Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 90 (2005) 141–150
Food Chemistry
www.elsevier.com/locate/foodchem

Physicochemical and biochemical changes during frozen storage of minced flesh of lizardfish (*Saurida micropectoralis*)

Kittima Leelapongwattana [a], Soottawat Benjakul [a,*], Wonnop Visessanguan [b], Nazalin K. Howell [c]

[a] *Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*
[b] *National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Phaholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand*
[c] *School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK*

Received 8 January 2004; received in revised form 11 March 2004; accepted 11 March 2004

Abstract

Physicochemical and biochemical changes of minced flesh of lizardfish (*Saurida micropectoralis*) kept in air and vacuum during frozen storage at −20 °C for 24 weeks were investigated. Formaldehyde and dimethylamine (DMA) contents increased with a concomitant decrease in trimethylamine oxide (TMAO) content as the storage time increased ($P < 0.05$). Ca^{2+}-ATPase activity decreased continuously with a coincidental decrease in the salt-soluble fraction. Disulfide bonds were increasingly formed throughout the storage ($P < 0.05$). Nevertheless, surface hydrophobicity increased and reached a maximum at week 4 with a subsequent decrease up to the end of storage. In general, greater changes were observed in lizardfish mince kept under vacuum than in air. A marked increase in trimethylamine-*N*-oxide demethylase (TMAOase) activities was observed up to 6 weeks, followed by continuous decrease up to 24 weeks of storage. TMAOase activity, as well as formaldehyde formation, could be reduced to some extent with packaging containing oxygen.

Keywords: Denaturation; Dimethylamine; Formaldehyde; Frozen; Lizardfish TMAOase; Trimethylamine-*N*-oxide

1. Introduction

Frozen storage is one of the most important techniques for long-term preservation of fish muscle. Nevertheless, structural and physicochemical changes still take place (Herrera, Pastoriza, & Sampedro, 2000). Alterations of fish muscle proteins during frozen storage include (1) partial dehydration of proteins during freezing, (2) changes in the environment of the proteins due to freeze-concentration of inorganic salts, (3) interaction of lipids, free fatty acids, and/or lipid oxidation products with proteins, and (4) the action of trimethylamine oxide demethylase (TMAOase). The last mechanism is believed to be important in fish that belong to the gadoid family (Krueger & Fennema, 1989). TMAOase catalyzes the breakdown of trimethylamine oxide (TMAO) to equimolar quantities of formaldehyde (FA) and dimethylamine (DMA) (Amano & Yamada, 1964; Lundstrom, Correia, & Wilhelm, 1982; Parkin & Hultin, 1982b). The formaldehyde produced has been suggested to cause cross-linking of the muscle proteins and toughening of the tissue (Castell, Smith, & Dale, 1973). Close correlations are generally observed between FA production and loss of protein solubility and/or the development of toughness in frozen stored gadoid fish muscle (Gill, Keith, & Smith, 1979). FA accelerates the formation of high molecular weight polymers from isolated myosin and actomyosin during freezing and frozen storage (Ang & Hultin, 1989).

The speed of TMAO degradation depends upon many factors, such as storage temperature, species, muscle integrity and reducing conditions (Parkin & Hultin, 1982a). Kidney of lizardfish contained a high

* Corresponding author. Tel.: +66-74-28-6334; fax: +66-74-212889.
E-mail address: soottawat.b@psu.ac.th (S. Benjakul).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.03.038

amount of TMAOase, which can contaminate fish muscle during processing (Benjakul, Visessanguan, & Tanaka, 2004). The absence of oxygen has also been shown to accelerate the rate of DMA and FA production (Lundstrom et al., 1982). Red hake, packed in oxygen-permeable film, showed decreased rates of DMA and FA formation during iced storage, compared to uncooked red hake packaged in cans purged with nitrogen and stored in ice. Furthermore, the production and reactivity of FA are enhanced in minced muscle by the rupture of cellular integrity in the muscle structure (Parkin & Hultin, 1982a).

Lizardfish (*Saurida* spp.) have been considered as a potential raw material for high-grade surimi production in Thailand, due to their high gel-forming ability and an appreciable whiteness of the flesh (Benjakul et al., 2004). However, gel-forming ability of this fish decreased rapidly during post-harvest handling. This was associated with the proteolysis as well as the formation of FA (Benjakul et al., 2004). Yasui and Lim (1987) also suggested that the decrease in gelling property of this fish during iced storage was due to the formation of FA and DMA. Recently, Benjakul, Visessanguan, Thongkaew, and Tanaka (2003b) reported an increase of FA in whole lizardfish during frozen storage. Apart from FA formation, lipid oxidation still occurs during frozen storage. Therefore, appropriate packaging is a promising way to retard the oxidation. However, packaging atmosphere with low oxygen might show a detrimental effect, especially the induction of FA formation. However, no information regarding the changes of minced flesh of lizardfish under different packaging atmospheres has been reported. Thus, the aims of this research were to examine the physicochemical and biochemical changes of minced flesh of lizardfish mince kept in air and under vacuum during extended frozen storage.

2. Materials and methods

2.1. Chemicals

All chemicals for TMAOase activity assay were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Trimethylamine-*N*-oxide was obtained from Aldrich Chemical Company, Inc (Milwaukee, WI, USA). Ammonium molybdate, 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), adenosine 5′-triphosphate (ATP), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and L-leucine were obtained from Sigma chemical Co. (St. Louis, MO, USA). Acetylacetone and Triton X-100 were purchased from Fluka (Messerchmittstr, Swizerland). Tris (hydroxymethyl) aminomethane, urea, sodium borohydride and Folin–Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany).

2.2. Fish sample preparation

Lizardfish (*Saurida micropectoralis*), between 200 and 250 g in weight, were purchased from the dock in Trang, Thailand. The fish, off-loaded approximately 36–48 h after catching, was transported in ice, with a fish/ratio of 1:2 (w/w), to the Department of Food Technology, Prince of Songkla University, Hat Yai within 3 h. They were headed, gutted and washed with water. The flesh was separated from skin and bone using a drum-type deboner with 4 mm diameter holes.

A sample (0.2 kg) was packed in a polyethylene bag and sealed, either under air or under vacuum. All samples were kept at −20 °C for 6 months. At definite time intervals (0, 1, 2, 4, 6, 8, 12, 16, 20 and 24 weeks), samples were removed, thawed with running water (26–28 °C) to obtain the core temperature of 0–2 °C and subjected to analyses.

