available. Therefore, in this study, four different thawing methods were compared to explore the quality of adductor muscle by comparing the changes in the biochemical properties including pH, ATP-related compounds and the microstructure of scallop adductor muscles after thawing and post cold storage.

1.1 Changes in the pH of pre-rigor scallop adductor muscle after thawing and storage

The changes in the pH of the scallop adductor muscle after different thawing methods and storage at 4 °C for 6 h and 24 h are shown in Figure 1-1. The pH of fresh scallop was 7.01±0.05, and the control group (C) changed little after storage for 1 day at 4 ℃. However, the pH values of the I, A and S groups decreased to 6.87, 6.78 and 6.88 after thawing (0 h of storage), respectively. While the pH of the scallops thawed by running water (R) decreased from 7.01 to 6.55, a change that was faster than that of the other three thawing groups. After 6 h of storage, the pH of each thawing group decreased, with values of 6.47±0.01 in group R, 6.74±0.05 in group I, 6.63±0.02 in group A and 6.74±0.03 in group S. The pH values of group I and group A were higher than those of group S and group R, which suggests that scallops thawed at low thawing temperatures will have good quality. After 24 h of refrigeration, the pH of the thawed group dropped to 6.22±0.05, and there was no significant difference ($p > 0.05$) between the groups, but the pH of the fresh scallops was still 7.0. The results show that different thawing methods will affect the pH change of scallops during thawing and short-term storage, but the difference decreases as the storage time increases. The rapid decline in pH might be the result of the accumulation of organic acids, such as lactic acid and succinic acid, and octopine, as mentioned by Hiltz and Dyer (1971). The decompounding of ATP-related compounds also lead to decrease in pH.

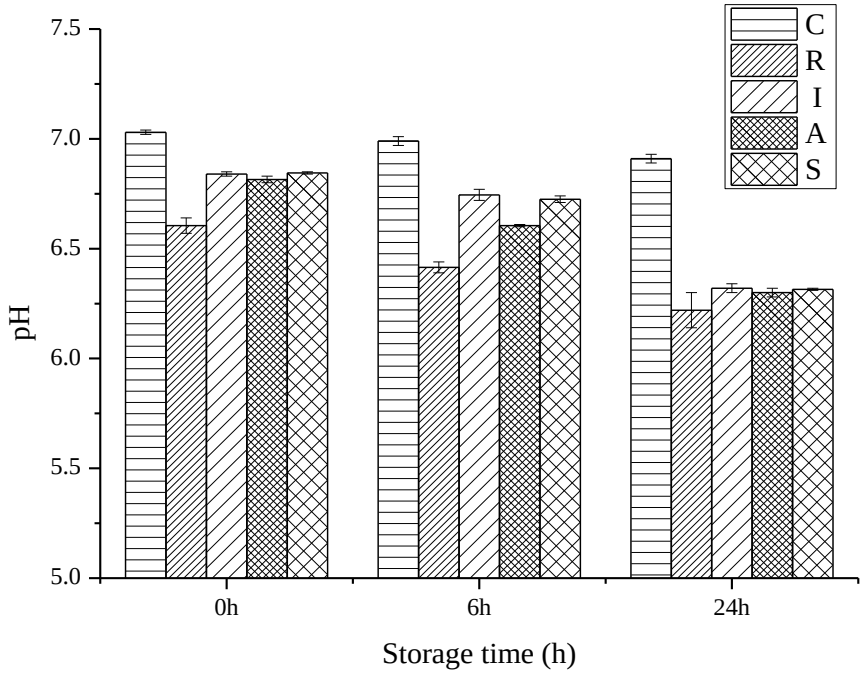

Figure 1-1. Changes in pH of pre-rigor scallop adductor muscle after different thawing method (0 h), storage 6 h and 24 h at 4 ℃.

R: running water thawing; I: ice-water thawing; A: air thawing; S: ice saltwater thawing.

1.2 Changes in ATP-related compounds of pre-rigor scallop adductor muscle during thawing and storage

The total concentration of ATP-related compounds in scallop adductor muscle before thawing was 10.2±1.0 μmol/g, including 7.0±0.6 μmol/g of ATP. For the fresh scallop, the ATP decreased from 7.1 to 6.9 at 4℃ storage for 24 h (data not shown). However, the ATP content of scallops decreased rapidly with AMP accumulation after thawing, as shown in Figure 1-2. During the thawing process, the ATP content of the R group declined logarithmically [y =-0.06x +1.96, r^2=0.95, $p < 0.001$] (y: ATP content, μmol/g; x: time, min) from 7.03 μmol/g to 4.30 μmol/g, but the decrease in ATP was not more than 80% due to the short thawing time. Compared to the I and A groups, the ATP content declined logarithmically [I: y=-0.02x+1.99, r2=0.99, $p < 0.001$; A:y=-0.02x+1.93, r2=0.97, $p < 0.001$], but the AMP accumulation of the A group after thawing was 3.69 μmol/g, which was higher than the 2.04 μmol/g in the I group because of the longer thawing time. For the S group, the ATP content decreased more quickly than that in the A and I groups [y=-0.06 Ln(x)+1.75, r^2=0.94, $p < 0.001$], and ATP was degraded by 95% in 40 min, which caused AMP accumulation at 4.7 μmol/g. The thawing temperature may have been near the freezing point of scallops, and the structure of the scallops was damaged during thawing, which caused the release of Ca^{2+} from the sarcoplasmic reticulum to accelerate the decomposition of ATP.

It was reported that the ATP content in the adductor muscle of the Catarina scallop was 6.6 μmol/g before thawing (Ocaño-higuera, Maeda-martínez, Lugo-sánchez, & Pacheco-aguilar, 2006), which is similar to the levels found in our study. However, these results were lower than the 10.2 μmol/g reported in the Japanese baking scallop (Wongso & Yamanaka, 1998). The differences in the initial ATP values of scallops may be due to differences in species, seasons and habitats (Pacheco-

Aguilar, et al., 2008). Kawashima and Yamanaka (1995) also reported that the ATP and its related compounds drastically changed by the thawing procedure. But in their research, the changes in the scallop adductor muscle by rapid thawing were smaller than those by slow thawing maybe due to slow thawing take longer time, so that AMP decomposed to HxR and Hx.

Changes in ATP-related compounds of fresh and frozen-thawed scallop during storage are shown in Figure 1-3. After storage for 24 h, ATP-related compounds in the I group still had a high AMP content of 21.39%, which was higher than that of R (10.78%), A (7.38%) and S (6.37%). Less IMP accumulated during thawing or storage, which may be due to a lack of AMP deaminase activity (Mendes, Quinta, & Nunes, 2001). A similar result was also shown in scallop and oyster (Yokoyama, et al., 1992; Wang, et al., 2007; Pacheco-Aguilar, et al., 2008). However, the reason for IMP accumulation is not clear.

The results of the K value determination are also shown in Figure. 1-3. After thawing, the K values of the A and S groups were 4.56% and 4.30%, respectively, which were higher than the 1.07% in the I group and the 0.66% in the R group. After storage for 6 h, the K value of the S group (32.62%) was higher than that R (20.65%), I (21.52%) and A (22.05%) groups. Because the S group accumulated a large amount of AMP after thawing, the AMP was easily decomposed into HxR and Hx during storage. Ice-water thawing is the best choice due to its slow ATP decomposition rate during thawing and low K value after storage for 24 h. Although the K value is widely accepted as a freshness index for many types of fish, for scallops, the K value rises rapidly after thawing, and a more than 50% increase within one day does not mean that the scallops cannot be eaten as sushi or sashimi. Wongso, et al. (1998) also reported that the K value is not a good freshness index for frozen-thawed shellfishes, which suggested that other comprehensive indexes were needed, such as the salt solubility,

Ca^{2+}-ATPase activity and microstructure.

1.3 Changes in biochemical properties of pre-rigor scallop adductor muscle myofibrils after thawing and storage

Salt solubility is a very important index to evaluate the myofibrils of scallop adductor muscles, as shown in Figure 1-4. The results suggested that the salt solubility of myofibrils increased with increasing NaCl concentration. The salt solubility of the C group (21.7% at 0.5 M NaCl concentration) was much lower than that of other thawing groups (Figure 1-4, A-0 h), which was completely different from the results seen in carp (Azuma & Konno, 1998), indicating that the myosin and actin of fresh scallops could not fully dissociate and dissolve at high salt concentrations (0.5 M NaCl). Perhaps because fresh scallops contain high ATP during the preparation of myofibrillar proteins, high levels of ATP cause muscle contraction during homogenization, and myosin and actin bind closely. The salt solubility of the R group was 70.8% at 0.5 M NaCl, while that of the I, A and S groups was 86.7%, 85.9% and 85.2%, respectively. After 24 h of storage (Figure 1-4, B-24 h), the salt solubility of the R group decreased significantly ($p < 0.05$), possibly due to the high running water temperature damaging the structure of the scallop, which led to the rod portions of the myofibrillar proteins being intertwined, making it difficult to dissolve and resulting in a decrease in salt solubility. Mg-ATP has a strong ability to dissociate myosin from actin, which causes myofibrils to easily dissolve in solution.

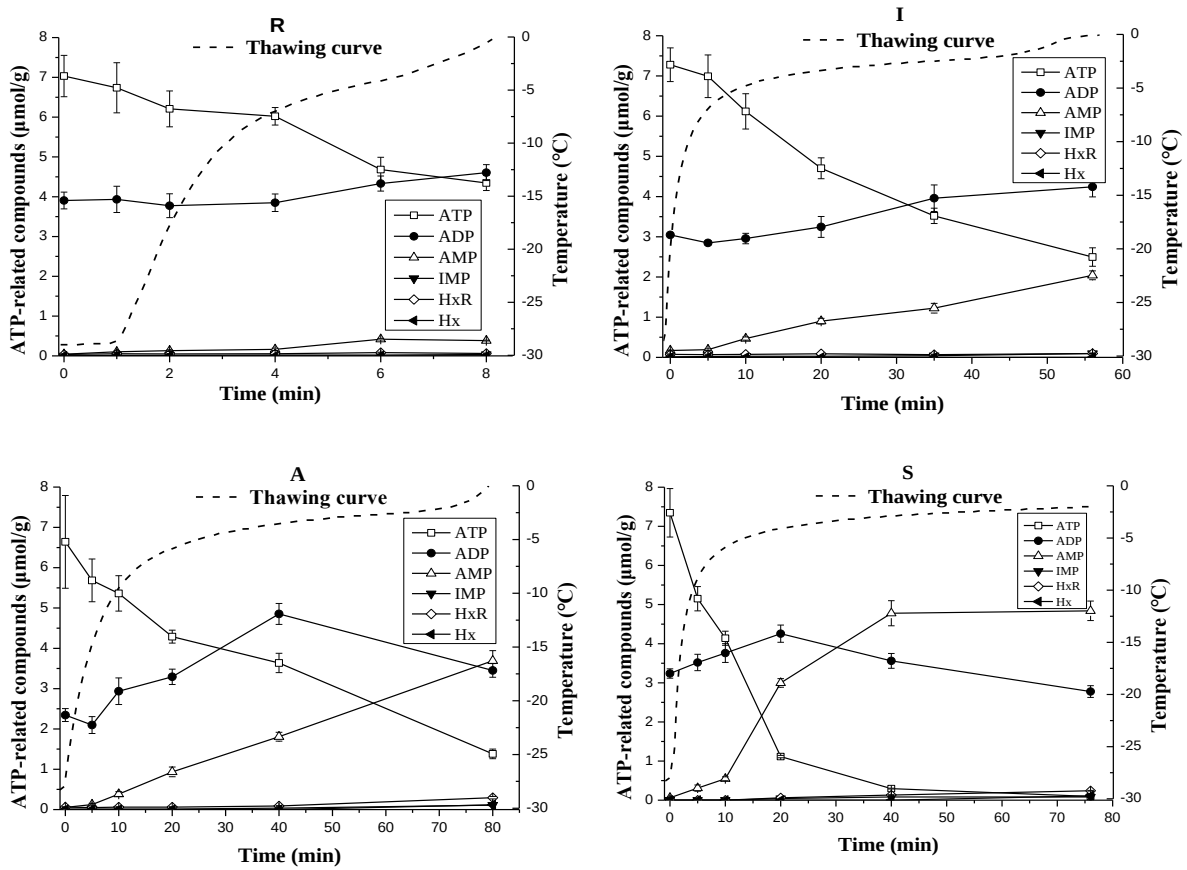

Figure 1-2 Changes in ATP-related compounds of pre-rigor scallop adductor muscle after during thawing.

R: running water thawing; I: ice-water thawing; A: air thawing; S: ice-saltwater thawing.

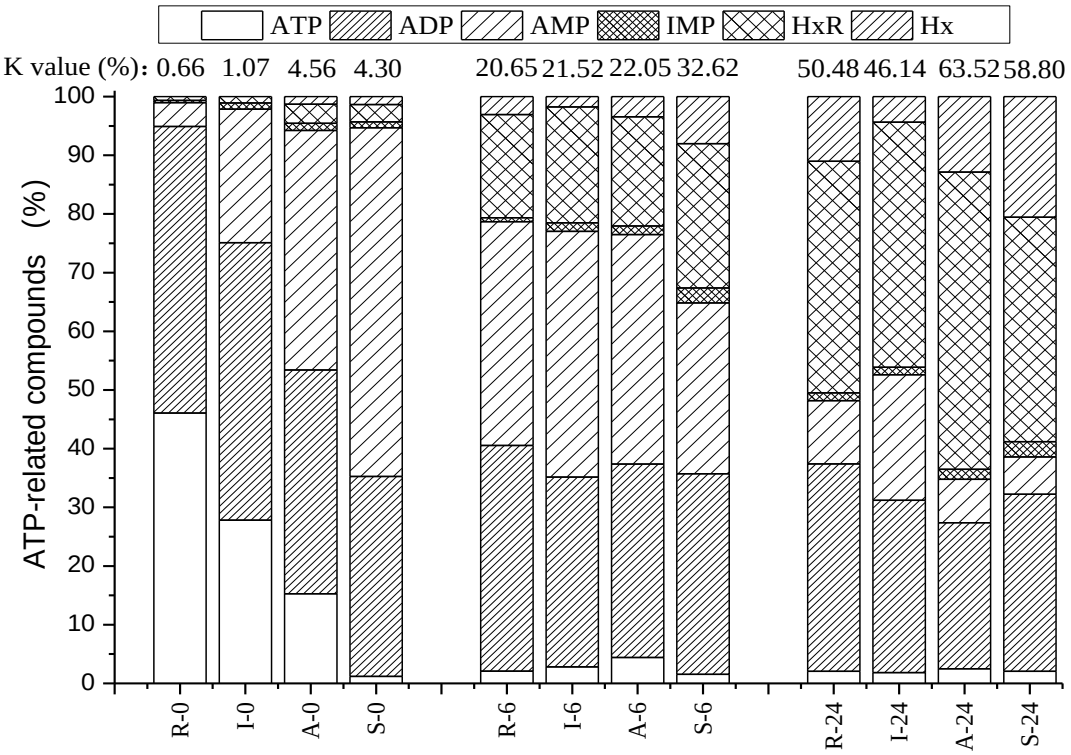

Figure 1-3 Changes in ATP-related compounds of pre-rigor scallop adductor muscle during storage at 4 ℃.

R: running water thawing; I: ice-water thawing; A: air thawing; S: ice-saltwater thawing.
The number (0, 6, 24) means the scallop storage at 4 ℃ for 0 h, 6 h, 24 h.
The number in the top of bar chart means K value (average value, n=3).

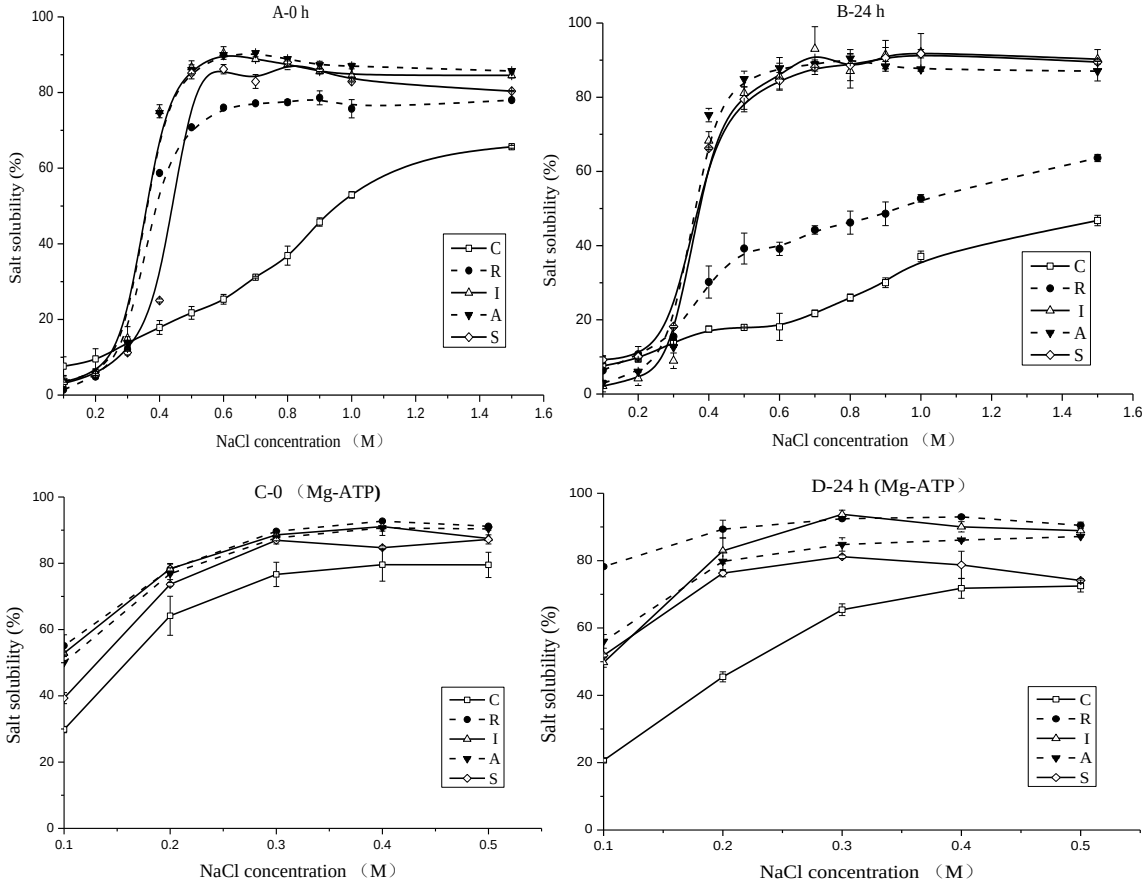

Figure 1-4 Changes in protein salt solubility of pre-rigor scallop after thawing (A and C) and storage at 4 ℃ for 24 h (B and D).