2.3. Trimethylamine-*N*-oxide determination

A sample (2.5 g) was added to 10 ml of 5% trichloroacetic acid and homogenised with a homogeniser (IKA Labortechnik, Malaysia) at a speed of 12,000 rpm for 2 min. The homogenate was centrifuged at 3000*g* for 15 min. The supernatant containing TMAO was used for analysis. TMAO was determined after reduction to TMA by the method of Benjakul et al. (2004). The supernatant (2 ml) was added to 2 ml of 1% $TiCl_3$ and incubated at 80 °C for 90 s, followed by cooling with running water. TMA was determined according to the method of Conway and Byrne (1936). TMAO was then calculated after subtracting the indigenous TMA content in the samples and expressed as μmol/g.

2.4. Dimethylamine and free formaldehyde determination

A sample (2.5 g) was added to 10 ml of 5% trichloroacetic acid and homogenised with a homogeniser at a speed of 12,000 rpm for 2 min. The homogenate was centrifuged at 3000*g* for 15 min and the supernatant was removed. To the pellet, a further 5 ml of 5% trichloroacetic acid were added and the mixture was homogenised as previously described. The supernatants were combined and neutralised to pH 6.0–6.5 and the final volume was made up to 25 ml using distilled water. The supernatant was then used for DMA and formaldehyde determinations as described by Benjakul et al. (2004).

2.5. TMAOase activity measurement

2.5.1. Preparation of TMAOase crude extract from lizardfishmuscle

The TMAOase extract was prepared according to method of Benjakul, Visessanguan, and Tanaka (2003a)

with some modifications. To prepare crude TMAOase extracts, finely chopped muscle of lizardfish was extracted with 2 volumes of chilled 20 mM Tris–acetate buffer, pH 7.0, containing 0.1 M NaCl and 0.1% Triton X-100. The mixture was homogenised for 3 min using a homogeniser at a speed of 12,000 rpm. The homogenate was centrifuged at 19,400*g* for 1 h at 4 °C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The supernatant obtained was referred to as "TMAOase crude extract".

2.5.2. TMAOase activity assay

TMAOase activity was assayed using TMAO as a substrate in the presence of selected cofactors (Benjakul et al., 2003a). To 2.5 ml of assay mixture (24 mM Tris–acetate containing 24 mM TMAO, 2.4 mM cysteine, 2.4 mM ascorbate and 0.24 mM $FeCl_2$, pH 7.0), 0.5 ml of enzyme solution was added to initiate the reaction. The reaction was conducted at 25 °C for 80 min and 1 ml of 10% trichloroacetic acid was added to terminate the reaction. The reaction mixture was then centrifuged at 8000*g* for 15 min and the supernatant was subjected to DMA determination. One unit of TMAOase is defined as the activity, which released 1 nmol DMA per min.

2.6. Ca^{2+}-ATPase activity

Natural actomyosin (NAM) was prepared by the method of Benjakul, Seymour, Morrissey, and An (1997) and Ca^{2+}-ATPase activity was assayed as described by Benjakul et al. (1997). Specific activity was expressed as μmol of inorganic phosphate (Pi) released per milligramme of protein within 1 min for the reaction at 25 °C.

2.7. Total sulfhydryl and disulfide bond contents

Total sulfhydryl content was measured using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Benjakul et al. (1997). Disulfide bond was determined by using 2-nitro-5-thiosulfobenzoate (NTSB) assay according to the method of Thannhauser, Konishi, and Scheraga (1987).

2.8. Surface hydrophobicity

Surface hydrophobicity was determined according to the method described by Benjakul et al. (1997) using 1-anilinonapthalene-8-sulfonic acid (ANS) as a probe. NAM dissolved in 10 mM phosphate buffer, pH 6.0, containing 0.6 M NaCl, at different concentrations was mixed with ANS. The fluorescence intensity of ANS-protein conjugates was measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm by RF-1501 spectrofluorometer (Shimadzu, Kyoto, Japan). The initial slope of the plot of fluorescence intensity versus NAM concentration was referred to as SoANS.

2.9. α-Amino acid

The α-amino acid was determined as described by Benjakul and Morrissey (1997). Diluted samples (125 μl) were mixed thoroughly with 2.0 ml of 0.2125 M phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01% TNBS solution. The mixture was then placed in a water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 1.0 M sodium sulfite. The mixture was cooled to room temperature for 15 min. The absorbance was measured at 420 nm and α-amino acid was expressed in terms of L-leucine.

2.10. Protein solubility

Solubility was determined according to the method of Hamada, Tsuji, Nakayama, and Niwa (1977) with some modifications. A sample was thawed with running water until the core temperature reached 0–2 °C. To 2 g sample, 20 ml of 0.6 M KCl were added and the mixture was homogenised for 3 min at speed of 12,000 rpm. The homogenate was centrifuged at 5000*g* for 30 min at 4 °C. The supernatant was defined as salt-soluble fraction (I), which is considered to be native proteins and the insoluble material was treated with 4 volumes of a mixture solution containing 8 M urea, 6 mM EDTA, and 0.6 M KCl solution. The mixture was stirred for 30 min at 25 °C. The supernatant, obtained after centrifuging at 15,000*g* at 15 °C for 1 h, was defined as the urea-soluble fraction (II) which is considered to be an aggregate stabilized by hydrogen and hydrophobic bonds. Finally, any remaining precipitate was treated with a mixture solution containing 0.5% $NaBH_4$, 6 mM EDTA, 8 M urea, and 0.6 M KCl and stirred as before. The supernatant obtained after centrifuging at 15,000*g* at 15 °C for 1 h was defined as the $NaBH_4$-soluble fraction (III), which is an aggregate caused by the formation of disulfides and the final residue was defined as the insoluble fraction (IV). Protein concentration in the fractions was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum albumin as standard. The solubility of each fraction was expressed as the percentage of the soluble protein, compared with total protein in the sample.

2.11. Statistical analyses

Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range Test (DMRT) (Steel & Torrie, 1980). Statistical analyses were performed using the

Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Changes in trimethylamine-N-oxide, formaldehyde and dimethylaniline contents of muscle of lizardfish during frozen storage

Changes in free formaldehyde content are presented in Fig. 1(a). In general, FA content in lizardfish mince increased dramatically as the storage time increased ($P < 0.05$). Greater changes were observed in the samples kept under vacuum, than in those stored in air, throughout the storage. After 24 weeks of storage, the FA contents in lizardfish mince kept in air and under vacuum were 2.04 and 2.99 μmol/g, respectively. Similarly, the DMA content also increased continuously as the storage time increased ($P < 0.05$). A sharp increase was observed at weeks 4 and 6 for those samples kept under vacuum and in air, respectively (Fig. 1(b)). At week 24, the DMA content increased 4.6- and 6.5-fold in samples kept in air and under vacuum, respectively. Careche, Del Mazo, Torrejon, and Tejada (1998) also found an increase in the contents of DMA and FA in cod fillets during frozen storage at −20 and −30 °C. The contents of DMA and FA in whole fish and fillets of horse mackerel and Mediterranean hake increased during frozen storage at −18 °C for 12 months (Simeonidou, Govaris, & Vareltzis, 1997).

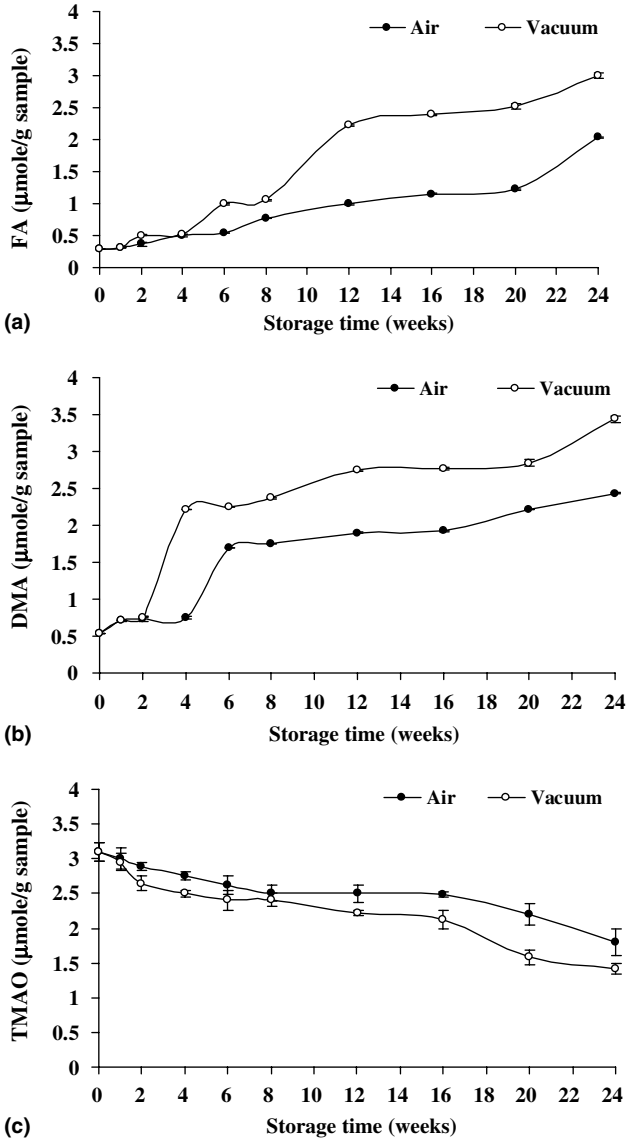

Fig. 1. Changes in FA (a), DMA (b) and TMAO contents (c) in lizardfish mince kept in air and under vacuum during frozen storage at −20 °C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

During frozen storage of several species of the Gadidae family, DMA and FA are formed from TMAO as a result of the action of the TMAOase enzymatic system (Amano & Yamada, 1964). This enzyme is distributed in various organs (Benjakul et al., 2004; Rehbein & Schreiber, 1984) and muscle (Kimura, Seki, & Kimura, 2000a, 2000b; Phillipy & Hultin, 1993). The speed of TMAO degradation depends on many factors, including storage temperature, species, muscle integrity and reducing conditions (Parkin & Hultin, 1982a). The absence of oxygen has also been shown to accelerate the rate of DMA and FA production. Lundstrom, Correia, and Wilhelm (1983) reported that minimal DMA production rates were obtained by storing fresh red hake fillets or mince exposed to air or to 100% oxygen. Maximal DMA production rates were obtained by storage under vacuum or in 100% nitrogen. From the result, DMA and FA contents were much higher in minced flesh of lizardfish kept under vacuum than that kept in air. Therefore, the absence of oxygen was shown to accelerate the rate of DMA and FA formation. Oxygen probably functioned as the inhibitor of TMAOase.

During frozen storage of fish muscle, DMA and FA are formed from TMAO in equimolar amounts (Parkin & Hultin, 1982b). However, detectable free FA content was much lower than DMA content (Fig. 1(a) and (b)). This was probably due to the reaction of FA with fish muscle components. Thus, the amount of free FA measured in lizardfish mince was lower than the theoretically expected amount. FA reacts with different functional groups of protein side chains, followed by the formation of intra- and inter-molecular methylene bridges (Sotelo, Pineiro, & Perez-Martin, 1995). These could increase protein denaturation during frozen storage. As FA molecules were bound to the proteins, unfolding and aggregation occurred, and the size of aggregates progressively increased, resulting in the greater loss in solubility.

Decrease in TMAO content was coincidental with the increases in the contents of DMA and free FA formed (Fig. 1(c)). A continuous decrease in the TMAO content was observed throughout the storage time ($P < 0.05$). It has been known that TMAOase demethylates TMAO to DMA and FA (Parkin & Hultin, 1982a). A high content of TMAO, as well as TMAOase, in this species might contribute to the great formation of DMA and FA. From the result, it was noted that TMAO content in samples kept under vacuum decreased to a greater extent, than in those stored in air. This result reconfirmed the role of oxygen as the TMAOase inhibitor.

3.2. Changes in TMAOase activity in lizardfish muscle during frozen storage

The changes in TMAOase activities of both samples were monitored throughout frozen storage (Fig. 2). No changes in TMAOase activity were observed in lizardfish mince kept in air within the first 2 weeks ($P > 0.05$). Conversely, a marked increase in activity was found in the samples kept under vacuum ($P < 0.05$). From the result, the highest TMAOase activity was observed at week 6 of storage. Thereafter, the activity decreased continuously up to 24 weeks of storage ($P < 0.05$) (Fig. 2). Higher activity was generally observed in lizardfish mince kept under vacuum, than when those stored in air. An increase in activity might result from the disruption of membranes of fish muscle subjected to frozen storage. The formation and accretion of ice crystals, dehydration, and increase in solute in unfrozen phase led to changes in muscle tissue as well as cell damage (Shenouda, 1980). Freezing and frozen storage cause the release of mitochondrial and lysosomal enzymes into the sarcoplasm (Hamm, 1979). As a result, more TMAOase could be extracted from muscle, particularly in the first 6 weeks of storage. The subsequent decrease in activities might be due to the denaturation of TMAOase. Additionally, more aggregate, possibly stabilized by disulfide bonds and methylene bridges was formed as shown by the loss in solubility. As a consequence, extraction efficacy of TMAOase from lizardfish mince would be lowered, especially with increasing storage time. Since less FA and DMA were produced in samples kept under air, it is likely that TMAOase activity was inhibited to some extent. Thus, lower TMAOase activity was found in mince stored under such a condition.