The number (0, 24) means the scallop storage at 4 ℃ for 0 h, 24 h.

C: control group; R: running water thawing; I: ice-water thawing; A: air thawing; S: ice-saltwater thawing.

However, the salt solubility of group C was still lower than that of the other thawing groups. The Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) results of scallop myofibrillar tissues after thawing in 0.1 M NaCl consistent with those of the salt solubility assays (data not shown).

Ca^{2+}-ATPase can be used as an indicator for the integrity of myosin molecules, and the globular heads of myosin are responsible for Ca^{2+}-ATPase activity (Benjakul, et al., 2003). In our study, the Ca^{2+}-ATPase activity of all frozen-thawed scallop myofibrils was maintained at 0.78-0.84 μmol Pi/min/mg in 0.1 M and 0.5 M NaCl solution and without a significant difference between the different groups (Table 1-1). The results show that the S-1 of scallop myosin was not damaged by thawing, and different thawing methods had little effect. However, there is a large error in the data of Ca^{2+}-ATPase activity between different groups. The main reason for this error is that scallops have high Ca^{2+}-ATPase activity, and there are large differences among different individuals. Later, the results of the chymotrypsin digestion model experiment also suggested that the rod of myosin was easily degraded, while the S-1 remained stable (data not shown), which can explain why the Ca^{2+}-ATPase activity did not change substantially during thawing and storage. Sriket, Benjakul, Visessanguan and Kijroongrojana (2007) examined on the effect of freeze-thawing cycles on the biochemical properties of shrimp, and the results showed that the Ca^{2+}-ATPase activity of black tiger shrimp and white shrimp decreased after one cycle of freezing-thawing, but no marked changes were found with increasing freeze-thaw cycles for up to five cycles.

Table 1-1 Changes in Ca^{2+}-ATPase activity of pre-rigor scallop adductor muscle during storage at 4 ℃ after thawing.

NaCl Concentration	Storage time (h)	C	R	I	A	S
0.1M	0	0.81±0.09[a]	0.80±0.07 [a]	0.81±0.08 [a]	0.79±0.07 [a]	0.80±0.07 [a]
	24	0.81±0.08 [a]	0.79±0.09 [a]	0.80±0.07 [a]	0.82±0.05 [a]	0.80±0.07 [a]
0.5M	0	0.78±0.11 [a]	0.77±0.10 [a]	0.84±0.10 [a]	0.79±0.06 [a]	0.81±0.10 [a]
	24	0.82±0.08 [a]	0.78±0.09 [a]	0.78±0.09 [a]	0.83±0.05 [a]	0.80±0.08 [a]

Value are given as mean ± standard deviation (n=3).

Different letters (a) within the same row indicate significant difference ($p < 0.05$)

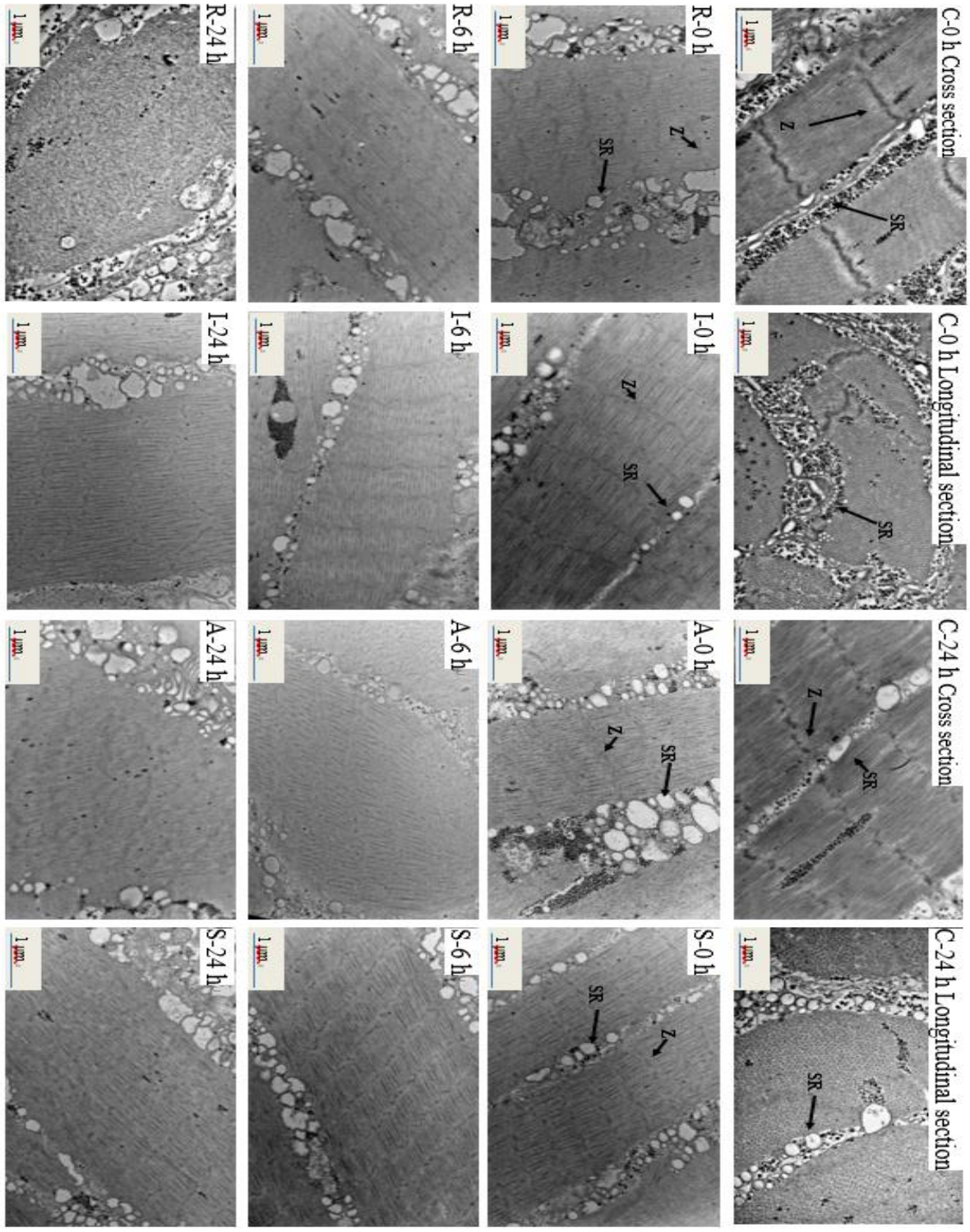

Figure 1-5. Changes in microstructural properties of pre-rigor scallop adductor muscle after thawing and storage.

C: control. R: running water thawing; I: ice- water thawing; A: air thawing; S: ice saltwater thawing. Thawing group (R, I, A, S) only cross section. SR: sarcoplasmic reticulum.

1.4 Changes in microstructural properties of pre-rigor scallop adductor muscle after thawing and storage

The TEM results (Figure 1-5) show that the scallop muscle structure remained relatively intact in the native state (control group) because of its clear Z line and small sarcoplasmic reticulum (SR). During the storage process, the Z line is gradually damaged, and the SR swells. The results show that after running water thawing (R group), due to the excessive rate of temperature rise and the high thawing temperature, the structure of the scallops was destroyed, the Z line became unclear and the SR swelled. The structures of group A and group S remained intact after thawing and storage for 6 h, but the structure was destroyed after 24 h. For group I, the microstructure of the scallops remained relatively intact and close to that of the control group after thawing and storage. Therefore, ice-water thawing is the best thawing method because it causes less damage to the scallop structure.

1.5 Conclusions

In this study, pre-rigor frozen scallops were thawed by running water, ice-water, air and ice-saltwater. The results showed that ice-water thawing is the best thawing method because it is associated with slow pH decreases and fewer changes in ATP-related compound. Changes in the free amino acids (FAAs) of scallops thawed by different thawing methods were also examined (Table 1-2). The results showed that scallops were rich in glycine, and taurine acid and alanine were also present at high levels. The content of FAAs in the I, A and S groups was similar to that in the control group, while that of the R group changed significantly. Ice-water thawing also has little effect on the biochemical properties of myofibrils and on the microstructure of scallop adductor muscle, which are

involving in maintaining the high-quality of scallop adductor muscle during cold storage after thawing.

In addition, whole scallops were also examined after these four thawing methods, and the results indicated that ice-water thawing is the best method due to the quality of the frozen-thawed scallops being close to that of fresh scallops, which suggested that ice-water thawing was also suitable for the whole scallop adductor muscle. Overall, the freezing and thawing process had a comprehensive effect on the quality of scallop adductor muscle, and ice-water thawing is the best thawing method because it is simple, easy to control and has less effect on the quality of scallop adductor muscle.

Table 1-2 Free amino acid changes of fresh scallop and frozen-thawed scallop.

Amino acid	C	R	I	A	S
Taurine	6.91±0.12 ^{b}	7.41±0.14 ^{a}	6.89±0.11^{bc}	6.73±0.11^{d}	6.98±0.17 ^{b}
Threonine	0.17±0.02 ^{ab}	0.20±0.01 ^{a}	0.17±0.01 ^{ab}	0.19±0.01 ^{a}	0.18±0.02 ^{ab}
Glutamic	0.71±0.06 ^{a}	0.77±0.06 ^{a}	0.68±0.05 ^{a}	0.66±0.06 ^{a}	0.67±0.05 ^{a}
Glycine	20.84±0.55 ^{b}	22.39±0.39 ^{a}	20.42±0.54 ^{b}	19.84±1.14 ^{c}	20.42±0.20 ^{b}
Alanine	2.94±0.07 ^{b}	3.59±0.04 ^{a}	3.04±0.02 ^{b}	3.49±0.09 ^{a}	3.50±0.14 ^{a}
Leucine	0.35±0.02 ^{a}	0.35±0.01 ^{a}	0.37±0.12 ^{a}	0.31±0.03 ^{a}	0.28±0.14 ^{a}
Lysine	0.17±0.02 ^{a}	0.20±0.01 ^{a}	0.18±0.01 ^{a}	0.18±0.01 ^{a}	0.18±0.01 ^{a}
Arginine	4.71±0.92 ^{a}	3.43±0.16 ^{b}	3.92±0.22 ^{ab}	2.57±0.55 ^{c}	3.14±0.36 ^{bc}
Proline	0.94±0.11 ^{a}	1.12±0.04 ^{a}	1.03±0.18 ^{a}	0.98±0.04 ^{a}	1.02±0.02 ^{a}

Value are given as mean ±standard deviation (n=3)

Different letters (a-f) within the same row indicate significant differences between treatments ($p < 0.05$)

C: control. R: running water thawing; I: ice- water thawing; A: air thawing; S: ice saltwater thawing.

Chapter 2 Condition-dependent Adenosine Monophosphate Decomposition Pathways by Endogenous Enzymes in Striated Adductor Muscle from Japanese Scallop (*Patinopecten yessoensis*)

With the improvement of cold-chain technology and lifestyle changes, the consumption of fresh aquatic products has increased greatly (Cheng, Sun, Han, & Zeng, 2014; Qin, et al., 2016), especially that of sashimi and sushi, which have become very popular throughout the world (Kawashima, et al., 1992; Yokoyama, Sakaguchi, Kawai, & Kanamori, 1994a; Ogata, et al., 2018). When the freshness of aquatic products decreases, the concentrations of major adenine nucleotides and their related compounds in the postmortem muscles of these aquatic organisms also change. In the muscles of fish, it is well-known that adenosine triphosphate (ATP) decomposition results in inosine monophosphate (IMP) accumulation, and nucleotide degradation proceeds as follows: ATP→ ADP → AMP → IMP→ HxR (inosine)→ Hx (hypoxanthine) (Saito, 1961). However, for marine invertebrate muscles, AMP accumulates instead of IMP, and is a major route for the conversion of AMP to HxR via adenosine (AdR), as follows: ATP→ADP→AMP→AdR→ HxR→Hx (Yokoyama, et al., 1992; Hatae, et al., 1995; Kawashima, et al., 1995; Vidode Mattio, et al., 2001) .

Some reports also indicated that both AMP decomposition pathways exist in invertebrates because of the detection of only small amounts of IMP and AdR in these organisms (Watanabe, et al., 1992; Kawashima & Yamanaka, 1994; Yokoyama, Sakaguchi, et al., 1994a). However, the process of IMP generation in scallops (*Mytilus galloprovincialis*) is controversial (Lazou, 1989), and many studies have drawn contradictory conclusions. Arai (1961) reported that IMP was unlikely to accumulate in scallop (*Patinopecten yessoensis*) due to the absence of AMP deaminase. Wongso, et

al. (1998) also reported that IMP was not detected during storage in scallops (*Pecten albicans*), but other studies indicated that small amounts of IMP accumulated in scallops (Kawashima, et al., 1992, 1994; Pacheco-Aguilar, et al., 2008; Wei, Tian, Yamashita, et al., 2020). Moreover, few studies on IMP decomposition have been reported, although Kawashima, et al. (1994) indicated that the accumulation of HxR and Hx in scallops was thought to be mainly due to endogenous enzymes rather than microbial growth.

As described in chapter 1, ATP degradation in fresh scallop was slow, but it was very fast in frozen-thawed scallop. This phenomenon may occur because the Ca^{2+} uptake ability of the sarcoplasmic reticulum decreases after the freeze-thaw process, which causes the concentration of Ca^{2+} to increase inside the myofibrils, resulting in the generation of Ca^{2+} ion-activated myofibrillar Mg^{2+}-ATPase and the acceleration of ATP decomposition (Kawashima, et al., 1992, 1995). The degree of AMP accumulation leads to HxR and Hx generation during future storage. However, the reason for HxR and Hx generation is unclear. Some studies suggested that the rapid conversion of AMP to HxR and Hx via AdR was found in scallop (Pacheco-Aguilar, et al., 2008), but whether HxR can be generated from IMP decomposition has not been determined. In addition, some studies focused on AMP, IMP and AdR enzyme decomposition have been reported in shellfish. Fujisawa, et al. (1987) indicated low activities of AMP deaminase, 5'-nucleotidase and adenosine deaminase in shellfish compared with those in fish by determining the decrease in the absorbance at 265 nm. Moreover, scallop adenosine deaminase was characterized via its molecular weight, optimum pH and enzyme activity after purification (Yoshida & Aikawa, 1993; Chen, Uchida, Migitaka, Hayashi, & Uwajima, 2000). However, there have been no studies focused on the products of AMP and AdR decomposition determined directly by HPLC. As the composition of ATP-related compounds can be used for quality

evaluation, the breakdown of major adenine nucleotides and their related compounds in postmortem muscles was correlated to the activity of various enzymes in each decomposition process. Therefore, the decomposition of AMP, IMP and AdR substrates by enzyme activity should be studied in detail in scallops.

Therefore, this work attempts to confirm the production of IMP in scallops. Moreover, the enzyme activity related to the breakdown of major adenine nucleotides in scallop adductor muscles was studied in detail by using IMP, AMP and AdR as respective substrates. Additionally, the effects of heat treatment and metal ions on AMP and AdR decomposition by enzyme activity were also investigated.

2.1 Effect of antibiotics and storage temperature on AMP decomposition and IMP accumulation in frozen-thawed scallop muscle.

The results of the AMP decomposition and IMP accumulation in frozen-thawed scallop muscle washed with sterilized seawater with or without 150 ppm antibiotics are shown in Figure 2-1. The IMP contents in all groups were approximately 0.18-0.22 μmol/g after thawing in ice-water (0°C) and remained constant at approximately 0.22-0.35 μmol/g during the 24-h storage period regardless of the storage temperature. The AMP decomposition rate of scallop at 20°C was faster than that at 4°C, possibly due to the higher enzyme activity at 20°C. No effects of antibiotics on AMP decomposition were observed.

Arai (1961) reported the presence of adenosine deaminase with high activity and the absence of AMP deaminase, which resulted in the absence of IMP, in Japanese scallop adductor muscle. Lazou

(1989) also reported that low AMP deaminase activity was found in most invertebrate tissues. However, Pacheco-Aguilar reported that a low concentration of IMP (0.2 μmol/g) was found in the Pacific lions-paw scallop during storage at 0°C for 15 days (Pacheco-Aguilar, et al., 2008). In addition, in a previous study, chopped scallop adductor muscle was mixed with 1% chloramphenicol at 5°C and 10°C for 10 days, and 1% IMP was detected at the beginning of storage, but the effect of temperature on the IMP generation rate was not discussed (Kawashima, et al., 1994). Kawashima, et al. (1992) suggested that AMP degraded to HxR through two pathways because both IMP and AdR were detected during storage. In our study, a low concentration of IMP was generated due to endogenous enzymes rather than bacterial enzymes regardless of storage temperature. However, AdR was not detected, probably because of the high activity of adenosine deaminase in scallop; once AdR was generated, it was immediately decomposed into HxR and Hx. Therefore, further research on the biochemical properties of AdR deaminase is necessary.

2.2 Decomposition of AMP, IMP and AdR in scallop muscle tissue solution, myofibrillar proteins and sarcoplasmic proteins

To detect the AdR deaminase activity, crude enzyme from scallop striated adductor muscle was prepared by homogenization with 10 mM sodium phosphate buffer (pH 7.0). The homogenate was separated into three parts by centrifugation as follows: 1) scallop muscle tissue solution (suspended solution); 2) myofibrillar proteins (precipitate) and 3) sarcoplasmic proteins (supernatant). Substrates containing 4 mM AMP and IMP were also used to compare the decomposition ability of AdR deaminase. The AMP, IMP and AdR contents in scallop muscle tissue solution (suspended solution),

myofibrillar proteins (precipitate) and sarcoplasmic proteins (supernatant) after 24 h of incubation at 25°C are shown in Figure 2-2. AMP decomposed in the three solution types, while HxR and Hx accumulated. The AMP contents in the suspended solution decreased from 4.35±0.26 mM to 1.82±0.30 mM, and this decrease was slower than that in the supernatant, which showed a decrease from 3.97±0.18 mM to 0.48±0.08 mM. AMP decomposed more slowly in the precipitate, in which the contents decreased from 3.98±0.25 mM to 3.05±0.12 mM. These results suggested that the AMP decomposition enzyme in scallops is mainly a water-soluble protein.

However, the IMP concentration remained at 4.06±0.04 mM in the suspended solution, precipitate (myofibrillar protein) and supernatant (sarcoplasmic protein) and did not significantly differ among them ($p > 0.05$). Wu, Zhang, Shi, Ebitani and Konno (2016) indicated that IMP decomposition was caused by endogenous enzymes and bacterial enzymes in fish muscle. In our study, the lack of IMP decomposition suggested that scallop IMP 5-nucleotidase has low activity. In contrast, AdR decomposed rapidly in all three solutions. The initial AdR concentrations in the suspended solution and supernatant were 3.93±0.27 mM and 3.92±0.25 mM, respectively, and the AdR decomposed completely within 1 h, leading to the accumulation of HxR (3.50±0.27 mM in the suspended solution and 3.37±0.31 mM in the supernatant) and Hx (0.51±0.09 mM in the suspended solution and 3.44±0.10 mM in the supernatant). In contrast, the total decomposition of AdR required 4 h in the precipitate. The residual amounts of the corresponding enzyme in the precipitate could be attributed to the slow decomposition of AdR. Nevertheless, the results suggested that AdR deaminase is a water-soluble protein exhibiting high activity in scallop. However, although no AdR was detected during AMP decomposition in our study, a high rate of AdR decomposition in the suspended solution and supernatant indicated that scallop adenosine deaminase has high activity, which suggested a rapid

conversion of AMP to HxR and Hx via AdR in scallop. Similar results have also been reported for muscle from Pacific lion-paw scallop (Pacheco-Aguilar, et al., 2008). According to the above results, there are two AMP decomposition reaction pathways that occur within postmortem scallop, and the AdR pathway is the faster of the two pathways.

2.3 Comparison of AdR decomposition rates in scallop muscle extracellular solution

To elucidate whether scallop AdR deaminase is intracellular or extracellular and to compare the differences in AdR deaminase activity, the AdR and HxR contents were measured in fresh and frozen-thawed scallop soaking solutions. After soaking for 1.5 h at 4°C, the AdR contents in fresh scallop soaking solution was not reduced (Figure 2-3A), and no HxR accumulated, which indicated that scallop AdR deaminase is an intracellular enzyme. However, in the frozen-thawed scallop soaking solution, after 1.5 h soaking (Figure 2-3B), the AdR contents decreased from 3.98±0.12 mM to 3.15±0.05 mM within 120 min.

After soaking scallops for 24 h at 4°C, the protein concentration in the soaking solution increased to 0.54±0.02 mg/mL for the fresh scallop group and 2.22±0.02 mg/mL for the frozen-thawed group due to cell damage during soaking. Consequently, AdR deaminase also increased in the soaking solution, which decomposed AdR from 3.99±0.11 mM to 1.72±0.23 mM with HxR accumulating to 0.94±0.05 mM for the fresh scallop soaking solution. The AdR decomposed completely within 120 min with HxR accumulating to 3.06±0.13 mM in the frozen-thawed scallop soaking solution. Therefore, the decomposition rate of AdR in the frozen-thawed scallop soaking solution was higher than that in the fresh scallop soaking solution, which might result from the increase in AdR deaminase

due to the damage to scallop muscle cells during soaking, especially for the frozen-thawed group. The above results indicated that AdR deaminase might be an intracellular enzyme that is released into the soaking solution.

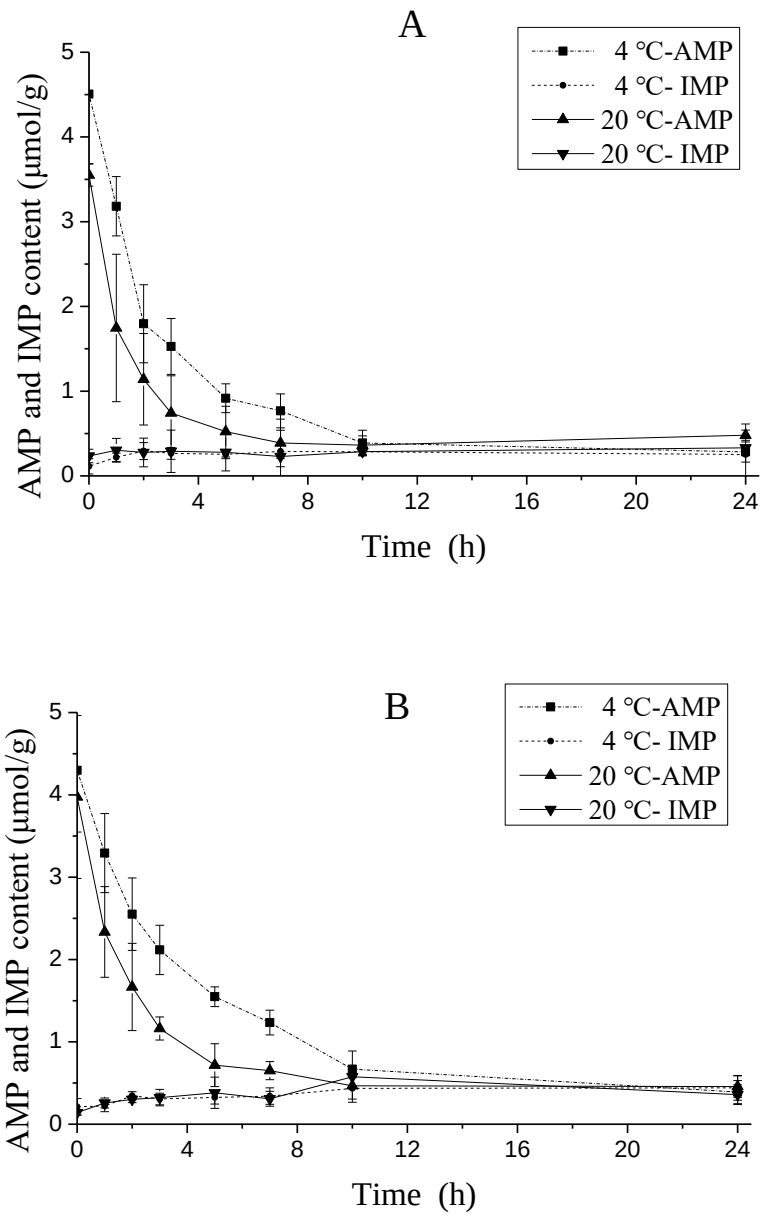

Figure 2-1 Effect of antibiotics and storage temperature on AMP decomposition and IMP accumulation in frozen-thawed scallop muscle during storage for 24 h.

A. The scallops were washed by sterilized seawater with 150 ppm antibiotics;
B. The scallops were washed by sterilized seawater.

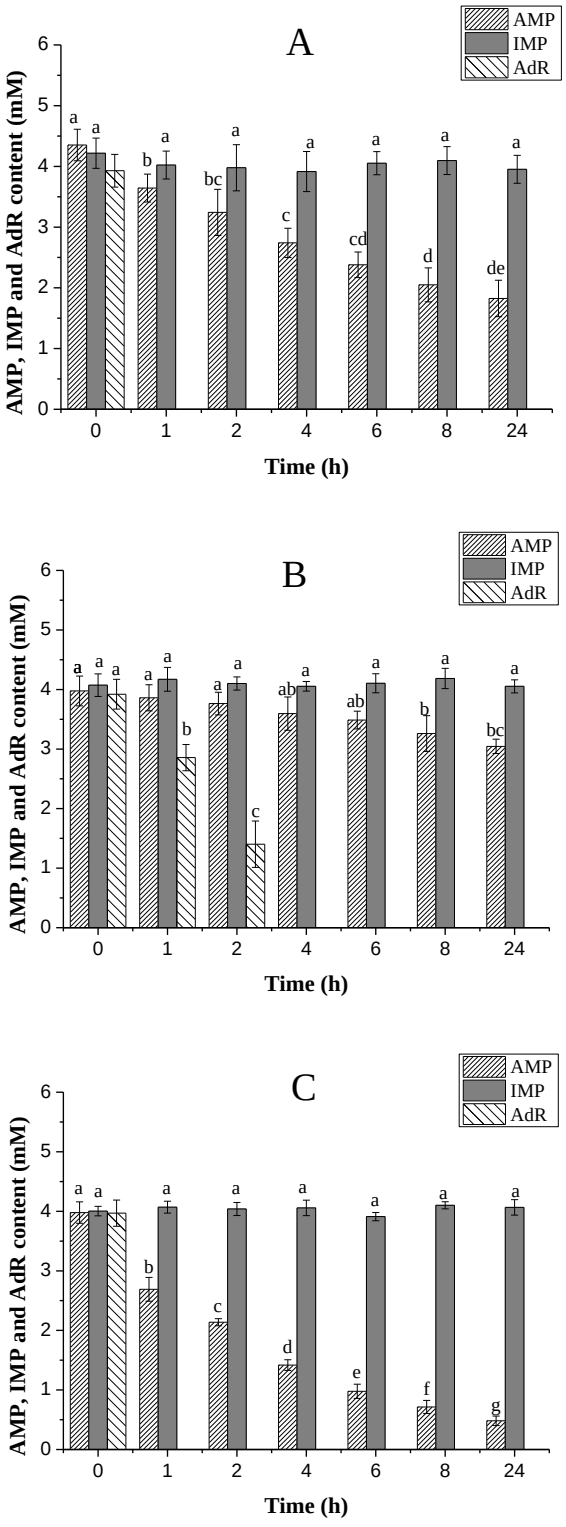

Figure 2-2 Decomposition of AMP, IMP and AdR in scallop solution during incubation at 25°C for 24 h.

Different letters (a-g) within the same group indicate significant differences. ($p < 0.05$)
A: Scallop muscle tissue solution (suspended solution); B: Myofibrillar proteins (precipitate); C: Sarcoplasmic proteins (supernatant).

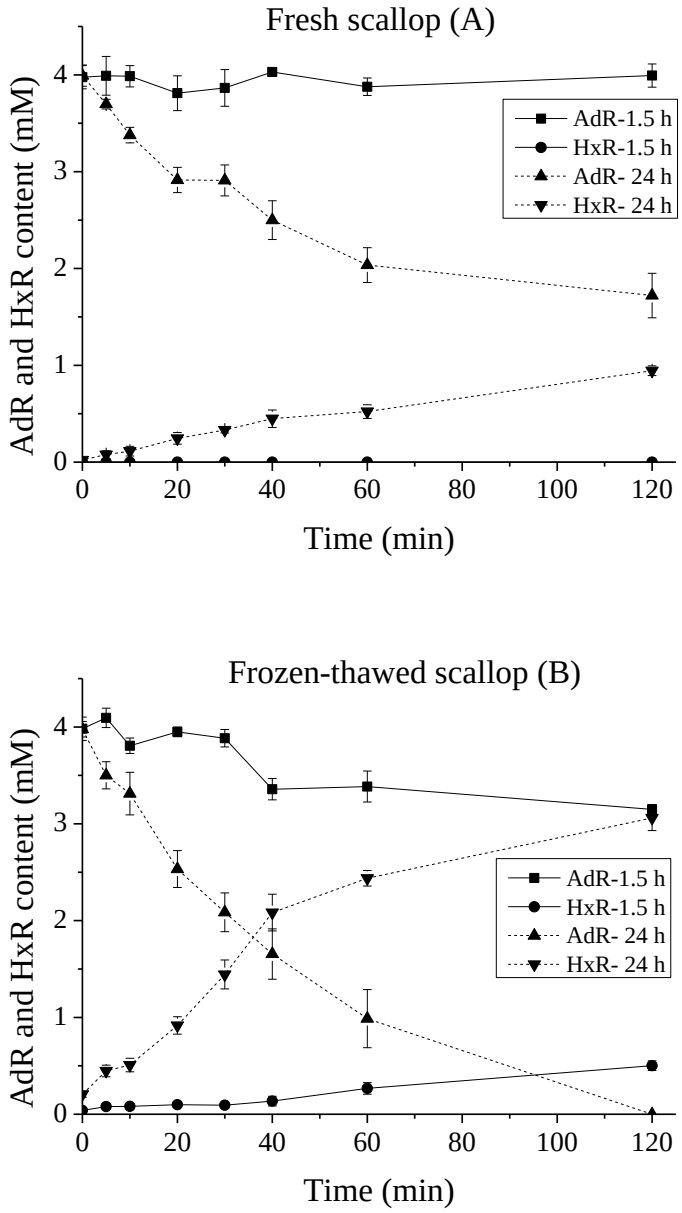

Figure 2-3 Comparison of AdR decomposition rates and HxR generation rate in scallop soaking solution during incubation at 4°C for 120 min.

A: Fresh scallop soaking solution for 1.5 h and 24 h;

B: Frozen-thawed scallop soaking solution for 1.5 h and 24 h;

2.4 Effect of EDTA addition and short-term heating on the AMP decomposition rate

As it is well-known, many cations, such as Na^+, Ca^{2+}, and Mg^{2+}, serve as essential activating ions for specific enzyme activity. Na^+ and Ca^{2+} are the major cations in extracellular fluid, while Mg^{2+} is an essential intracellular cation. Nearly 99% of the total body magnesium is located in the bone or intracellular space (Mccarthy & Kumar, 1999). Physiological metal ions such as Mg^{2+} and Ca^{2+} play a role in kinase activity and regulation. In the above soaking experiments, we found that frozen-thawed scallop muscle cells suffered damage more easily than fresh ones during soaking. We wondered how the divalent metal ions affected the AMP decomposition. To examine the effect of metal ions on the AMP decomposition enzyme in scallop and the enzyme thermal stability, the AMP, IMP, AdR and Hx contents in scallop muscle tissue solution were investigated after the addition of 5 mM EDTA or the application of short-term heating (Figure 2-4). Since AdR could not be detected due to the high AdR decomposition rate, HxR accumulation was measured to determine whether the decomposition of AMP in the crude enzyme solution occurred through the AdR pathway. In the control, the AMP contents decreased from an initial amount of 3.25±0.21 mM to 0.68±0.12 mM over 8 h, while HxR increased to 2.05±0.19 mM at 4 h and then decreased to 1.76±0.16 mM at 8 h. The decrease in the HxR concentration was due to the conversion of HxR to Hx. However, no IMP was detected during the incubation. The AMP concentration changed to 2.36±0.18 mM after the addition of 5 mM EDTA to the crude enzyme solution, which was lower than that of the control, indicating that ADP was generated from AMP at the beginning of the incubation (data not shown). During incubation, AMP decreased to 1.04±0.01 mM within 8 h and IMP increased to 2.94±0.22 mM. The lack of HxR accumulation suggested that AdR generation from AMP decomposition did not occur.

Although a low concentration of HxR was detected at the beginning of incubation, it almost disappeared during storage. These results suggest that AMP decomposes into IMP instead of AdR in the absence of bivalent metal ions; hence, no HxR accumulated during incubation due to the low IMP nucleotidase activity. Some studies have reported that IMP is associated with the umami taste (a pleasant savory taste) associated with fish and shellfish (Howgate, 2006; Hong, et al., 2017), and HxR and Hx are important contributors to off-flavor in fish muscle (Li, Zhang, Lu, Song, & Luo, 2017). Therefore, a suitable process should be developed to obtain high IMP contents to produce scallops with good flavor.

Short-term heating of the crude enzyme solution was performed to test the thermal stability of the enzyme and the possibility of AMP decomposition when the enzymes are denatured (Figure 2-4C, 2-4D). After 1 min of heating, the temperature of the crude enzyme solution increased to 45-50°C, and the AMP contents decreased from 3.08±0.12 mM to 0.88±0.10 mM; the AMP decomposition profile was similar to that of the control solution. However, the AMP contents did not change (3.24±0.04 mM) in the crude enzyme solution after 5 min of heating, and no HxR or IMP accumulated due to enzyme denaturation in all the heated groups.

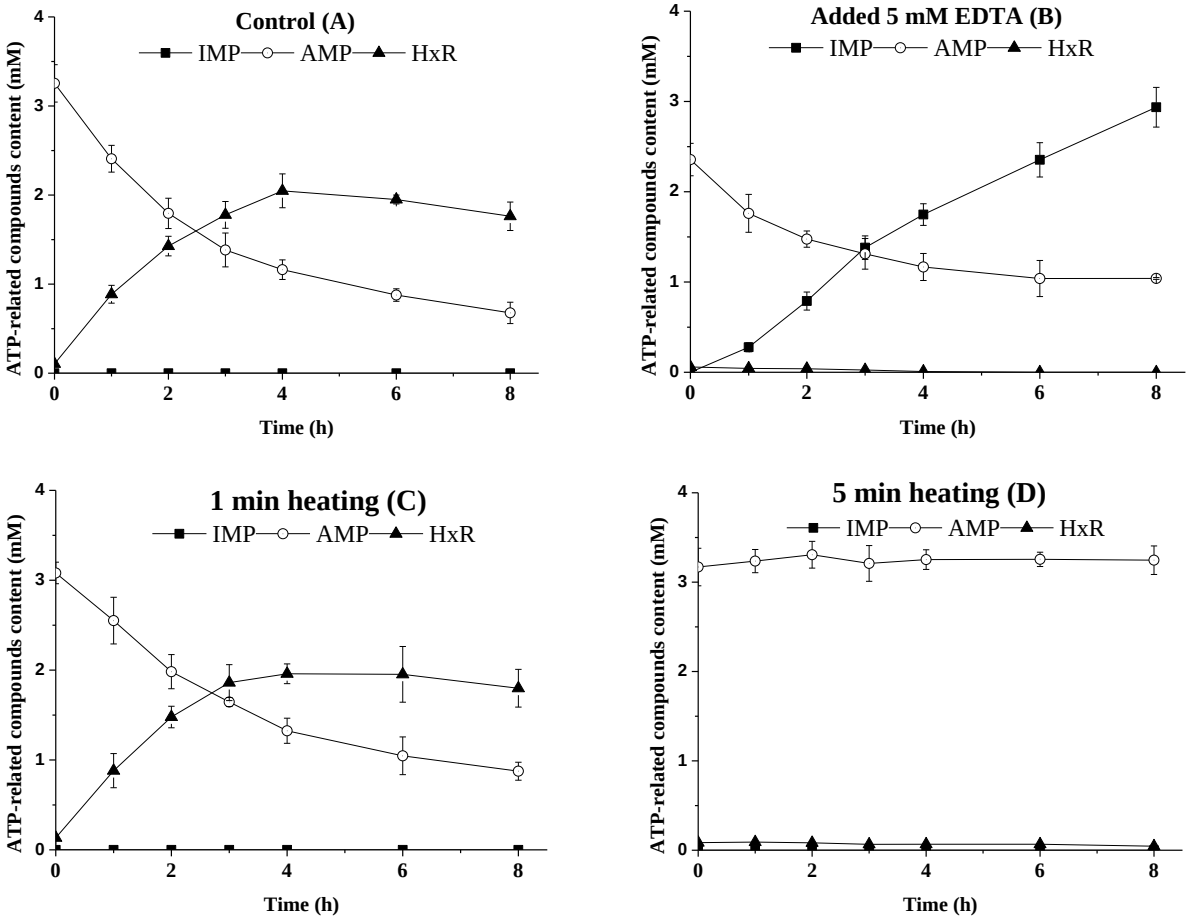

Figure 2-4 Effect of EDTA addition and short-term heating on AMP decomposition rate during incubation at 25°C for 8 h.