Although lizardfish mince was kept under frozen storage, TMAOase activity still continued, due to the low activation energy needed for this enzyme (Parkin & Hultin, 1982b). Benjakul et al. (2003a) reported that the activation energy of TMAOase from lizardfish kidney was 30.5 kJ mol^{-1} K^{-1}. Activation energy of TMAOase from Alaska pollack muscle was 38.4 kJ mol^{-1} K^{-1} (Kimura et al., 2000b), while an activation energy of 5.2 kJ mol^{-1} K^{-1} was found in TMAOase from red hake muscle (Phillipy & Hultin, 1993). Therefore, lizardfish TMAOase might play an important role in the FA production during extended frozen storage, which possibly induced the aggregation and denaturation of lizardfish muscle proteins during frozen storage.

3.3. Changes in Ca^{2+}-ATPase activity of NAM extracted from lizardfish mince during frozen storage

The Ca^{2+}-ATPase activities of the extracted NAM from lizardfish mince kept in air or under vacuum decreased with increasing frozen storage time ($P < 0.05$) (Fig. 3). The decreasing rate of Ca^{2+}-ATPase activity was much greater in samples kept under vacuum than in those kept in air. Mincing might result in breakdown of

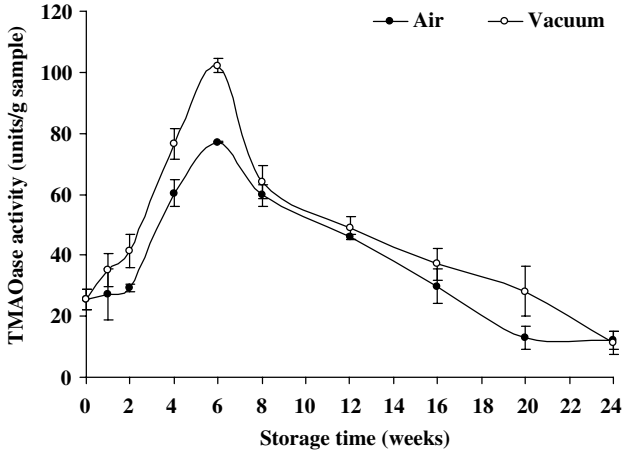

Fig. 2. Changes in TMAOase acitivity in lizardfish mince kept in air and under vacuum during frozen storage at −20 °C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

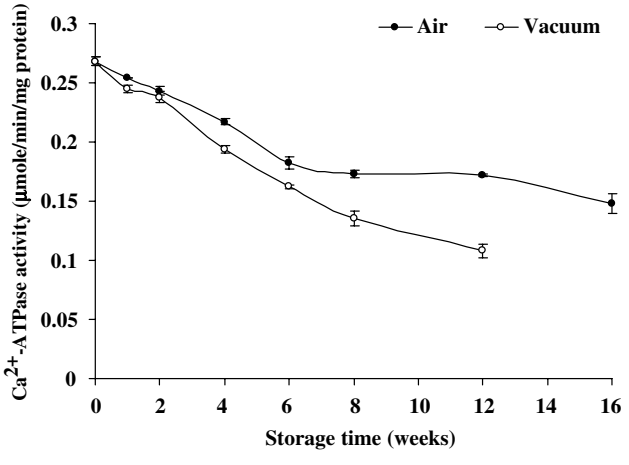

Fig. 3. Changes in Ca^{2+}-ATPase activity of natural actomyosin extracted from lizardfish mince kept in air and under vacuum during storage at −20 °C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

tissue and the release of TMAOase. As a result, FA might be produced to a greater extent and cause the denaturation of Ca^{2+}-ATPase. After 12 weeks of frozen storage, Ca^{2+}-ATPase activity of lizardfish mince, kept in air and under vacuum, decreased by 35.8% and 59.7%, respectively, compared to that obtained in fresh muscle. Ca^{2+}-ATPase activity can be used as an indicator for the integrity of myosin molecules (Benjakul et al., 1997). The globular heads of myosin are responsible for Ca^{2+}-ATPase activity (Benjakul et al., 2003b). Loss of Ca^{2+}-ATPase activity was associated with denaturation of myosin (Del Mazo, Torrejon, Careche, & Tejada, 1999). Suzuki (1967) and Hatano (1968) postulated that during frozen storage, the loss of enzyme activity of NAM was due to the tertiary structural changes caused by ice crystallization. Jiang, Ho, and lee (1985) also reported a decrease in Ca^{2+}-ATPase activity of mackerel and amberfish actomyosin during frozen storage. Ca^{2+}-ATPase activity of carp myofibrils decreased slowly during frozen storage (Azuma & Konno, 1998). Recently, Benjakul et al. (2003b) reported that Ca^{2+}-ATPase activity of NAM extracted from croaker, threadfin bream and lizardfish, stored at −20 °C, decreased as the storage time increased. It was noted that the decreasing rate of ATPase activity in lizardfish mince observed in this study was greater than that found in the whole fish reported by Benjakul et al. (2003b). Continuous decrease in Ca^{2+}-ATPase activity of minced flesh kept under vacuum was observed throughout the storage up to 12 weeks. For the samples stored in air, a marked decrease in Ca^{2+}-ATPase activity was found up to 6 weeks of storage. Thereafter, no changes were observed within 12 weeks. However, a slight decrease at week 16 was found.

From the result, the decreasing rate of Ca^{2+}-ATPase activity of the sample kept under vacuum, which had a higher FA content, was greater than that kept in air. FA, known as an effective cross-linker via methylene bridges (Sikorski, Kolokowski, & Burt, 1990), probably induced the aggregation of protein, particularly in the myosin head region. As a consequence, a concomitant marked decrease in Ca^{2+}-ATPase activity was observed in those samples containing a higher content of FA.

3.4. Changes in sulfhydryl and disulfide bond contents of NAM extracted from lizardfish mince during frozen storage

Total sulfhydryl content of both samples decreased gradually during frozen storage ($P < 0.05$) (Fig. 4(a)). A sharp decrease in sulfhydryl content was observed in the first 2 weeks of storage. Greater decrease in total sulfhydryl content was observed in the samples kept under vacuum, than in those kept in air. At week 12, sulfhydryl content in lizardfish mince kept in air and under vacuum decreased by 41.4% and 45.1%, compared to that found

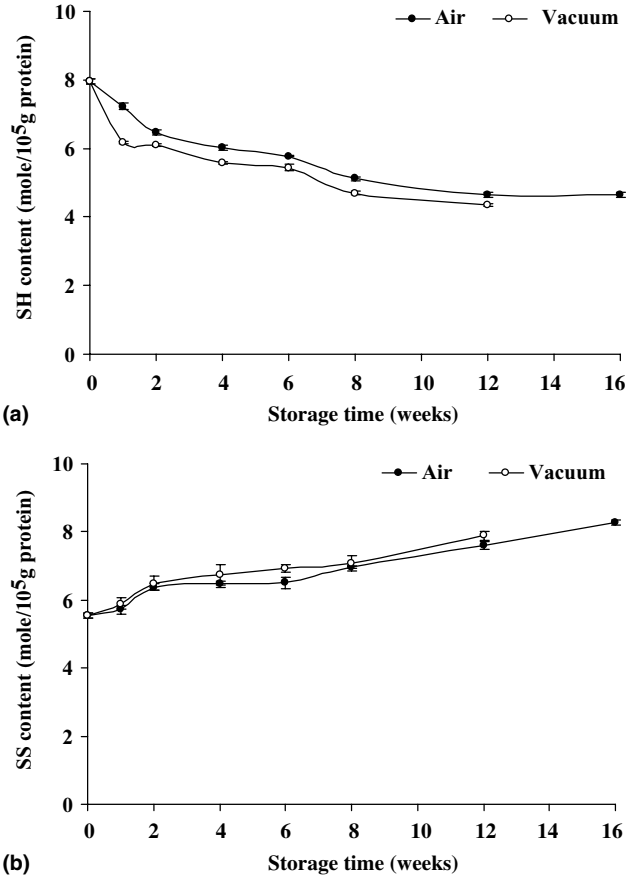

Fig. 4. Changes in total sulfhydryl (a) and disulfide bond (b) contents of natural actomyosin extracted from lizardfish mince kept in air and under vacuum during storage at −20 °C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

in fresh sample, respectively. The decrease in sulfhydryl groups generally resulted from the formation of disulfide bonds through oxidation of sulfhydryl groups or disulfide interchanges (Hayagawa & Nakai, 1985). Sulfhydryl groups on the myosin head portion, named SH_1 and SH_2, were reported to be involved in the ATPase activity of myosin (Kielley & Bradley, 1956). The reduction in the sulfhydryl content might be explained by the following two reasons: the sulfhydryl groups of inter- or intra-proteins formed cross-linkage (Huidobro, Mohamed, & Tejada, 1998), or the exposed sulfhydryl groups in protein interacted with additives or small molecular weight compounds (e.g., peptides) in the water-soluble protein fraction (Owusu-Ansah & Hultin, 1987). From the results, the decrease in sulfhydryl groups was coincidental with the decrease in Ca^{2+}-ATPase activity (Fig. 3). Therefore, it is postulated that conformational changes of myosin, especially in the head region, occurred rapidly in the first 2 weeks. Del Mazo et al. (1999) also found that the sulfhydryl groups of NAM extracted from hake fillets decreased with increasing storage time at −20 and −30 °C. Ramirez,

Martin-Polo, and Bandman (2000) reported that reactive sulfhydryl groups decreased to 55% of the initial value after 5 days of frozen storage. Additionally, Jiang, Hwang, and Chen (1988a) reported that, during the first 2 weeks of frozen storage of milkfish actomyosin, the reactive sulfhydryl groups decreased significantly ($P < 0.01$). During prolonged storage at −20 °C, the tertiary structure of actomyosin was changed by the formation of disulfide, hydrogen and hydrophobic bonds. Consequently, the reactive sulfhydryl groups, masked in molecules, were gradually exposed and oxidised to disulfides. The rate of exposure and oxidation of reactive sulfhydryl groups might reach an equilibrium state during storage when the amount of the reactive sulfhydryl groups no longer changed (Jiang et al., 1988a).

From the result, a greater decrease in sulfhydryl groups was found in samples kept under vacuum throughout storage, than in those stored in air. It was assumed that a higher content of FA formed in the former samples could induce the conformational changes of proteins, in which SH groups were exposed to oxidation. The disulfide bond content of NAM increased throughout the frozen storage with a concomitant decrease in sulfhydryl groups ($P < 0.05$) (Fig. 4(b)). In general, the amounts of disulfide bonds formed in samples kept under vacuum were slightly higher than in those kept in air (Fig. 4(b)). After 12 weeks of storage, the disulfide bond contents of samples stored in air and under vacuum increased by 37.3% and 42.5%, compared to that of fresh sample, respectively. The accelerated denaturation of myosin molecules, especially the conformational changes, in which the reactive sulfhydryl groups were exposed to oxidation, might result in the increased disulfide bond formation. Sultanbawa and Li-Chan (2001) found that the NAM and surimi from ling cod without cryoprotectants had increased amounts of disulfide bonds after freezing.

The decrease in sulfhydryl groups with a concomitant disulfide bond formation (Fig. 4(a) and (b)) was generally in accordance with the decreased Ca^{2+}-ATPase activity (Fig. 3). Fish myosin molecule contains many sulfhydryl groups (Buttkus, 1970). Sulfhydryl groups located in the head portion (SH_1 and SH_2) play an essential role in ATPase activity (Kielley & Bradley, 1956). Furthermore, sulfhydryl groups localized in light meromyosin (SH_a) also contribute to oxidation (Sompongse, Itoh, & Obataka, 1996). The oxidation of sulfhydryl groups, especially in the head region, causes decrease in Ca^{2+}-ATPase activity. Buttkus (1971) and Hamada et al. (1977) concluded that the decrease in Ca^{2+}-ATPase activity was closely related to the oxidation of sulfhydryl groups. Decrease in the total number of sulfhydryl groups indicates that the loss of Ca^{2+}-ATPase activity might be due to the oxidation of sulfhydryl groups on the active site of actomyosin (Jiang, Hwang, & Chen, 1988b).

3.5. Changes in surface hydrophobicity of NAM extracted from lizardfish mince during frozen storage

The surface hydrophobicity of NAM from both samples is shown in Fig. 5. Surface hydrophobicity increased up to 4 weeks, with a subsequent decrease up to 16 weeks. During the first 4 weeks, surface hydrophobocity of sample kept under vacuum increased to a greater extent, than in those kept in air. However, the surface hydrophobicity of the former was lower than that of the latter during week 4–16. Increase in surface hydrophobicity of proteins during frozen storage can be attributed to the unfolding of proteins and the exposure of hydrophobic aliphatic and aromatic amino acids (Baddii & Howell, 2001). An initial increase in surface hydrophobicity was observed during frozen storage of isolated hake NAM (Del Mazo, Huidobro, Torrejon, Tejada, & Careche, 1994). The subsequent decrease in surface hydrophobicity with the extended storage time might be due to the aggregation of exposed proteins via hydrophobic interaction. This leads to the masking of previously exposed hydrophobic portions. As a result, decrease in surface hydrophobicity may be observed. Careche and Li-Chen (1997) suggested that, in the presence of FA during frozen storage of cod myosin, more extensive protein denaturation results in more interactions between hydrophobic groups and formation of covalent bonds.