A: Scallop muscle tissue solution without any treatment;
B: Scallop muscle tissue solution with 5 mM EDTA;
C: Scallop muscle tissue solution for 1 min heating;
D: Scallop muscle tissue solution for 5 min heating.
Heating temperature: 100 °C

2.5 Effect of EDTA addition and short-term heating on the AdR decomposition rate

As the soaking solution of frozen-thawed scallop showed high AdR deaminase activity, it was used as the scallop crude enzyme to detect the AdR decomposition rate after EDTA addition or short-term heating (Figure 2-5). In the control solution, the initial AdR content was 3.98±0.21 mM, and complete decomposition occurred within 120 min while the HxR contents increased from 0.48±0.09 mM to 4.12±0.33 mM. However, the AdR decomposition rate became slower than that in the control solution after the addition of 5 mM EDTA, with the AdR contents decreasing from 4.15±0.21 mM to 0.61±0.08 mM while the HxR contents increased to 3.49±0.19 mM. This result suggested that bivalent ions can improve the AdR decomposition rate in the crude enzyme solution. The change in AdR in the crude enzyme solution after 1 min of heating was similar to that observed for the control group, while the AdR in the solution remained at 3.95±0.13 mM after 5 min of heating due to the denaturation of AdR deaminase.

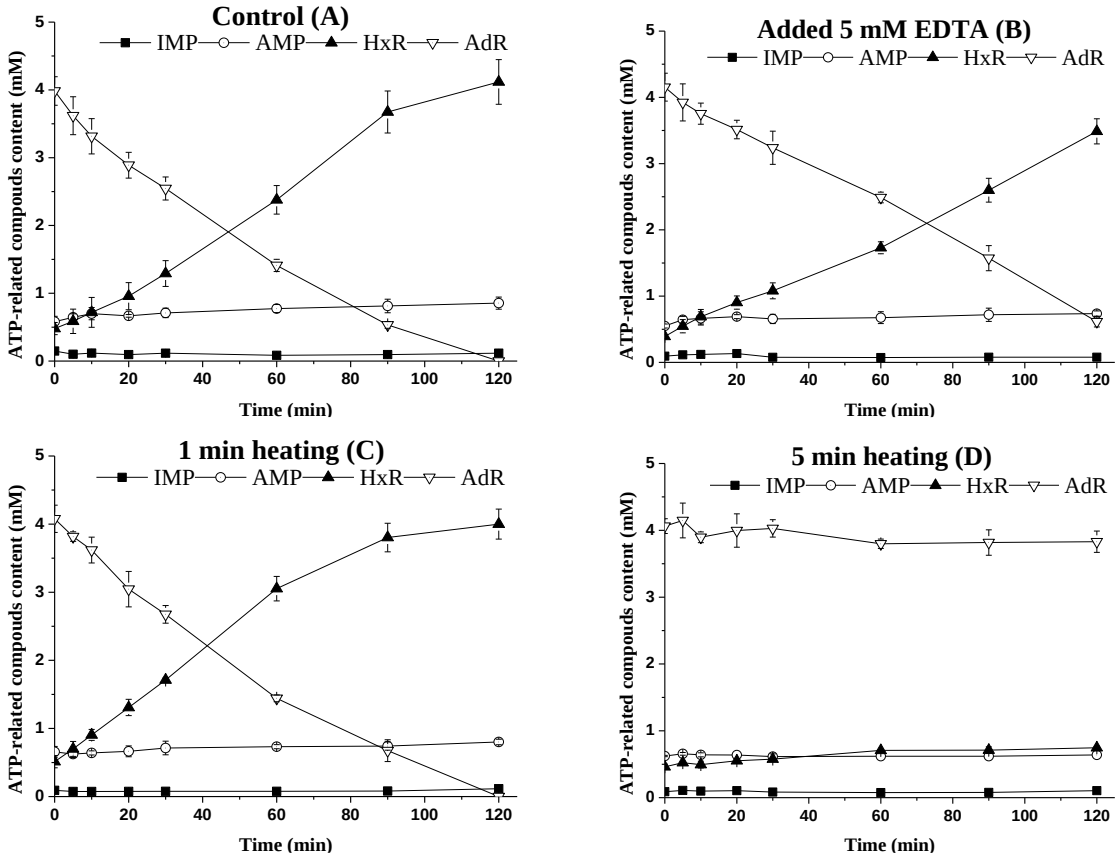

Figure 2-5 Effect of EDTA addition and short-term heating on AdR decomposition rate during incubation at 4°C for 120 min.

A: Scallop muscle soaking solution without any treatment;
B: Scallop muscle soaking solution with 5 mM EDTA;
C: Scallop muscle soaking solution for 1 min heating;
D: Scallop muscle soaking solution for 5 min heating.
Heating temperature: 100°C

2.6 Conclusions

In this study, we confirmed that IMP generation was caused by endogenous enzymes rather than bacterial enzymes. In scallop crude enzyme solution (supernatant), AMP and AdR decomposed simultaneously with HxR generation, while IMP decomposition did not occur during incubation. Because AdR deaminase rapidly converts AdR into HxR, AdR is difficult to detect when AMP decomposes. However, after the addition of 5 mM EDTA, AMP decomposed into IMP in the absence of bivalent metal ions. This is the first study to identify IMP accumulation with the addition of EDTA. Therefore, it is suggested that there are two AMP decomposition pathways in scallop striated muscle. Under natural conditions, AMP decomposes into AdR, but when divalent ions are removed, AMP is converted to IMP. In addition, no IMP 5'-nucleotidase activity was detected in scallop, and it was difficult to generate HxR from IMP in scallop, suggesting that good scallop flavor can be obtained during storage if the AdR pathway is inhibited. Therefore, the influence of metal ions on the AMP decomposition pathway remains to be further studied to improve IMP accumulation and obtain better flavors of scallop products.

Chapter 3 Effect of EGTA Addition and Different Ions on Pathways of AMP Decomposition in Japanese Scallop (*Patinopecten yessoensis*)

Compared to other muscle foods, fish and shellfish are fast-deteriorating or perishable materials (Cheng, Sun, Zeng, & Liu, 2015). Fish or shellfish will enter into rigor mortis within a few hours or several days, correlated with flexibility lose and ATP-related compounds decomposition (Hong, et al., 2017). The concentrations of ATP and its breakdown products are used extensively to calculate a number of different specific indices of freshness (Saito, Arai, & Matsuyoshi, 1959), such as K value (Blafsdbttir, et al., 1997), AEC value (Yokoyama, et al., 1992), Hx/AMP (Yokoyama, Takahashi, Sakaguchi, Kawai, & Kanamori, 1994) etc. In the muscle of fish, AMP was converted to HxR and Hx via IMP. (Saito, 1961). But for the marine invertebrates muscle, AMP was accumulated instead of IMP and a major route for conversion of AMP to HxR via adenosine (AdR) (Kawashima, et al., 1995; Vidode Mattio, et al., 2001). Moreover, both IMP pathway and AdR pathway are co-existed in some marine invertebrates, such as scallop (Pacheco-Aguilar, et al., 2008), abalone (Watanabe, et al., 1992), prawn (Matsumoto, et al., 1990), et al.

For scallop, even if there are two pathways of ATP decomposition, IMP accumulation is still very small (Wei, et al., 2020). IMP, as a kind of umami substances, have been widely used as enhancers to make foods more palatable (Kuchiba-manabe, Matoba, & Hasegawa, 1991). Development of a suitable produce good flavor scallops with high IMP contents is necessary. However, few studies focus on IMP accumulation in scallop.

As described in chapter 2, IMP generation rate increased dramatically in scallop crude enzyme solution containing 5 mM EDTA, which suggested that AMP will decompose to IMP if metal ions

concentration change. But there are few studies focused on the effect of different ions and microbial activity during incubation on AMP decomposition pathway. In addition, some studies reported that the molecular weight of adenosine deaminase (ADA, EC 3.5.4.4) in scallop adductor muscle has been estimated to be 35-38 kDa by SDS-PAGE (Sato & aikawa, 1991; Yoshida, et al., 1993), but there was no characterization by proteomic analysis directly. Whether the cause of IMP accumulation is due to the inhibition of adenosine deaminase activity and the cessation of the AdR pathway is still unclear.

Therefore, in this study, effect of EGTA on AMP pathway was investigated. Different ions (K^+, Ca^{2+} and Mg^{2+}) related to the breakdown of AMP in non-ions scallop enzyme solution was studied in detail. Moreover, the protein composition of scallop enzyme solution during dialysis and incubation was detected by SDS-PAGE, and 40 kDa fraction was detected by LC-MS/MS directly.

3.1 Effect of EGTA addition on AMP decomposition

To examine the effect of ions except Ca^{2+} on the AMP decomposition rate in scallop, the ATP-related compounds contents in scallop muscle tissue solution was investigated after the addition of 5 mM EGTA (Figure. 3-1). Since AdR could not be detected due to the high activity of adenosine deaminase (Arai, 1961), HxR and Hx accumulation was measured to judge whether the decomposition of AMP in the crude enzyme solution occurred through the AdR pathway. For the control group, AMP content decreased from the initial 3.01±0.12 mM to 1.98±0.19 mM over 8 h, while HxR content increased to 1.25±0.08 mM. Hx content also increased to 1.25±0.12 mM due to the conversion of HxR at 24 h. However, little IMP (less than 0.2 mM) was detected during incubation. For the CP group, AMP content decreased from 3.08±0.11 mM to 2.38±0.21 mM at 4 h with. However, IMP concentration became 0.74±0.15 mM due to AMP decomposition within 4 h and keep stable in

latter incubation, which is higher than that of control group. HxR and Hx content increased to 0.98±0.19 mM and 0.52±0.06 mM at 8 h suggested that removal of calcium ions has little effect on the AdR pathway. But the accumulation of HxR and Hx was lower than that of control group suggested that IMP generation will decrease the AdR generation rate. However, Wei, Tian, Lin, et al. (2020) reported that IMP content increased to 2.94±0.22 mM correlate with AMP content decreased to 1.04±0.01 mM in 10 mM sodium phosphate buffer (pH 7.0) containing 5 mM EDTA suggested that the absence of bivalent metal ions will affect the AMP pathway. But how the exact ions affected the AMP decomposition pathway needs further study.

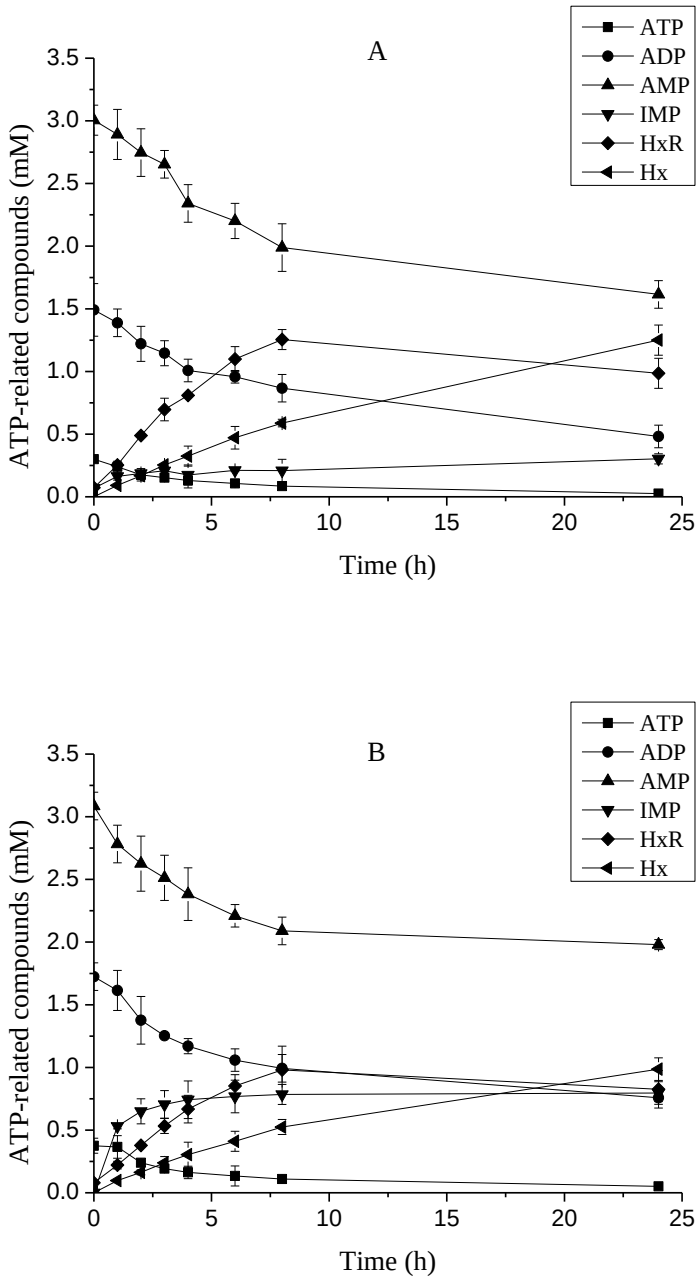

Figure 3-1 Effect of EGTA addition on AMP decomposition.

A: Scallop muscle tissue solution without any treatment;
B: Scallop muscle tissue solution with 5 mM EGTA.

3.2 Changes in protein composition of crude enzyme during dialysis and incubation

Changes in protein content of scallop crude enzyme solution during dialysis was examined and shown in Figure 3-2A. The initial protein concentration was 9.31±0.04 mg/mL and 9.29±0.02 mg/mL in whole enzyme solution and supernatant, and without significant different ($p > 0.05$). After 8 h, protein content decreased to 7.75±0.27 mg/mL was caused by the sodium phosphate buffer passes through the membrane into the enzyme solution. However, protein content in supernatant was 6.36±0.14 mg/mL, which was lower than crude enzyme solution, suggested that protein proteins precipitated during dialysis. The final protein content was 7.44±0.04 mg/mL and 5.22±0.34 mg/mL in crude enzyme solution and supernatant after dialysis, respectively.

The result of changes in protein content of crude enzyme solution and supernatant were shown in Figure 3-2B. At the end of dialysis, the crude enzyme solution (D24) was divided into two groups: one without any treatment (C) and one with 0.1% chloramphenicol (CP), and storage at 25℃ for 24 h. Protein content of crude enzyme solution in C group and CP group was 7.35±0.27 with no significant difference ($p > 0.05$). For the supernatant of these two group, protein content kept 5.22±0.34 mg/mL before incubation 8 h in C group and CP group. However, protein content of supernatant of C group decreased to 2.91±0.04 mg/mL, while that of CP group decreased to 5.26±0.24 mg/mL. This phenomenon might be caused by microbial activity in C group, which led to protein denaturation. Therefore, the protein composition of crude enzyme solution during dialysis and incubation need to be studied. Moreover, this decreases of protein content in supernatant will affect its AMP decomposition pathway.

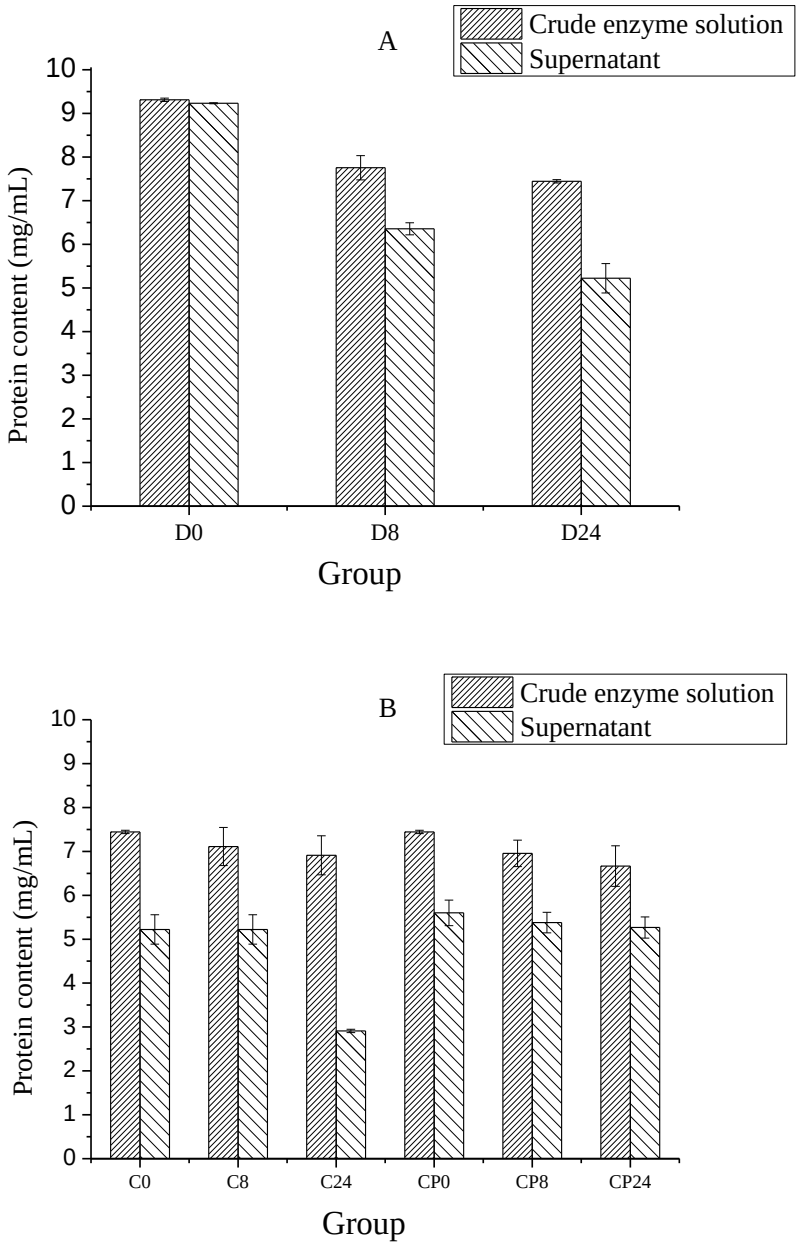

Figure 3-2 Changes in protein content of scallop tissue solution during dialysis and incubation.