Hydrophobic interactions between the exposed groups results in a decrease in solubility of proteins (Buttkus, 1974). In general, the denaturation and aggregation of protein starts from the formation of

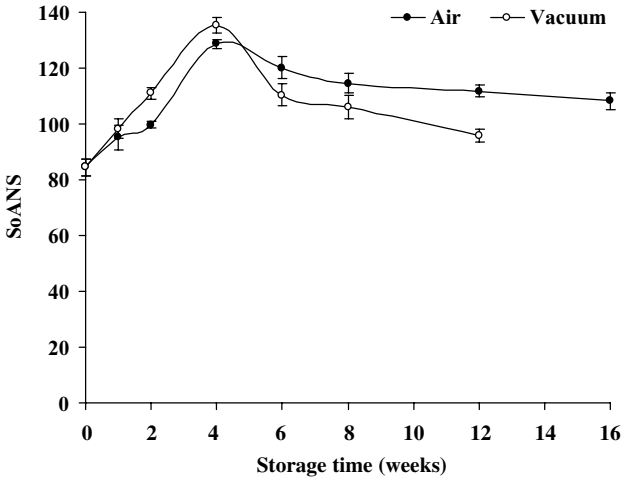

Fig. 5. Changes in surface hydrophobicity of natural actomyosin extracted from lizardfish mince kept in air and under vacuum during storage at −20 °C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

disulfide bonds, followed by a rearrangement of hydrophobicmolecular bonds basis (Buttkus, 1974). From the result, the drastic decrease in surface hydrophobicity after 4 weeks possibly resulted from the great extent of cross-links induced by FA between the methyl groups of hydrophobic amino side chain, leading to the considerable decrease of free hydrophobic groups.

3.6. Changes in α-amino acid content of NAM extracted from lizardfish mince during frozen storage

Fig. 6 shows the changes in α-amino acid content of NAM from lizardfish mince during frozen storage. TNBS-reactive amino groups of both samples decreased drastically as the storage time increased ($P < 0.05$). No changes were found after 8 and 12 weeks of storage for the samples kept under vacuum or in air. The rate of loss in α-amino groups was generally higher in samples kept under vacuum than in those kept in air. The decrease in the α-amino acid content was possibly associated with the higher amount of FA produced by TMAOase during the storage (Fig. 1(a)). FA is known to react with a wide spectrum of amino acyl side chains in proteins (Ang & Hultin, 1989). The decrease in TNBS-reactive amino groups was used as a measure of the reaction between FA and proteins (Ang & Hultin, 1989). Lysine is one of the more reactive side chain groups with FA (Tome, Kozlowski, & Mabon, 1985). Moreover, Ang and Hultin (1989) proposed that a relatively small number of side chain modifications can markedly affect the stability of some proteins, especially when they are subjected to stress conditions, e.g., freezing and thawing. Ang and Hultin (1989) also found a loss of amino groups of partially purified cod myosin during storage at −80 °C.

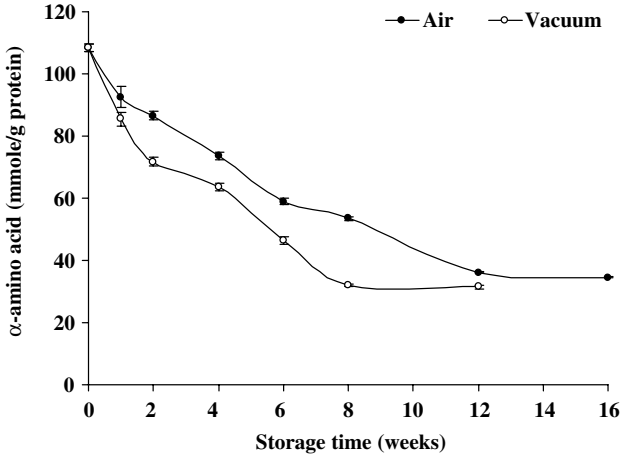

Fig. 6. Changes in α-amino acid content of natural actomyosin extracted from lizardfish mince kept in air and under vacuum during storage at −20 °C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

From the result, the loss of α-amino groups might be due to the formation of aggregate induced by FA. Masking of α-amino groups, caused by the aggregate stabilized by other bonds, such as disulfide and hydrophobic interaction was also presumed.

3.7. Changes in protein solubility of lizardfish muscle during frozen storage

The solubility of lizardfish mince is shown in Fig. 7. During extended storage, the salt-soluble fraction (I) of lizardfish mince kept under vacuum (Fig. 7(b)) decreased to a greater extent, than in that kept in air (Fig. 7(a)). During the early stage of freeze denaturation of fish proteins, both myosin and actin apparently form an insoluble fraction that accounts for the observed decrease in protein solubility (Jiang & Lee, 1985). On the other hand, the urea-soluble fraction (II) increased during the first 8 weeks of storage with a subsequent decrease during prolonged storage. The increase in the urea-soluble fraction indicated the formation of hydrogen and hydrophobic bonds during frozen storage (Jiang et al., 1988a). After 8 weeks of storage, the decrease

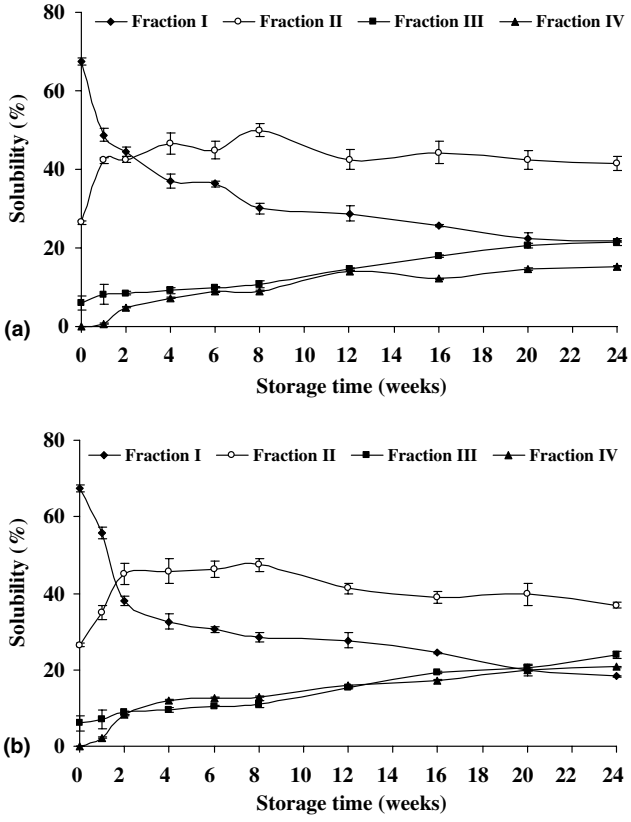

Fig. 7. Changes in protein solubility of lizardfish mince kept in air (a) and under vacuum (b) during storage at −20 °C for 24 weeks. Bars indicate standard deviation from triplicate determinations. The solubility in each fraction was expressed as the percentage of soluble protein relative to total protein content in the sample.

in urea-soluble proteins might be due to increased formation of the disulfides, which consequently caused incomplete disruption of hydrogen and hydrophobic bonding by urea. The $NaBH_4$-soluble (III) and insoluble (IV) fractions of both samples increased gradually throughout storage up to 24 weeks, suggesting increase in disulfide bonds formed and non-disulfide aggregate. The increase in the $NaBH_4$-soluble (III) fraction was in accordance with the increase in disulfide bond formation (Fig. 4(b)). For the insoluble fraction, the FA formed during extended storage might contribute to the aggregate, which could not be solubilised by all denaturants tested. From the result, $NaBH_4$-soluble (III) and insoluble (IV) fractions were higher in lizardfish mince kept under vacuum than in that kept in air (Fig. 7(a) and (b)). FA has been known as an effective cross-linker. Addition of FA to cod myosin causes loss of solubility (Careche & Li-Chen, 1997). Therefore, loss in solubility of muscle proteins in the minced flesh of lizardfish was possibly caused by the formation of disulfide, hydrogen and hydrophobic bonds as well as by methylene bridges. Huidobro et al. (1998) found that the amount of protein linked by covalent bonds in hake mince increased during one year of storage at −20 °C.

4. Conclusion

Muscle proteins underwent physicochemical and biochemical changes during frozen storage of minced flesh of lizardfish. Greater changes were observed in the samples kept under vacuum than in those in air. It is likely that those changes were influenced by FA produced by TMAOase. The absence of oxygen was shown to accelerate the formation of FA and DMA during frozen storage. Therefore, packaging of minced flesh of lizardfish, under conditions lacking in oxygen, might induce a quality loss during frozen storage.

Acknowledgements

This work was supported by the Thailand Research Fund under the Royal Golden Jubilee Ph.D., Program to Kittima Leelapongwattana (PHD/0145 /2545).

References

Amano, K., & Yamada, K. (1964). A biological formation of FA in the muscle tissue of gadoid fish. *Nippon Suisan Gakkaishi, 30*, 430–435.

Ang, J. F., & Hultin, H. O. (1989). Denaturation of cod myosin during freezing after modification with formaldehyde. *Journal of Food Science, 54*, 814–818.

Azuma, Y., & Konno, K. (1998). Freeze denaturation of carp myofibril compared with thermal denaturation. *Fisheries Science, 64*, 287–290.

Baddii, F., & Howell, N. K. (2001). A comparison of biochemical changes in cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) during frozen storage. *Journal of the Science of Food and Agriculture, 81*, 1–11.

Benjakul, S., & Morrissey, M. T. (1997). Protein hydrolysates from Pacific whiting solid wastes. *Journal of Agricultural and Food Chemistry, 45*, 3423–3430.

Benjakul, S., Seymour, T. S., Morrissey, M. T., & An, H. (1997). Physicochemical changes in Pacific whiting muscle proteins during iced storage. *Journal of Food Science, 62*, 729–733.

Benjakul, S., Visessanguan, W., & Tanaka, M. (2004). Induced formation of dimethylamine and formaldehyde by lizardfish kidney trimethylamine-*N*-oxide demethylase. *Food Chemistry, 84*, 297–305.

Benjakul, S., Visessanguan, W., & Tanaka, M. (2003a). Partial purification and characterization of trimethylamine-*N*-oxide demethylase from lizardfish kidney. *Comparative Biochemistry Physiology Part B, 135*, 359–371.

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

Buttkus, H. (1970). Accelerated denaturation of myosin in frozen solution. *Journal of Food Science, 35*, 558–562.

Buttkus, H. (1971). The sulfhydryl content of rabbit and trout myosins in relation to protein stability. *Canadian Journal of Biochemistry, 49*, 97–102.

Buttkus, H. (1974). On the nature of the chemical and physical bonds which contribute to some structural properties of protein foods: A hypothesis. *Journal of Food Science, 39*, 484–489.

Careche, M., & Li-Chen, E. (1997). Structural changes and Raman spectroscopic studies of cod myosin upon modification with formaldehyde or frozen storage. *Journal of Food Science, 62*, 717–723.

Careche, M., Del Mazo, M. L., Torrejon, P., & Tejada, M. (1998). Importance of frozen storage temperature in the type of aggregation of myofibrillar proteins in cod (*Gadus morhua*) fillets. *Journal of Agricultural and Food Chemistry, 46*, 1539–1546.

Castell, C. H., Smith, B., & Dale, J. (1973). Comparison of changes in trimethylamine, dimethylamine and extractable protein in iced and frozen gadoid fillets. *Journal of the Fisheries Research Board of Canada, 30*, 1246–1248.

Conway, E. J., & Byrne, A. (1936). An absorption apparatus for the micro determination of certain volatile substances. I. The micro-determination of ammonia. *Biochemical Journal, 27*, 419–429.

Del Mazo, M. L., Huidobro, A., Torrejon, P., Tejada, M., & Careche, M. (1994). Role of formaldehyde in formation of natural actomyosin aggregates in hake during frozen storage. *Zeitschrift fur Lebensmittel-Untersuchung und-Forschung, 198*, 459–464.

Del Mazo, M. L., Torrejon, P., Careche, M., & Tejada, M. (1999). Characteristics of the salt-soluble fraction of hake (*Merluccius merluccius*) fillets stored at −20 and −30 °C. *Journal of Agricultural and Food Chemistry, 47*, 1372–1377.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry Biophysics, 82*, 70–77.

Gill, T. A., Keith, R. A., & Smith, L. B. (1979). Textural deterioration of red hake and haddock muscle in frozen storage as related to chemical parameters and changes in the myofibrillar proteins. *Journal of Food Science, 44*, 661–667.

Hamada, I., Tsuji, K., Nakayama, T., & Niwa, E. (1977). Oxidative denaturation of actomyosin. *Bulletin of Japanese Society of Scientific Fisheries, 43*, 1105–1109.

Hamm, R. (1979). Delocalization of mitochondrial enzymes during freezing and thawing of skeletal muscle. In O. R. Fennema (Ed.), *Protein at Low Temperatures*, *Advances in Chemistry Series* (Vol. 180, pp. 191). Washington, DC: American Chemical Society.

Hatano, S. (1968). Effect of freezing and storage on the enzyme activities. *Refrigeration (Tokyo), 43*, 14–20.

Hayagawa, S., & Nakai, S. (1985). Contribution of hydrophobicity, net charge and sulfhydryl groups to thermal properties of ovalbumin. *Canadian Institute of Food Science and Technology Journal, 18*, 290–295.