A: Changes in protein content of crude enzyme solution and supernatant during dialysis;
B: Changes in protein content of crude enzyme solution and supernatant during dialysis; C: control group (without any treatment); CP: with 0.1% chloramphenicol
Number 0, 8 and 24 means storage for 0, 8 and 24 h.

3.3 Changes in protein composition of crude enzyme solution, supernatant and precipitate during dialysis and incubation

The SDS-PAGE results of scallop crude enzyme, supernatant and precipitate were shown in Figure 3-3. The crude enzyme were mainly water-soluble protein and the molecular weight were near 89kDa, 50kDa, 40kDa, 30kDa and 20kDa. During dialysis, the composition of crude enzyme solution and supernatant were not changed during dialysis (Figure 3-3A). Maybe due to less microbial activities in low temperature (4 ℃). The protein composition of crude enzyme solution, supernatant and precipitate were not change at 0, 4 and 12 h incubation (Figure 3-3 C group and Figure 3-3 CP group). However, when incubation time more than 16 h, 40 kDa fragment of supernatant in C group was decreased gradually light in supernatant with the same part of precipitate increased gradually dark, while that of CP group was not. Which suggested that protein denaturation might cause by microbial activity. Some studies reported that molecular weight of adenosine deaminase (EC 3.5.4.4) in scallop adductor muscle was near 40 kDa (Yoshida, et al., 1993; Chen, et al., 2000). However, the reason that changes in protein composition and 40 kDa fragment disappear need subsequent analysis.

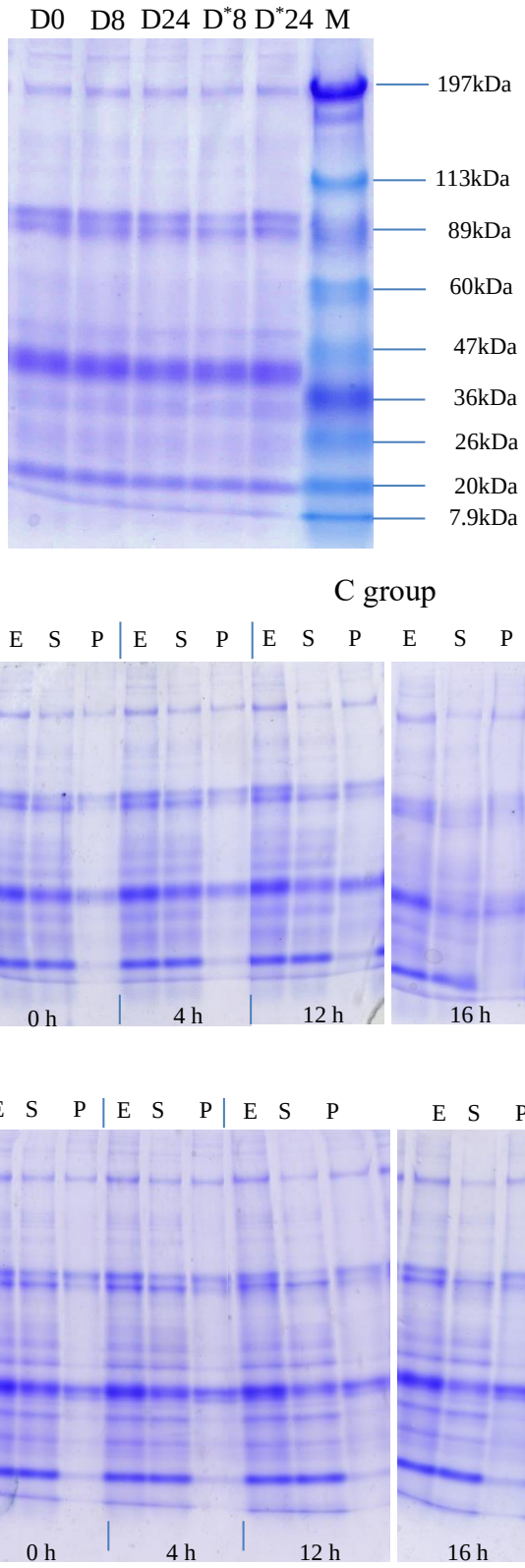

Figure 3-3 Changes in protein compounds of crude enzyme solution during dialysis and incubation.

M: protein marker; D: Protein during dialyzing; " * ": supernatant protein after centrifugation; E: crude enzyme solution; S:supernatant; P: precipitate; Number means storage time. Protein marker (Nacalal Tesque, Inc. Japan): myosin (197 kDa); β- galactosidase (113 kDa); bovine serum albumin (89 kDa); glutamate dehydrogenase (60 kDa); ovalbumin (47 kDa); Carbonic anhydrase (36 kDa); myoglobin (26 kDa); lysozyme (20 kDa); aprotinin (7.9 kDa).

3.4 Effect of microbial activities and K^+ ions on AMP decomposition

The results of effect of microbial activities and K^+ ions on AMP decomposition was shown in Table 3-1. Since AdR could not be detected due to the high AdR decomposition rate, HxR accumulation was measured to determine whether the decomposition of AMP in the crude enzyme solution occurred through the AdR pathway (Wei, Tian, Lin, et al., 2020). When the phosphate buffer (pH 7.0) containing 10 mM sodium ions, the AMP content decreased from initial 3.01±0.12 mM to 2.82±0.21 mM with 0.25±0.04 mM IMP generated at 8 h in Na^+-C group. However, IMP accumulation reached to 2.09±0.12 mM at 24 h due amount of AMP decomposition, and no HxR was detected. Therefore, the decomposition of AMP to IMP might be caused by microbial activity. The same phenomenon occurred in K^+-C group but IMP accumulation was 2.93±0.12 mM, which is higher than that of Na^+-C group. However, in phosphate buffer (pH 7.0) containing 0.1% chloramphenicol solution, AMP content of Na^+-CP group decreased from 3.00±0.19 mM to 2.55±0.21 mM with 0.57±0.13 mM IMP accumulation. AMP content of K^+-CP group decreased from 2.99±0.15 mM to 2.06±0.18 mM, but IMP accumulation reached 1.47±0.26 mM. IMP generation in K^+-CP group was higher than that of Na^+-CP suggested that K^+ can promote the decomposition of AMP to IMP. A small amount (less than 0.5 mM) of HxR and Hx in this two CP group were detected indicated that IMP pathway and AdR pathway co-exist in the absence of microbial activity.

Table 3-1 Effect of microbial activities on ATP-related compounds decomposition.

Group	Time (h)	ATP	ADP	AMP	IMP	HxR	Hx
	0	N/A	N/A	3.01±0.12 ^{a}	N/A	N/A	N/A
Na^{+}-C	8	0.03±0.01^{a}	0.19±0.04 ^{a}	2.82±0.21 ^{b}	0.25±0.04 ^{b}	0.15±0.02 ^{a}	0.09±0.01 ^{b}
	24	0.02±0.01 ^{a}	0.08±0.04 ^{b}	1.18±0.15 ^{c}	2.09±0.12 ^{a}	N/A	0.33±0.10 ^{a}
	0	N/A	N/A	3.00±0.19 ^{a}	N/A	N/A	N/A
Na^{+}-CP	8	0.02±0.01 ^{a}	0.16±0.03 ^{a}	2.73±0.18 ^{b}	0.24±0.02 ^{b}	0.14±0.03 ^{b}	0.02±0.01 ^{b}
	24	0.02±0.01 ^{a}	0.09±0.03 ^{b}	2.55±0.21 ^{c}	0.57±0.13 ^{a}	0.21±0.05 ^{a}	0.14±0.03 ^{a}
	0	N/A	N/A	3.07±0.20 ^{a}	N/A	N/A	N/A
K^{+}-C	8	0.07±0.01 ^{a}	0.17±0.06 ^{ab}	2.73±0.11 ^{b}	0.42±0.11 ^{b}	0.30±0.08 ^{a}	N/A
	24	0.07±0.02 ^{a}	0.23±0.10 ^{a}	0.86±0.31 ^{c}	2.93±0.43 ^{a}	N/A	0.46±0.14 ^{a}
	0	N/A	N/A	2.99±0.15 ^{a}	N/A	N/A	N/A
K^{+}-CP	8	0.06±0.01 ^{a}	0.10±0.06 ^{ab}	2.71±0.21 ^{ab}	0.35±0.12 ^{b}	0.27±0.17 ^{ab}	0.24±0.10 ^{a}
	24	0.06±0.02 ^{a}	0.17±0.03 ^{a}	2.06±0.18 ^{b}	1.47±0.26 ^{a}	0.37±0.15 ^{a}	0.24±0.05 ^{a}

*Different letters (a-c) within the same column in the same group indicate significant differences ($p < 0.05$)

C: control group (without any treatment); CP: with 0.1% chloramphenicol

3.5 Effect of different metal ions on AMP decomposition

The effects of metal ions on AMP decomposition of dialysis scallop crude enzyme were shown in Table. 3-2. The initial AMP content in each group was 2.92±0.11 mM (Ca group), 2.97±0.16 mM (Mg group) and 2.97±0.21 mM (mix group), and without significant difference ($p > 0.05$), while other ATP-related compounds were not detected. A small amount of ATP and ADP were detected in three groups because of AMP conversion at 8 h incubation. AMP content decreased to 2.88±0.17 mM, 2.04±0.11 mM and 2.22±0.11mM in Ca group. Less IMP (less than 0.1 mM) generated in this three groups suggested that Ca^{2+} and Mg^{2+} had no effect on AMP converts to IMP. However, the HxR content in Mg group was 0.60±0.09 mM, which was higher than that in mix group (0.43±0.09 mM) and in Ca group (0.15±0.02 mM), indicated that magnesium ions promote the AdR pathway while calcium ions do not. After incubation for 24 h, AMP content in Mg group was 1.20±0.11 mM, which was lower than that in mix group (1.78±0.16 mM) and in Ca group (2.43±0.26 mM). Moreover, the generation amount of HxR and Hx of Mg group is also higher than that of the other two groups while IMP content were similar within these three groups, which indicated that calcium ion has an inhibitory effect on the decomposition of AMP. Wei, Tian, Lin, et al. (2020) also reported that the AMP decomposition rate in supernatant of homogenized scallop adductor muscle was higher that suspended solution, which may be due to the large amount of calcium ions attached to myofibrils that affect the decomposition rate of AMP.

3.6 Identification of adenosine deaminase in crude enzyme solution by LC-MS/MS

The quantification of 40 kDa fragment of scallop crude enzyme solution was performed by LC-

MS/MS technique (Table 3-3). The results shown that adenosine deaminase was detected in this fraction which molecular weight was 41.5 kDa. Moreover, adenosine kinase was also detected with 46.9 kDa of molecular weight. Exist of adenosine deaminase and adenosine kinase suggested that AdR can be decomposed in scallop. Many studies reported high adenosine deaminase activity in scallop (Arch & Newsholme, 1987; Sato, et al., 1991; Wei, Tian, Lin, et al., 2020). Pacheco-Aguilar, et al. (2008) also indicated a rapid conversion of AMP to HxR and Hx via AdR in Pacific lion-paw scallop. Therefore, another reason of a large amount of IMP accumulated in crude enzyme solution might be cause by denaturation of adenosine deaminase.

3.7 Conclusions

In this study, in the crude enzyme solution of scallop without calcium ion, IMP pathway and AdR pathway coexist, and a small amount of IMP accumulates in a short time due to the decomposition of AMP. Moreover, effect of different ions and microbial activity on decomposition of AMP was studied in dialyzed scallop crude enzyme solution. The results indicated that microbial growth led to a large amount of IMP accumulated after incubating for more than 16 h incubation at 25 ℃. Potassium ions can promote IMP pathway, magnesium ions can promote the decomposition AdR pathway, but calcium ions can slightly inhibit the decomposition of AMP. During dialysis and incubation, the enzyme gradually denatured due to the growth of microorganisms, which result in a reduction of protein content in supernatant of scallop crude enzyme solution, and the denatured protein contain adenosine deaminase was confirmed by LC-MS/MS with 41.5 kDa of molecular weight. Therefore, changing the ions concentration or inhibiting the activity of adenosine deaminase in scallop will improve IMP accumulation and obtain better flavors of scallop products.

Table 3-2 Effect of $CaCl_2$ and $MgCl_2$ on ATP-related compounds decomposition.

Group	Time (h)	ATP	ADP	AMP	IMP	HxR	Hx
Added 1 mM $CaCl_2$	0	N/A	N/A	2.92±0.11 [a]	N/A	N/A	N/A
	8	0.02±0.01 [a]	0.09±0.02 [b]	2.88±0.17 [ab]	0.10±0.01 [b]	0.13±0.03 [a]	N/A
	24	0.02±0.01 [a]	0.16±0.02 [a]	2.43±0.26 [b]	0.28±0.07 [a]	0.15±0.02 [a]	0.15±0.02 [a]
Added 6 mM $MgCl_2$	0	N/A	N/A	2.97±0.16 [a]	N/A	N/A	N/A
	8	0.02±0.01 [a]	0.14±0.02 [a]	2.04±0.11 [b]	0.08±0.01 [b]	0.60±0.09 [a]	0.31±0.06 [b]
	24	0.02±0.01 [a]	0.11±0.03 [a]	1.20±0.11 [c]	0.13±0.03 [a]	0.67±0.11 [a]	0.95±0.21 [a]
Added 1 mM $CaCl_2$ and 6 mM $MgCl_2$	0	N/A	N/A	2.97±0.21 [a]	N/A	N/A	N/A
	8	0.02±0.01 [a]	0.16±0.05 [a]	2.22±0.11 [b]	0.07±0.01 [b]	0.43±0.09 [a]	0.20±0.03 [b]
	24	0.02±0.01 [a]	0.12±0.03 [ab]	1.78±0.16 [c]	0.14±0.02 [a]	0.48±0.07 [a]	0.61±0.08 [a]

*Different letters (a-c) within the same column in the same group indicate significant differences ($p < 0.05$)

Table 3-3 Identification of 40 kDa fragment of scallop crude enzyme solution by LC-MS/MS.

Accession	Number of detected peptides	Mass (Da)	Description
ACT_PLAMG	17	42077	Actin, adductor muscle OS=Placopecten magellanicus OX=6577 PE=2 SV=1
ACT3A_HELAM	16	42148	Actin, cytoplasmic A3a OS=Helicoverpa armigera OX=29058 GN=actA3a PE=2 SV=1
OCDH_MIZYE	16	43845	Octopine dehydrogenase OS=Mizuhopecten yessoensis OX=6573 GN=odh PE=2 SV=1
ACT_MAYDE	15	42132	Actin OS=Mayetiola destructor OX=39758 PE=2 SV=1
KARG_HALMK	2	40245	Arginine kinase OS=Haliotis madaka OX=81897 PE=2 SV=1
Adenosine deaminase	4	41503	Adenosinedeaminase [*Patinopecten yessoensis*]
Adenosine kinase	1	46903	Adenosinekinase [*Patinopecten yessoensis*]

Chapter 4 Changes in Biochemical Properties and Microstructure of Freeze-dried Scallop Adductor Muscle during Room Temperature Storage and Rehydration Process

In general, aquatic products are highly susceptible to mechanical damage and microbial spoilage thus perishable due to its high moisture content (Hong, et al., 2017). To improve the shelf-life of aquatic products, fish and marine invertebrate was stored in frozen or low temperature (Lee & Park, 2016). However, storage in low temperature or frozen for long time is very expensive. Therefore, developed new methods which was low cost is necessary.

Nowadays, drying technology has been successfully applied to food products due to reduction of moisture content, so that the food can be safe storage for a long time even in room temperature. Moreover, Reduction in weight and volume resulting in lower costs of package, storage and transportation (Mayor, et al., 2004; Taheri-Garavand, et al., 2011; Zhang, et al., 2017). Many drying technology were used for vegetables (Chen, Guo, & Wu, 2016), fruits (Asami, Hong, Barrett, & Mitchell, 2003) and aquatic products (Ma, Qu, & Sun, 2017). However, unsuitable drying process can induce quality degradation, such as oxidation, loss of nutritional and functional properties, changes in structure, et al (Miranda, Maureira, Rodríguez, & Vega-Gálvez, 2009). Some studies reported that freeze-drying is one of dehydration methods which can obtain highest quality products (little structure change, good rehydration capacity and minimal loss in nutritional qualities) compared with other methods (Rahman, 2006; Abbasi & Azari, 2009; Crapo, et al., 2010). However, there are few studies on the quality change of aquatic products after freeze-dried. Moreover, rehydration is one of the most important quality properties for dehydration products (Deng, et al., 2014), but few studies

focus on the changes in biochemical properties of freeze-dried aquatic production during rehydration process.

Therefore, in this study, the ATP-related compounds of freeze-dried scallop was investigated during room temperature storage and rehydration process. In addition, salt solubility, Ca^{2+}-ATPase activity and denaturation profiles of myofibrillar protein of fresh scallop, frozen-thawed scallop and rehydrated scallop were compared. At last, the microstructure of fresh and rehydrated scallops was also observed by transmission electron microscopy.