Herrera, J. J., Pastoriza, L., & Sampedro, G. (2000). Inhibition of formaldehyde production in frozen-stored minced blue whiting (*Micromesistius poutassou*) muscle by cryostabilizers: An approach from the glassy state theory. *Journal of Agricultural and Food Chemistry, 48*, 5256–5262.

Huidobro, A., Mohamed, G. F., & Tejada, M. (1998). Aggregation of myofibrillar proteins in hake, sardine and mixed minces during frozen storage. *Journal of Agricultural and Food Chemistry, 46*, 2601–2608.

Jiang, S. T., & Lee, T. C. (1985). Changes in free amino acids and protein denaturation of fish muscle during frozen storage. *Journal of Agricultural and Food Chemistry, 33*, 839–844.

Jiang, S. T., Hwang, B. S., & Chen, C. S. (1988a). Effect of storage temperatures on the formation of disulfides and denaturation of milkfish actomyosin (*Chanos chanos*). *Journal of Food Science, 53*, 1333–1335.

Jiang, S. T., Hwang, B. S., & Chen, C. S. (1988b). Denaturation and change in SH group of actomyosin from milkfish (*Chanos chanos*) during storage at −20 °C. *Journal of Agricultural and Food Chemistry, 36*, 433–437.

Jiang, S. T., Ho, M. L., & lee, T. C. (1985). Optimization of the freezing conditions on mackerel and amberfish for manufacturing minced fish. *Journal of Food Science, 50*, 727–732.

Kielley, W. W., & Bradley, L. B. (1956). The relationship between sulfhydryl groups and the activation of myosin adenosinetriphosphatase. *The Journal of Biological Chemistry, 218*, 653–659.

Kimura, M., Seki, N., & Kimura, I. (2000a). Occurrence and some properties of trimethylamine-*N*-oxide demethylase in myofibrillar fraction from walleye pollack muscle. *Fisheries Science, 66*, 725–729.

Kimura, M., Seki, N., & Kimura, I. (2000b). Purification and characterization of trimethylamine-*N*-oxide demethylase from walleye pollack muscle. *Fisheries Science, 66*, 967–973.

Krueger, D. J., & Fennema, O. R. (1989). Effect of chemical additives on toughening of fillets of frozen storage Alaska pollack (*Theragra chacogramma*). *Journal of Food Science, 54*, 1101–1106.

Lowry, Q. H., Rosebrough, N. J., Farr, L. A., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 256–275.

Lundstrom, R. C., Correia, F. F., & Wilhelm, K. A. (1982). Enzymatic dimethylamine and formaldehyde production in minced American plaice and backbone flounder mixed with a red hake TMAOase active fraction. *Journal of Food Science, 47*, 1305–1310.

Lundstrom, R. C., Correia, F. F., & Wilhelm, K. A. (1983). Dimethylamine production in fresh red hake (*Urophycis chuss*): The effect of packaging material oxygen permeability and cellular damage. *Journal of Food Biochemistry, 6*, 229–241.

Owusu-Ansah, Y. J., & Hultin, H. O. (1987). Effect of in situ formaldehyde production on solubility and crosslinking of proteins of minced red hake muscle during frozen storage. *Journal of Food Biochemistry, 11*, 17–39.

Parkin, K. L., & Hultin, H. O. (1982a). Some facts influencing the production of dimethylamine and formaldehyde in minced and intact red hake muscle. *Journal of Food Processing and Preservation, 6*, 73–97.

Parkin, K. L., & Hultin, H. O. (1982b). Fish muscle microsomes catalyze the conversion of trimethylamine oxide to dimethylamine and formaldehyde. *FEBS Letters, 139*, 61–64.

Phillipy, B. Q., & Hultin, H. O. (1993). Distribution and some characteristics of trimethylamine-*N*-oxide (TMAO) demethylase activity of red hake muscle. *Journal of Food Biochemistry, 17*, 235–250.

Ramirez, J. A., Martin-Polo, M. O., & Bandman, E. (2000). Fish myosin aggregation as affected by freezing and initial physical state. *Journal of Food Science, 65*, 556–560.

Rehbein, H., & Schreiber, W. (1984). TMAOase activity in tissues of fish species from the Northeast Atlantic. *Comparative Biochemistry Physiology Part B, 79*, 447–452.

Shenouda, S. Y. K. (1980). Theories of protein denaturation during frozen storage of fish flesh. In C. O. Chichester (Ed.), *Advance in Food Research* (p. 275). New York: Academic Press.

Sikorski, Z. E., Kolokowski, A., & Burt, J. R. (1990). Postharvest biochemical and microbial changes. In Z. E. Sikorski (Ed.), *Seafood Resources Nutritional Composition and Preservation* (pp. 55–85). USA: CRC Press, Inc..

Simeonidou, S., Govaris, A., & Vareltzis, K. (1997). Effect of frozen storage on the quality of whole fish and fillets of horse mackerel (*Trachurus trachurus*) and Mediterranean hake (*Merluccius mediteraneus*). *Zeitschrift fur Lebensmittel-Untersuchung und-Forschung, 204*, 405–410.

Sompongse, E., Itoh, Y., & Obataka, A. (1996). Effect of cryoprotectants and reducing reagent on the stability of actomyosin during ice storage. *Fisheries Science, 62*, 110–113.

Sotelo, G. C., Pineiro, C., & Perez-Martin, R. I. (1995). Denaturation of fish proteins during frozen storage: Role of formaldehyde. *Zeitschrift fur Lebensmittel-Untersuchung und-Forschung, 200*, 14–23.

Steel, R. G. D., & Torrie, J. H. (1980). *Principles and procedures of statisics: A biometrical approach* pp. 862. New York: McGraw-Hill.

Sultanbawa, Y., & Li-Chan, E. C. Y. (2001). Structural changes in natural actomyosin and surimi from ling cod (*Ophiodon elongates*) during frozen storage in the absence or presence of cryoprotectants. *Journal of Agricultural and Food Chemistry, 49*, 4716–4725.

Suzuki, T. (1967). Freezing denaturation of fish proteins. *Refrigeration (Tokyo), 42*, 46–51.

Tome, D., Kozlowski, A., & Mabon, F. (1985). Carbon-13 NMR study on the combination of formaldehyde with bovine serum albumin. *Journal of Agricultural and Food Chemistry, 33*, 449.

Thannhauser, T. W., Konishi, Y., & Scheraga, H. A. (1987). Analysis for disulfide bonds in peptides and proteins. *Methods in Enzymology, 143*, 115–118.

Yasui, A., & Lim, P. Y. (1987). Changes in chemical and physical properties of lizardfish meat during iced and frozen storage. *Nippon Shokuhin Kyogo Gakkaishi, 34*, 54–60.