4.1 Changes in ATP-related compounds content during room temperature storage

Variations in ATP-related compounds of freeze-dried scallop were shown in Table 4-1. Less ATP decomposed in high freshness scallop during freezing and freeze-drying process. The moisture content of scallop was 2.13±0.42% in scallops after freeze-drying process. The ATP content were 3.46±0.11 μmol/g, 3.30±0.65 μmol/g and 3.66±0.17 μmol/g in day 0, 15 and 30, and with no significant difference ($p > 0.05$). Little changes in ADP and AMP content were also observed in freeze-dried scallop during storage. Moreover, less than 0.1 μmol/g IMP and no HxR and Hx were detected. The results showed that the ATP-related compounds of freeze-dried scallops during normal temperature storage did not change much when the humidity was less than 10%, so that scallops can still maintain a high freshness. This may be due to the lower activity of enzymes involved in the decomposition of ATP-related compounds at lower water activities. The similar results was reported by (Arai, et al., 1968), who found that the ATP content in scallop muscle kept 4.52 μmol/g when storage in desiccator for 5 months.

Table 4-1 Changes in ATP-related compounds content (μmol/g) of freeze-dried scallop during room temperature storage.

Time (day)	ATP	ADP	AMP
0	3.46 ±0.11 [ab]	2.16 ±0.08 [a]	0.38 ±0.01 [a]
1	3.86 ±0.34 [a]	2.68 ±0.04 [a]	0.41 ±0.09 [a]
2	3.70 ±0.07 [a]	2.40 ±0.30 [a]	0.38 ±0.01 [a]
3	3.75 ±0.22 [a]	2.36 ±0.08 [a]	0.37 ±0.10 [a]
5	3.82 ±0.03 [a]	2.35 ±0.13 [a]	0.39 ±0.01 [a]
7	3.49 ±0.32 [ab]	2.15 ±0.10 [a]	0.35 ±0.01 [a]
15	3.30 ±0.65 [ab]	1.91 ±0.27 [a]	0.23 ±0.02 [b]
30	3.66 ±0.17 [a]	2.13 ±0.09 [a]	0.28 ±0.01 [b]

Value are given as mean ± standard deviation (n = 3).

*Different letters (a-b) within the same column indicate significant differences ($p < 0.05$)

Storage condition: room temperature (18±1.5℃), humidity less than 10%.

4.2 Changes in ATP-related compounds content of freeze-dried scallop adductor muscle during rehydration and storage.

Table 4-2 shows the changes in ATP-related compounds content of freeze-dried scallop adductor muscle during rehydration and storage. The scallop rehydration rate increased from the initial 21.01±0.35% to 57.73±11.38% after rehydration for 1.5 min. Then the quality of the scallops slowly increased with the extension of the rehydration time, and the rehydration rate reached 74.86±15.33% at 60 min. Before rehydration, ATP and AMP content in freeze-dried scallop were 3.44±0.21 μmol/g and 0.41±0.05 μmol/g, and IMP, HxR and Hx were not detected. After 1.5 minutes of rehydration, the ATP in scallops rapidly decomposed to 0.56±0.21 μmol/g, resulting in AMP content increased to 2.35±0.58 μmol/g, and a small amount of IMP (0.20±0.08 μmol/g) and HxR (0.38±0.04 μmol/g) were also detected. With the rehydration time increasing, AMP content decreased to 1.17±0.35 μmol/g for 60 min. And IMP, HxR and Hx accumulation was 0.45±0.05 μmol/g, 1.12±0.14 μmol/g and 0.46±0.07 μmol/g, respectively. Moreover, for the scallop which rehydrated for 20 min, AMP content decreased to 0.54±0.10 μmol/g, with IMP, HxR and Hx accumulation was 0.47±0.06 μmol/g, 1.69 μmol/g and 0.93 μmol/g. This result suggested that rehydration will not affect AMP decomposition pathway. However, no AdR was detected during rehydration and storage.

Some studies reported that IMP pathway and AdR pathway coexist in invertebrates for both IMP and AdR were detected (Watanabe, et al., 1992; Kawashima, et al., 1994; Yokoyama, Sakaguchi, Kawai, & Kanamori, 1994b). Wei, Tian, Yamashita, et al. (2020) also indicated that AMP decomposed to HxR via AdR. AdR in scallop was difficult to detected because of high activity of adenosine deaminase. During the freeze-drying and rehydration process, the sarcoplasmic reticulum was broken, which caused Ca^{2+} released from sarcoplasmic reticulum to accelerate the decomposition (Watabe,

Ushio, Iwamoto, Yamanaka, & Hashimoto, 1989). The increase of Ca^{2+} concentration in scallop muscles triggers the activation of actin-activated Mg^{2+}-ATPase activity of myosin and resulted in rapid breakdown of ATP.

4.3 Changes in protein salt solubility of freeze-dried scallop during rehydration and storage.

To evaluate the quality of scallop adductor muscle myofibrils protein, salt solubility is a very important index, as shown in Figure 4-1. The results suggested that the salt solubility of myofibrils increased with increasing NaCl concentration. The salt solubility of myofibrils in day 1 and storage 1 day group were 37.84±0.11% and 41.62±0.74% at 1.0 M NaCl. After storage for 15 and 30 day, the solubility of the day 15 and day 30 group were 60.67±3.82% and 52.75±2.73% at 1.0 NaCl. The salt solubility of these four groups was similar to that of fresh scallop but much lower than that of frozen-thawed scallop (Wei, Tian, Yamashita, et al., 2020), indicating that myosin and actin of freeze-dried scallop could not fully dissociate and dissolve at high salt concentrations. Mg-ATP has a strong ability to dissociate myosin from actin, which cause myofibrils to easily dissolve in solution. The results showed that the salt solubility of day 1, 1 day-storage, day 15 and day 30 group with the presence of Mg-ATP at 0.4 M NaCl concentration were 65.82±0.34%, 67.52±3.19%, 73.70±0.53% and 74.51±2.11%, respectively. Moreover, the results suggested that the low salt solubility in freeze-dried scallop without Mg-ATP addition was caused by freeze-drying and rehydration process, and it did not change during refrigeration.

Table 4-2 Changes in ATP-related compounds content (μmol/g) of freeze-dried scallop adductor muscle during rehydration and storage.

Time (min)	ATP	ADP	AMP	IMP	HxR	Hx
0	3.04 ±0.01 [a]	2.25 ±0.43 [a]	0.41 ±0.05 [de]	N/A	N/A	N/A
1.5	0.56 ±0.21 [b]	1.91 ±0.42 [ab]	2.35 ±0.58 [ab]	0.20 ±0.08 [b]	0.38 ±0.04 [e]	N/A
5	0.35 ±0.01 [c]	1.92 ±0.33 [ab]	2.93 ±0.34 [a]	0.19±0.18 [b]	0.52 ±0.03 [d]	N/A
10	0.31 ±0.01 [d]	1.78 ±0.42 [ab]	2.66 ±0.23 [ab]	0.19 ±0.07 [b]	0.65 ±0.11 [c]	N/A
20	0.29 ±0.01 [de]	1.45 ±0.51 [bc]	1.92 ±0.06 [b]	0.17 ±0.15 [b]	0.69 ±0.18 [c]	0.20 ±0.28 [c]
60	0.51 ±0.12 [b]	1.47 ±0.28 [bc]	1.17 ±0.35 [c]	0.45 ±0.05 [a]	1.12 ±0.14 [b]	0.46 ±0.07 [b]
Day 1	0.28 ±0.01 [de]	1.42 ±0.55 [bc]	0.54 ±0.10 [d]	0.47 ±0.06 [a]	1.69 ±0.18 [a]	0.93 ±0.52 [a]

Value are given as mean ± standard deviation (n = 3).

*Different letters (a-c) within the same column indicate significant differences ($p < 0.05$)

Rehydration temperature: 20℃.

Day 1: the rehydrated scallop stored in 5℃ for day 1.

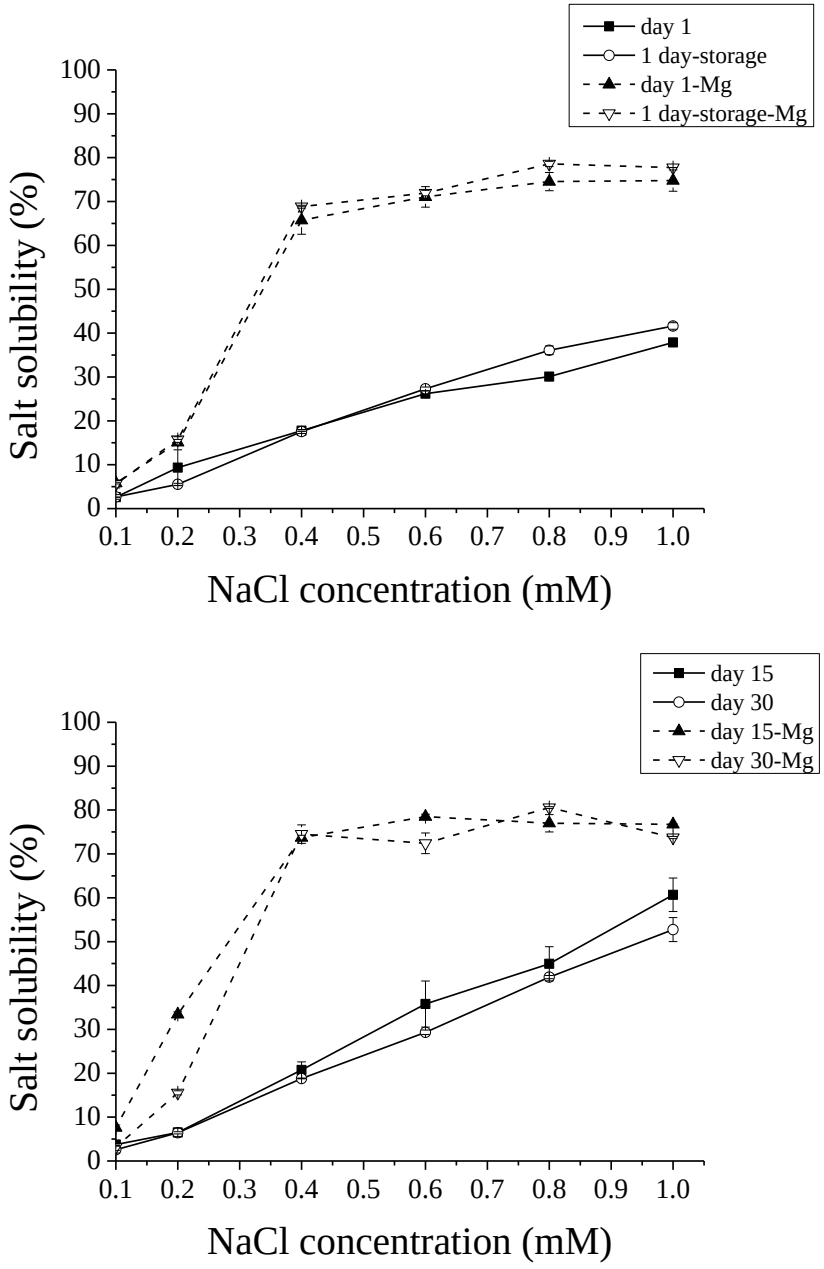

Figure 4-1. Changes in protein salt solubility of freeze-dried scallop during rehydration and storage.

Day 1, 15 and 30 mean the freeze-dried scallop storage in room temperature for 1, 15 and 30 day. “1 day storage” means the rehydration scallop stored in 5 ℃ for 1 day.

4.4 Changes in Ca^{2+}-ATPase activity of freeze-dried scallop during rehydration and storage

Ca^{2+}-ATPase can be used as an indicator for the integrity of myosin molecules, and the globular heads of myosin are responsible for Ca^{2+}-ATPase activity (Benjakul, et al., 2003). In our study (Figure 4-2), the myofibril Ca^{2+}-ATPase activity of all freeze-dried group was maintained at 0.74±0.03 μmol Pi/min/mg in 0.1 M NaCl solution and without a significant difference ($p > 0.05$). Wei, Tian, Yamashita, et al. (2020) reported that the Ca^{2+}-ATPase activity of fresh and frozen-thawed scallop was 0.80 ± 0.02 μmol Pi/min/mg in 0.1 M NaCl, which was similar to that in our study. The results suggested that the S-1 of scallop myosin was stable and had no damage during freeze-drying and rehydration process.

4.5 Comparison of protein digestion patterns among fresh, frozen-thawed and freeze-dried scallop adductor muscle.

The myofibrillar protein digestion model experiment of fresh scallops, frozen-thawed scallops and freeze-dried scallops was shown in Figure 4-3. The myosin of fresh scallop, frozen-thawed scallop and freeze-dried scallops with three kinds of treatment (Rehydrated scallop, rehydrated scallop storage for 1 day, and freeze-dried scallop storage in desiccator for 30 days) were all selectively cleaved into S-1 and rod without heating. After heating at 40℃ for 5 and 20 min, there was no obvious difference in the amount of S-1 and rod produced in fresh scallops and frozen-thawed scallops, and it was similar to that one without heating. For the freeze-dried samples (storage at day 0 and day 1 group), myosin was cleaved into S-1 and rod by chymotrypsin, which are the same to fresh scallop and frozen-thawed scallop. However, after storage for 30 days, the myosin was cleaved

into S-1, rod and 100 kDa fragment. Moreover, the production of rod reduced after heating 5 and 20 min in myosin of three freeze-dried scallop group, and the rod for freeze-dried scallops stored for 30 days had an even greater degradation, while S-1 fragment did not change. The difference in denaturation profiles of fresh scallop, frozen-thawed scallop and three kinds of freeze-dried scallop indicated that the S-1 portion of myosin was stable during freeze-drying and rehydration process while myosin rod was unstable. The about results explained why the salt solubility of freeze-dried scallop was low, while Ca^{2+}-ATPase activity was the same to the that of fresh scallop.

4.6 Comparison of microstructural properties of fresh scallop and freeze-dried scallop during storage

The TEM results (Figure. 4-4) show that the fresh scallop muscle structure remained relatively intact for clear Z line and small sarcoplasmic reticulum (SR), but Z line gradually damaged and the SR swelled with increasing storage time. The similar result was reported by Wei, Tian, Yamashita, et al. (2020). However, the Z line was unclear in rehydrated scallops, but SR showed no swelling. The damage of Z line might be caused by water entering the cell during rehydration, caused the cells to swell. After storage at 5℃ for 24 h, the SR of rehydrated scallop also swelled. Therefore, in the process of rehydration and storage, the microstructure of scallop will be destroyed, which finally affected its texture.

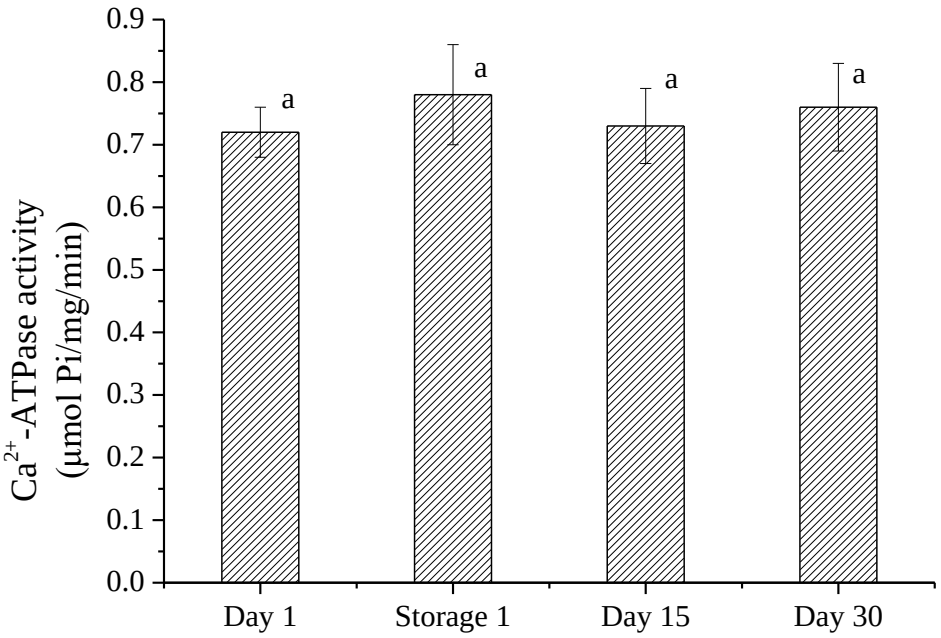

Figure 4-2 Changes in Ca^{2+}-ATPase activity of freeze-dried scallop during rehydration and storage

Value are given as mean ± standard deviation (n = 3).
Different letters (a) within the same row indicate significant difference ($p < 0.05$).

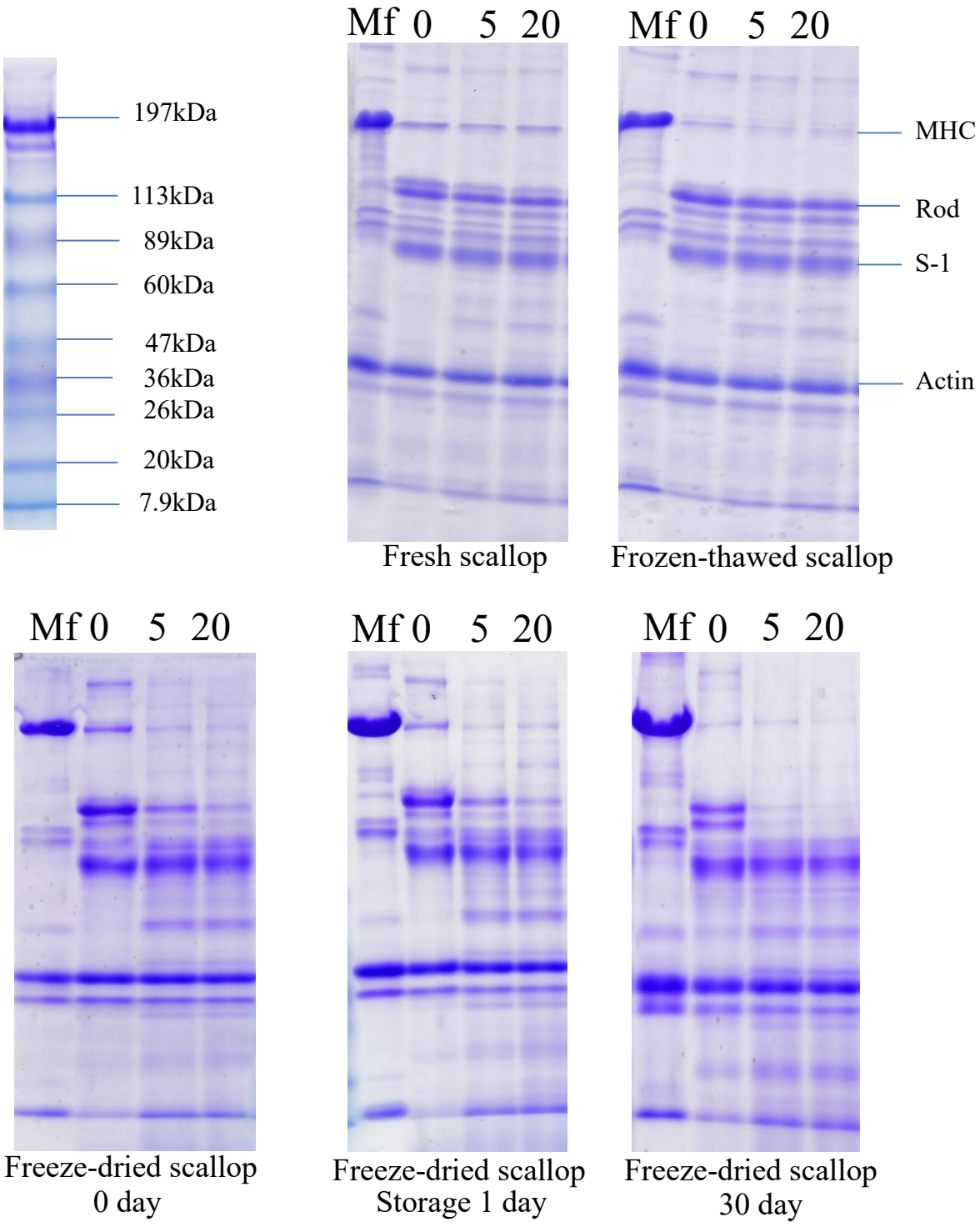

Figure 4-3. Changes in myofibrillar protein after digestion in fresh, frozen-thawed and freezing-dried scallop adductor muscle.

M: Marker; Mf: myofibrillar protein; MHC: myosin heavy chain.
Thawed scallop: Frozen scallop thawed by ice-water;
0, 5 and 20 means heating time at 40 ℃
Protein marker (Nacalai Tesque, Inc. Japan): myosin (197 kDa); β- galactosidase (113 kDa); bovine serum albumin (89 kDa); glutamate dehydrogenase (60 kDa); ovalbumin (47 kDa); Carbonic anhydrase (36 kDa); myoglobin (26 kDa); lysozyme (20 kDa); aprotinin (7.9 kDa).

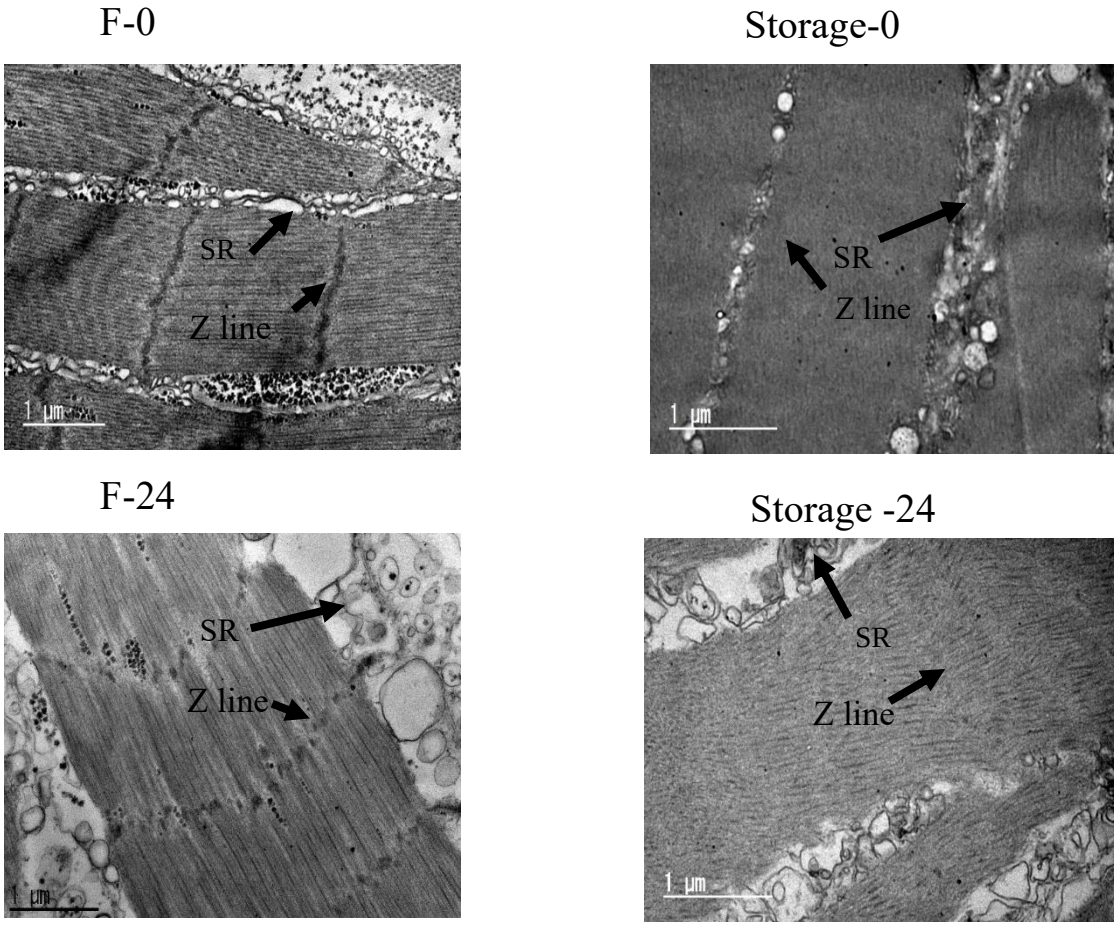

Figure 4-4. Comparison of properties of fresh scallop and freeze-dried scallop during storage

F: fresh scallop. Storage: rehydrated scallop storage at 5℃.
0 and 24 mean storage time was 0 and 24 h.

4.7 Conclusions

In this study, biochemical properties and microstructure of freeze-dried scallop were investigated during rehydration and storage. The results showed that ATP content of freeze-dried scallop did not change during room temperature storage at low humidity condition, while that decreased rapidly after rehydration for 1.5 min with AMP accumulation. With the increasing rehydration time, AMP gradually decomposed to IMP, HxR and Hx. Both IMP pathway and AdR pathway co-existed in scallop during rehydration. Rod of rehydrated scallop myosin was unstable while the S-1 fragment stable, which resulted to low salt solubility. But the Ca^{2+}-ATPase activity of rehydrated scallop myofibrils was similar with that of fresh scallop and frozen-thawed scallop. TEM results also showed that the microstructure of scallop adductor muscle was damaged during freeze-drying and rehydration process.

In addition, the scallop surimi products were prepared from fresh scallop, frozen scallop and three kinds of rehydrated scallop (shown in Figure 4-5) as materials. The results indicated the gel strength of rehydrated scallop was slightly lower that that made by fresh scallop and frozen scallop. Therefore, freeze-drying is a good treatment for scallop products for long time storage and reduction in weight without freshness degradation. And good rehydration method should be developed to get good quality of dehydrated scallop.

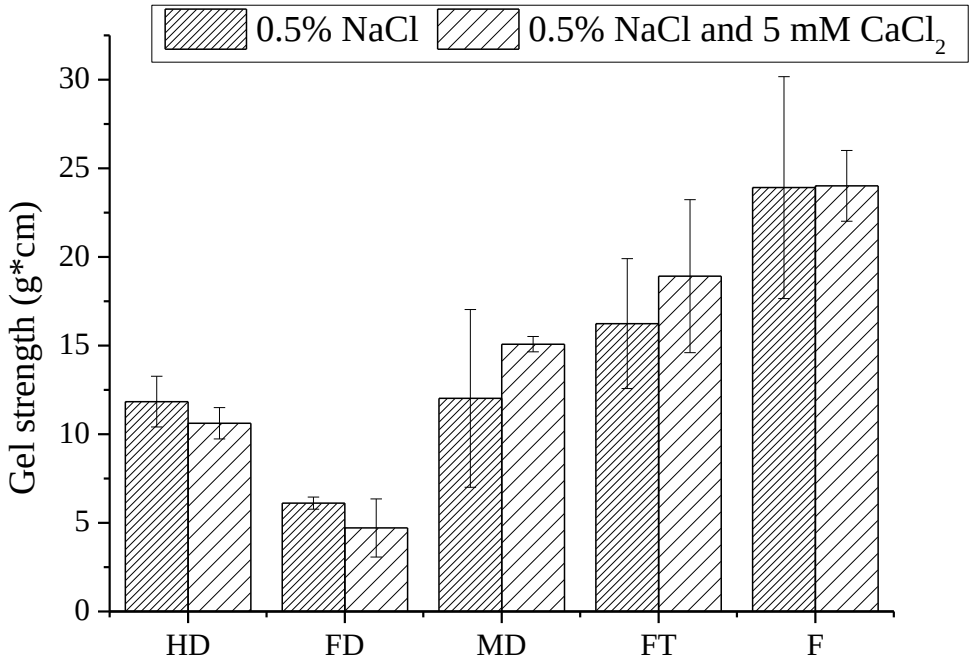

Figure 4-5. Comparison of gel strength of scallop surimi products made by different type of scallop adductor muscle.

Freeze-drying group: HD: high freshness scallop; FD: frozen scallop bought from Kamaishi; MD: minced scallop meat after homogenization;
FT: frozen-thawed scallop; F: freshness scallop.

General conclusion

In this thesis, changes in biochemical properties of scallop adductor muscle during freeze-thawing, freeze-drying and rehydration process was clarified. Moreover, the AMP decomposition pathway in scallop adductor muscle and its influencing factors was investigated, such as EDTA or EGTA addition, heating, metal ions concentration change, et al. The conclusions are shown as follows:

In Chapter 1, the effects of thawing methods on the biochemical properties and microstructure of pre-rigor frozen scallop striated adductor muscle was examined. Postmortem biochemical properties (pH, salt solubility, Ca^{2+}-ATPase activity, ATP-related compounds) and microstructural changes in the striated adductor muscle of pre-rigor frozen scallop were studied after thawing and during storage at 4℃. Four thawing methods were used: running water (18℃, R); ice-water (0℃, I); air (4℃, A) and ice-saltwater (-2℃, S). The pH values and salt solubility of R group were lower than the other three thawing groups while I group was highest after thawing. However, no significant difference ($p > 0.05$) in Ca^{2+}-ATPase activity were detected among 4 groups. The microstructure results indicated that the structure of I group was close to that of fresh scallop. Moreover, ATP decomposition rate was the slowest. Therefore, ice-water thawing is the best method because it induced the least changes in the biochemical properties and microstructures of scallop adductor muscle.

In chapter 2, condition-dependent adenosine monophosphate decomposition pathways by endogenous enzymes in striated adductor muscle from scallop was investigated. The purpose of this study was to confirm inosine monophosphate (IMP) generation and to clarify the decomposition pathway of adenosine monophosphate (AMP) by investigating the properties of AMP, IMP and

adenosine (AdR) decomposition enzymes in scallop. The results showed that IMP accumulated due to AMP decomposed by endogenous enzymes in scallops when stored at both 4°C and 20°C. The AMP decomposition rate was highest in the supernatant of homogenized scallop adductor muscle, follows are the suspended solution and precipitate, while IMP could not be decomposed in scallop. The results indicated that the activity of adenosine deaminase was very high, and this enzyme was involved in an intracellular process in scallop. Moreover, one minute of heating exerted little influence on the AMP and AdR decomposition rates, while 5 min of heating induced enzyme denaturation. The IMP generation rate increased dramatically in scallop crude enzyme solution containing 5 mM EDTA. This suggests that the major pathway of AMP decomposition might change with variations in metal ion concentrations in Japanese scallop.

In chapter 3, based on the above results, the effect of EGTA addition and different ions on the pathways of AMP decomposition in scallop was further studied. Scallop adductor muscle tissue solution was divided into control group (without any treatment) and CP group (containing 0.1% chloramphenicol). These two groups were dialyzed at 4℃ and then incubated at 25℃. Changes in ATP-related compounds of scallop adductor muscle tissue solutions with 5 mM EGTA, 10 mM K^+, 1 mM Ca^{2+} and 6 mM Mg^{2+} additions during incubation were detected by HPLC, and changes in protein composition of both groups were examined by SDS-PAGE during dialysis and incubation period. The results indicated that IMP generated rapidly in scallop crude enzyme solution containing 5 mM EGTA within 2 h. For the control group, the protein in the scallop enzyme solution gradually denatured due to microbial activity during incubation, while CP group did not. Adenosine deaminase and adenosine kinase were detected in denatured protein fraction (40 kDa) by LC-MS/MS. The results suggested that K^+ promoted the decomposition of AMP to IMP, Mg^{2+} promoted the decomposition of AMP to

AdR, and Ca^{2+} slightly inhibit the decomposition of AMP.

Furthermore, in Chapter 4, Changes in biochemical propertied and microstructure of Japanese scallop adductor muscle during storage, freeze-drying and rehydration process was investigated. The biochemical properties (ATP-related compounds, salt solubility, Ca^{2+}-ATPase activity) and microstructural changes in freeze-dried scallop adductor muscle of pre-rigor Japanese scallops during room temperature (18±2℃) storage and rehydration process were studied. The results showed that ATP and ADP contents were maintained or kept at 3.63±0.20 μmol/g and 2.27±0.23 μmol/g in freeze-dried scallop which stored at low humidity (less 10%) and room temperature for 30 days and with no significant difference ($p > 0.05$), and AMP and IMP contents were detected less than 0.42 μmol/g and 0.1 μmol/g, respectively. ATP was decomposed rapidly within 1.5 min of rehydration, accompanied by the accumulation of AMP. As the rehydration time increased, AMP was gradually decomposed into HxR and Hx. Salt solubility of freeze-dried scallop was lower than that of freeze-thawed scallop. However, no significant difference ($p > 0.05$) in Ca^{2+}-ATPase activity of freeze-dried scallop were detected during storage and rehydration process. Digestion results suggested that the rod of myosin was unstable after freeze-drying process while S-1 part was stable, compared with fresh scallop and freeze-thawed scallop. Moreover, the microstructure results indicated that the Z-line of scallop was broken during freeze-drying process.

According to this research, AMP decomposition pathway was affected by ions in scallop adductor muscle, maybe a good and safe way to obtain the scallop which have better flavor by IMP generation can be developed in the future. The rehydration method (rehydration time, temperature and different ions solution) also should be modified to get high quality rehydrated scallop. Moreover, new types of healthy scallop surimi products which are suitable for different people (old people,

children, teenagers and so on) can be developed from different kinds of materials, such as high freshness frozen-thawed and freeze-dried scallops.

References

Abbasi, S., & Azari, S. (2009). Novel microwave-freeze drying of onion slices. *International Journal of Food Science & Technology, 44*(5), 974-979.

Abe, S., Osako, K., Watanabe, M., & Suzuki, T. (2009). The Effect of Thawing Condition for Frozen Fish Meats―The Thawing Speed Dependence on Fish Muscle Protein Denaturation and Drip Amount. *Japan Society of Refrigerating and Air Conditioning Engineers, 26*(2), 149-158.

Ando, M., Oishi, K., Mochizuki, S., Tsukamasa, Y., & Makinodan, Y. (2002). Effect of inhabited sea area on meat firmness and its post-mortem change in chub mackerel during chilled storage. *Fisheries Science, 68*, 1337-1343.

Arai, K., Kobayashi, K., & Saito, T. (1968). On the Acid Soluble Nucleotides of the Freeze-Dried Muscle of Some Marine Invertebrates. *Japan Society of Nutrition and Food Science, 20*(5), 65-67.

Arai, K. (1961). Acid-soluble nucleotides in muscle of marine invertebrates: Effects of storing temperature upon the content of muscular nucleotides of some seashells (II). *Bulletin of Fisheries Sciences, Hokkaido University, 11*, 225-229.

Arch, J. R. S., & Newsholme, E. A. (1978). Activities and some properties of 5′-nucleotidase, adenosine kinase and adenosine deaminase in tissues from vertebrates and invertebrates in relation to the control of the concentration and the physiological role of adenosine. *Biochemical Journal, 174*(3), 965-977.

Asami, D. K., Hong, Y.-J., Barrett, D. M., & Mitchell, A. E. (2003). Comparison of the Total Phenolic and Ascorbic Acid Content of Freeze-Dried and Air-Dried Marionberry, Strawberry, and Corn Grown

Using Conventional, Organic, and Sustainable Agricultural Practices. *Journal of Agriculture and Food Chemistry, 51*(5), 1237-1241.

Azuma, Y., & Konno, K. (1998). Freeze Denaturation of Carp Myofibrils Compared with Thermal Denaturation. *Fisheries Science, 64*(2), 287-290.

Babić, J., Cantalejo, M. J., & Arroqui, C. (2009). The effects of freeze-drying process parameters on Broiler chicken breast meat. *LWT-Food Science and Technology, 42*(8), 1325-1334.

Benjakul, S., Visessanguan, W., Hongkaew, C., & Tanaka, M. (2003). Comparative study on physicochemical changes of muscle proteins from some tropical fish during frozen storage. *Food Research International, 36*(8), 787-795.

Olafsdóttir, G., E. Martinsdbttir, Oehlenschbger, J., Dalgaard, P., B. Jensen, Undeland, I., Mackie, I. M., G. Henehan, Nielsen, J., & Nilsen, H. (1997). Methods to evaluate fish freshness in research and industry. *Trends Food Science Technology, 8*(8), 258-265.

Boonsumrej, S., Chaiwanichsiri, S., Tantratian, S., Suzuki, T., & Takai, R. (2007). Effects of freezing and thawing on the quality changes of tiger shrimp (Penaeus monodon) frozen by air-blast and cryogenic freezing. *Journal of Food Engineering, 80*(1), 292-299.

Chen, Y., Uchida, H., Migitaka, Y., Hayashi, T., & Uwajima, T. (2000). Isolation and characterization of adenosine deaminase in the adductor muscle of marine bivalve molluscs. *Fisheries Science, 66*, 748-754.

Chen, Z. G., Guo, X. Y., & Wu, T. (2016). A novel dehydration technique for carrot slices implementing ultrasound and vacuum drying methods. *Ultrasonics Sonochemistry, 30*, 28-34.

Cheng, J.-H., Sun, D.-W., Han, Z., & Zeng, X.-A. (2014). Texture and Structure Measurements and Analyses for Evaluation of Fish and Fillet Freshness Quality: A Review. *Comprehensive Reviews in Food Science and Food Safety, 13*(1), 52-61.

Cheng, J.-H., Sun, D.-W., Zeng, X.-A., & Liu, D. (2015). Recent Advances in Methods and Techniques for Freshness Quality Determination and Evaluation of Fish and Fish Fillets: A Review. *Critical Reviews in Food Science and Nutrition, 55*(7), 1012-1225.

Crapo, C., Himelbloom, B., Pfutzenreuter, R., & Lee, C. (1999). Causes for soft flesh in giant grenadier (*Albatrossia pectoralis*) fillets. *Journal of Aquatic Food Product Technology, 8*(3), 55-68.

Crapo, C., Oliveira, A. C., Nguyen, D., Bechtel, P. J., & Fong, Q. (2010). Development of a method to produce freeze-dried cubes from 3 Pacific salmon species. *J Food Sci, 75*(5), E269-275.

Deng, Y., Luo, Y., Wang, Y., Yue, J., Liu, Z., Zhong, Y., Zhao, Y., & Yang, H. (2014). Drying-induced protein and microstructure damages of squid fillets affected moisture distribution and rehydration ability during rehydration. *Journal of Food Engineering, 123*, 23-31.

Dunajski, E. (1980). Texture of fish muscle. *Journal of Texture Studies, 10*(4), 301-318.

Ersoy, B., Aksan, E., & Ozeren, A. (2008). The effect of thawing methods on the quality of eels (*Anguilla anguilla*). *Food Chemistry, 111*(2), 377-380.

Fujisawa, K., & Yoshino, M. (1987). Activities of adenylate-degrading enzymes in muscles from vertebrates and invertebrates. *Comparative Biochemistry and Physiology, 86*(1), 109-112.

Godiksen, H., Morzel, M., Hyldig, G., & Flemming, J. (2009). Contribution of cathepsins B, L and D to muscle protein profiles correlated with texture in rainbow trout (*Oncorhynchus mykiss*). *Food*

Chemistry, 113(4), 889-896.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949). Determination of serum proteins by means of biuret reaction. *Journal of Biological Chemistry, 177*, 751-766.

Hatae, K., Nakai, H., Shimada, A., Murakami, T., Takada, K., Shirojo, Y., & Watabe, S. (1995). Abalone (*Hariltis discus*): seasonal variations in chemical composition and textural properties. *Journal of Food Science, 60*(1), 32-35.

Hiltz, D. F., & Dyer, W. J. (1971). Octopine in postmortem adductor muscle of the sea scallop (*Placopecten magellanicus*). *Journal of Fisheries Research Board of Canada, 28*(8), 869-874.

Hong, G.-P., Shim, K.-B., Choi, M.-J., & Min, S.-G. (2009). Effects of air blast thawing combined with infrared radiation on physical properties of pork. *Korean Society for Food Science of Animal Resources, 29*(3), 302-309.

Hong, H., Regenstein, J. M., & Luo, Y. (2017). The importance of ATP-related compounds for the freshness and flavor of post-mortem fish and shellfish muscle: A review. *Critical Reviews in Food Science and Nutrition, 57*(9), 1787-1798.

Howgate, P. (2006). A review of the kinetics of degradation of inosine monophosphate in some species of fish during chilled storage. *International Journal of Food Science and Technology, 41*(4), 341-353.

Hu, Y., Zhang, J., Ebitani, K., & Konno, K. (2013). Development of Simplified Method for Extraction ATP-related Compounds From Fish Meat. *Nippon Suisan Gakkaishi, 79*(2), 219-225.

Hynek, R., Svensson, B., Jensen, O. N., Barkholt, V., & Finnie, C. (2006). Enrichment and

Identification of Integral Membrane Proteins from Barley Aleurone Layers by Reversed-Phase Chromatography SDS-PAGE and LC−MS MS. *Journal of proteome research, 5*(11), 3105-3113.

Iwamoto, M., Yamanaka, H., Watabe, S., & Hashimoto, K. (1991). Changes in ATP and Related Breakdown Compounds in the Adductor Muscle of “Itayagai” Scallop Pecten Albicans During Storage at Various Temperatures. *Nippon Suisan Gakkaishi, 57*(1), 153-156.

Jeacocke, R. E. (1977). Continuous measurements of the pH of beef muscle in intact beef carcases. *International Journal of food technology, 12*(4), 375-386.

Kawashima, K., & Yamanaka, H. (1992). Effects of storage temperature on the post-mortem biochemical changes in scallop adductor muscle. *Nippon Suisan Gakkaishi, 58*(11), 2175-2180.

Kawashima, K., & Yamanaka, H. (1994). Effects of Chloramphenicol on post-mortem biochemical changes in scallop adductor muscle. *Fisheries Science, 60*(4), 461-465.

Kawashima, K., & Yamanaka, H. (1995). Effect of Freezing and Thawing on Post-mortem Biochemical Changes in Scallop Adductor Muscle. *Fisheries Science, 61*(4), 691-695.

Kimura, I., Takahashi, M., Nagahisa, E., & Fujita, T. (1982). Preparation of Monomeric Myosin from Alaska Pollock Frozen Surimi. *Bulletin of the Japanese Society of Scientific Fisherie, 48*(2), 251.

Kimura, M., Narita, M., Nomata, H., Kaneko, H., & Yamanaka, H. (1997). Effects of storage temperature on the rigor of scallop adductor muscle. *Nippon Suisan Gakkaishi, 63*(4), 621-626.

Kuchiba-manabe, M., Matoba, T., & Hasegawa, K. (1991). Sensory Changes in Umami Taste of Inosine 5′-Monophosphate Solution after Heating. *Journal of Food Science, 56*(5), 1429-1432.

Lazou, A. (1989). Adenylate metabolizing enzymes in invertebrate tissues. *Comparative*

Biochemistry and Physiology, 92(1), 175-180.

Lee, J., & Park, J. W. (2016). Pacific whiting frozen fillets as affected by postharvest processing and storage conditions. *Food Chemistry, 201*, 177-184.

Li, B., & Sun, D.-W. (2002). Novel methods for rapid freezing and thawing of foods-a review. *Journal of Food Engineering, 54*(3), 175-182.

Li, Q., Zhang, L., Lu, H., Song, S., & Luo, Y. (2017). Comparison of postmortem changes in ATP-related compounds, protein degradation and endogenous enzyme activity of white muscle and dark muscle from common carp (*Cyprinus carpio*) stored at 4 °C. *LWT - Food Science and Technology, 78*, 317-324.

Lujan-Acosta, J., Moreira, R. G., & Seyed-Yagoobi, J. (1997). Air-impingement drying of tortilla chips. *Drying Technology, 15*(3-4), 881-897.

Ma, J., Qu, J.-H., & Sun, D.-W. (2017). Developing hyperspectral prediction model for investigating dehydrating and rehydrating mass changes of vacuum freeze dried grass carp fillets. *Food and Bioproducts Processing, 104*, 66-76.

Makri, M. (2009). The biochemical textural and sensory properties of frozen stored (-22°C) king scallop (*Pecten maxinus*) meats. African *Journal of Biotechnology*, *8*(16), 3893-3903.

Martinez, I., Slizyte, R., & Dauksas, E. (2007). High resolution two-dimensional electrophoresis as a tool to differentiate wild from farmed cod (*Gadus morhua*) and to assess the protein composition of klipfish. *Food Chemistry, 102*(2), 504-510.

Matsumoto, M., & Yamanaka, H. (1990). Post-mortem biochemical changes in the muscle of kuruma

prawn during storage and evaluation of the freshness. *Nippon Suisan Gakkaishi, 56*(7), 1145-1149.

Mayor, L., & Sereno, A. M. (2004). Modelling shrinkage during convective drying of food materials: a review. *Journal of Food Engineering, 61*(3), 373-386.

Mccarthy, J. T., & Kumar, R. (1999). Divalent Cation Metabolism: Magnesium. *In Robert W. Schrier (Series Ed.), The Schrier Atlas of Diseases of the Kidney. Volume 1.*, (pp 4.1-4.12).Wiley-Blackwell; Hoboken, NJ, USA.

Mendes, R., Quinta, R., & Nunes, M. L. (2001). Changes in baseline levels of nucleotides during ice storage of fish and crustaceans from the Portuguese coast. *European Food Research and Technology, 212*(2), 141-146.

Miranda, M., Maureira, H., Rodríguez, K., & Vega-Gálvez, A. (2009). Influence of temperature on the drying kinetics, physicochemical properties, and antioxidant capacity of Aloe Vera (Aloe Barbadensis Miller) gel. *Journal of Food Engineering, 91*(2), 297-304.

Murata, Y., Ogiwara, M., Funahashi, H., Ueno, K., Okazaki, E., Kimura, I., & Fukuda, Y. (2008). Development of thawing method for frozen whale meat with high concentration of ATP. *Journal of Fisheries Technology, 1*(1), 37-41.

Nam, M.-M., Lee, C., Moon, T.-S., & Huh, M.-K. (2012). Genetic Diversity and Population Structure of the Scallop Patinopecten yessoensis in Korea, China, and Japan by Random Amplified Polymorphic DNA Markers. *Journal of Life Science, 22*(4), 466-471.

Nawirska, A., Figiel, A., Kucharska, A. Z., Sokół-Łętowska, A., & Biesiada, A. (2009). Drying kinetics and quality parameters of pumpkin slices dehydrated using different methods. *Journal of*

Food Engineering, 94(1), 14-20.

Nimmol, C., Devahastin, S., Swasdisevi, T., & Soponronnarit, S. (2007). Drying and heat transfer behavior of banana undergoing combined low-pressure superheated steam and far-infrared radiation drying. *Applied Thermal Engineering, 27*(14-15), 2483-2494.

Nowotny, A. (1979). Basic Exercises in Immunochemistry. In A. Nowotny (Ed.), . *Protein Determination by the Biuret Method*, (pp 168-169). Springer , Berlin, Heidelberg.

Ocaño-higuera, V. m., Maeda-martínez, A. n., Lugo-sánchez, M. e., & Pacheco-aguilar, R. (2006). Postmortem biochemical and textural changes in the adductor muscle of catarina scallop stored at 0 °C. *Journal of Food Biochemistry,, 30*(4), 373-389.

Ogata, Y., Iwane, R., & Kimura, I. (2018). Properties of muscle protein of freeze-thawed olive flounder containing a high concentration of ATP when being frozen. *Nippon Suisan Gakkaishi, 84*(5), 835-842.

Ogata, Y., Shindo, J., & Kimura, I. (2012). Suppression effect of ATP on freeze denaturation of fish myofibrillar protein. *Nippon Suisan Gakkaishi, 78*(3), 461-467.

Pacheco-Aguilar, R., Marquez-Ríos, E., Lugo-Sánchez, M. E., García-Sanchez, G., Maeda-Martínez, A. N., & Ocaño-Higuera, V. M. (2008). Postmortem changes in the adductor muscle of Pacific lions-paw scallop (*Nodipecten subnodosus*) during ice storage. *Food Chemistry, 106*(1), 253-259.

Qin, N., Li, D., Hong, H., Zhang, Y., Zhu, B., & Luo, Y. (2016). Effects of different stunning methods on the flesh quality of grass carp (*Ctenopharyngodon idellus*) fillets stored at 4 degrees C. *Food Chemistry, 201*, 131-138.

Rahman, M. S. (2006). Drying of fish and seafood. In *Handbook of industrial drying*, (pp. 572-587): CRC Press.

Rouille, J., Lebail, A., Ramaswamy, H. S., & Leclerc, L. (2002). High pressure thawing of fish and shellfish. *Journal of Food Engineering, 53*(1), 83-88.

Saito, T. (1961). Adenosinetriphosphate and the related compounds in the muscles of aquatic animals. *Bulletin of the Japanese Society of Scientific Fisherie, 27*(6), 461-465.

Saito, T., Arai, K.-i., & Matsuyoshi, M. (1959). A new method for estimating the freshness of fish. *Bulletin of the Japanese Society of Scientific Fisherie, 24*(9), 749-750.

Sato, Y., & aikawa, T. (1991). Adenosine deaminase in the adductor muscle of the scallop, *Patinopecten yessoensis*. *Comparative Biochemistry and Physiology, 99B*(1), 221-232.

Sriket, P., Benjakul, S., Visessanguan, W., & Kijroongrojana, K. (2007). Comparative studies on the effect of the freeze–thawing process on the physicochemical properties and microstructures of black tiger shrimp (*Penaeus monodon*) and white shrimp (*Penaeus vannamei*) muscle. *Food Chemistry, 104*, 113-121.

Taheri-Garavand, A., Rafiee, S., & Keyhani, A. (2011). Study on Effective moisture diffusivity, activation energy and mathematical modeling of thin layer drying kinetics of bell pepper. *Australian Journal of Crop Science, 5*(2), 128-131.

Vaughan, J. G. (1979). Food Microscopy.In P. Howgate (Eds), Chapter fish (pp. 243-292). Academic Press London UK, New York, San Francisco. .

Vidode Mattio, N. D., Paredi, M. E., & Crupkin, M. (2001). Postmortem Changes in the Adductor

Muscle of Scallop (*Chlamys tehuelchus*) in Chilled and Frozen Storage. *Journal of Aquatic Food Product Technology, 10*(3), 49-60.

Wang, H., Luo, Y., Shi, C., & Shen, H. (2015). Effect of Different Thawing Methods and Multiple Freeze-Thaw Cycles on the Quality of Common Carp (*Cyprinus carpio*). *Journal of Aquatic Food Product Technology, 24*(2), 153-162.

Wang, Q., Xue, C., Li, Z., Fu, X., Xu, J., & Xue, Y. (2007). Changes in the contents of ATP and its related breakdown compounds in various tissues of oyster during frozen storage. *Journal of Ocean University of China, 6*(4), 407-412.

Watabe, S., Ushio, H., Iwamoto, M., Yamanaka, H., & Hashimoto, K. (1989). Temperature-dependency of rigor-mortis of fish Muscle Myofibrillar Mg^{2+}-ATPase activity and Ca^{2+} uptake by sarcoplasmic reticulum. *Journal of Food Science*, *54*(5), 1107-1110.

Watanabe, H., Yamanaka, H., & Yamakawa, H. (1992). Post-mortem biochemical changes in the muscle of disk abalone during storage. *Nippon Suisan Gakkaishi, 58*(11), 2081-2088.

Wei, H., Tian, Y., Lin, Y., Maeda, H., Yamashita, T., Yu, K., Takaki, K., & Yuan, C. (2020). Condition-dependent adenosine monophosphate decomposition pathways in striated adductor muscle from Japanese scallop (Patinopecten yessoensis). *Journal of Food Science, 85*(5), 1462-1469.

Wei, H., Tian, Y., Yamashita, T., Ishimura, G., Sasaki, K., Niu, Y., & Yuan, C. (2020). Effects of Thawing Methods on the Biochemical Properties and Microstructure of Pre-rigor Frozen Scallop Striated Adductor Muscle. *Food Chemistry*, Advance online publication.

Wongso, S., & Yamanaka, H. (1998). Extractive components of the adductor muscle of Japanese

baking scallop and changes during refrigerated storage. *Journal of Food Science, 63*(5), 772-776.

Wu, Y., Zhang, J., Shi, J., Ebitani, K., & Konno, K. (2016). Biphasic IMP decomposition during the storage of flounder muscle at low temperature. *Nippon Suisan Gakkaishi, 82*(3), 342-348.

Yokoyama, Y., Sakaguchi, M., Kawai, F., & Kanamori, M. (1992). Changes in Concentration of ATP-related Compounds in Various Tissues of Oyster during Ice Storage. *Nippon Suisan Gakkaishi*, *58*(1), 2125-2136.

Yokoyama, Y., Sakaguchi, M., Kawai, F., & Kanamori, M. (1994a). Chemical Indices for assessing freshness of shellfish during storage. *Fisheries Science, 60*(3), 329-333.

Yokoyama, Y., Sakaguchi, M., Kawai, F., & Kanamori, M. (1994b). Effects of storage temperature on postmortem changes of ATP and its related compounds and freshness indices in oyster tissues. *Fisheries Science, 60*(2), 217-223.

Yokoyama, Y., Takahashi, S., Sakaguchi, M., Kawai, F., & Kanamori, M. (1994). Postmortem Changes of ATP and Its Related Compounds and Freshness Indices in Spear Squid Doryteuthis bleekeri Muscles. *Fisheries Science, 60*(5), 583-581.

Yoneda, C., Awazuhara, M., & Hatae, K. (2008). Quality of frozen tuna meat thawed by soaking in warm salt water. *Journal of Cookery Science of Japan, 41*(5), 337-343.

Yoneda, C., Kasai, M., Hatae, K., Hirota, Y., & Nakamura, J. (2006). Quality of frozen tuna meat thawed by three different methods. *Journal of Cookery Science of Japan, 39*(1), 16-21.

Yoshida, S., & Aikawa, T. (1993). Partial characterization of the adenosine deaminase purified from the scallop (*Pationpecten Yessoensis*) midgut gland. *Comparative Biochemistry & Physiology,*

105B(1), 51-61.

Yuan, C., Wang, X., Chen, S., Qu, Y., & Konno, K. (2011). Structural stability of myosin rod from silver carp as affected by season. *Journal of Food Science, 76*(5), 686-693.

Zhang, M., Chen, H., Mujumdar, A. S., Tang, J., Miao, S., & Wang, Y. (2017). Recent developments in high-quality drying of vegetables, fruits, and aquatic products. *Critical Reviews in Food Science and Nutrition, 57*(6), 1239-1255.

Zheng, L., Yu, K., Yuan, C., Wang, X., Chen, S., Kimura, I., & Konno, K. (2012). Characterization of myosin subfragment-1 of summer and winter silver carp (*Hypophthalmichthys molitrix*) muscle. *Journal of Food Science, 77*(9), 914-920.