CHAPTER 2

PHYSICOCHEMICAL AND BIOCHEMICAL CHANGES DURING FROZEN STORAGE OF MINCED FLESH OF LIZARDFISH (*SAURIDA TUMBIL*)

2.1 Abstract

Physicochemical and biochemical changes of minced flesh of lizardfish (*Saurida tumbil*) kept in air and under vacuum during frozen storage at -20°C for 24 weeks were investigated. Formaldehyde (FA) and dimethylamine (DMA) contents increased with a concomitant decrease in trimethylamine oxide (TMAO) content as the storage time increased ($P < 0.05$). The Ca^{2+}-adenosine 5′-triphosphatase (Ca^{2+}-ATPase) activity and TNBS-reactive amino groups decreased continuously with a coincidental decrease in the salt-soluble fraction. Loss in salt-soluble proteins was caused by the formation of protein cross-links stabilized by hydrogen, hydrophobic, disulfide and non-disulfide covalent bonds. 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 FA formation, could be reduced to some extent with packaging containing oxygen.

2.3 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 *et al.*, 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-*N*-oxide demethylase (TMAOase). The last mechanism is believed to be important in fish that belong to the gadoid family (Krueger and Fennema, 1989). TMAOase catalyzes the breakdown of trimethylamine oxide (TMAO) to equimolar quantities of formaldehyde (FA) and dimethylamine (DMA) (Amano and Yamada, 1964; Lundstrom *et al*., 1982; Parkin and Hultin, 1982b). The FA produced has been suggested to cause cross-linking of the muscle proteins and toughening of the tissue (Castell *et al*., 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 *et al*., 1979). FA accelerates the formation of high molecular weight polymers from isolated myosin and actomyosin during freezing and frozen storage (Ang and Hultin, 1989). The speed of TMAO degradation depends upon many factors, such as storage temperature, species, muscle integrity and reducing conditions (Parkin and Hultin, 1982a). Kidney of lizardfish contained a high amount of TMAOase, which can contaminate fish muscle during processing (Benjakul *et al*., 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 and 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 *et al*. (2003c) 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.

2.3 Materials and Methods

Chemicals

All chemicals for TMAOase activity assay were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). TMAO 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 (Buchs, Switzerland). Tris (hydroxymethyl) aminomethane, urea, sodium borohydride and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany).

Fish sample preparation

Lizardfish (*Saurida tumbil*), 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. 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 in 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.

Determination of trimethylamine oxide (TMAO)

The samples (2.5 g) were added with 10 ml of 5% (w/v) trichloroacetic acid and homogenized with a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 12,000 rpm for 2 min. The homogenate was centrifuged at 3,000 × g for 15 min. The supernatant containing the TMAO was used for analysis. The TMAO was determined after reduction to trimethylamine (TMA) by the method of Benjakul *et al.* (2004). To 2 ml of the supernatant, 2 ml of 1% $TiCl_3$ was added. The mixture was incubated at 80°C for 90 s, followed by cooling under running water. The TMA was determined according to the method of Conway and Byrne (1936). The TMAO was then calculated after subtracting the indigenous TMA content in the samples and expressed as micromoles per gram.

Determination of dimethylamine (DMA) and free formaldehyde (FA)

The samples (2.5 g) were added with 10 ml of 5% (w/v) trichloroacetic acid, then homogenized at a speed of 12,000 rpm for 2 min. The homogenate was centrifuged at 3,000 × g for 15 min. Five milliliters of 5% (w/v) trichloroacetic acid were added to the pellet, then homogenized as previously described. The supernatant was combined and neutralized 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 FA determination as described by Benjakul *et al.* (2004).

TMAOase activity measurement

Preparation of TMAOase crude extract from lizardfish muscle

The TMAOase extract was prepared according to method of Benjakul *et al.* (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 homogenized for 3 min using a homogenizer 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''.

TMAOase activity assay

The TMAOase activity was assayed using TMAO as a substrate in the presence of selected cofactors (Benjakul *et al.*, 2004). To 2.5 ml of the 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 the enzyme solution was added to initiate the reaction. The reaction was conducted at 25°C for 80 min, and 1 ml of 10% (w/v) trichloroacetic acid was added to terminate the reaction. The reaction mixture was then centrifuged at 8,000 × g for 15 min, and the supernatant was subjected to DMA determination. One unit of TMAOase was defined as the activity that released 1 nmol DMA per min.

Protein solubility

The solubility was determined according to the method of Hamada *et al.* (1977) with some modifications. The samples were thawed under running water until the core temperature (0-2°C) had been reached. To 2 g of the sample, 20 ml of 0.6 M KCl was added and the mixture was homogenized for 3 min at a speed of 12,000 rpm. The homogenate was centrifuged at 5,000 × g for 30 min at 4°C. The supernatant was defined as salt-soluble fraction (I), which is considered to be native proteins. The insoluble material was treated with four volumes of a mixture solution containing 8 M urea, 6 mM ethylenediaminetetraacetic acid (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 urea-soluble fraction (II), which is considered to be the aggregate stabilized by the hydrogen and hydrophobic bonds. Finally, any remaining precipitate was treated with a mixture solution containing 0.5% (w/v) $NaBH_4$, 6 mM EDTA, 8 M urea and 0.6 M KCl, then 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 the aggregate caused by the formation of disulfides, while the final residue was defined as

the insoluble fraction (IV). The protein concentration in the fractions was measured by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard. The solubility of each fraction was expressed as the percentage of the soluble protein compared with the total protein in the sample. The samples were solubilized in 0.5 M NaOH and the protein content was measured and used as a reference value (100%).

Preparation of natural actomyosin (NAM)

The NAM was prepared by the method of Benjakul *et al.* (1997). Briefly, 20 g of the lizardfish muscle was homogenized in 200 ml of chilled (4°C) 0.6 M KCl, pH 7.0, for 5 min using a homogenizer at a speed of 12,000 rpm. The extract was centrifuged at 5,000 × g for 30 min at 4°C. Three volumes of chilled distilled water were added to precipitate the NAM. The NAM was collected by centrifuging at 5,000 × g for 20 min at 4°C. The pellet was then dissolved by stirring in an equal volume of chilled 1.2 M KCl, pH 7.0, for 30 min at 4°C. The undissolved debris was removed by centrifuging at 5,000 × g for 30 min at 4°C. The supernatant obtained was referred to as the NAM.

Surface hydrophobicity

The surface hydrophobicity was determined according to the method described by Benjakul *et al.* (1997) using 1-anilinonaphthalene-8-sulfonic acid (ANS) as a probe. The NAM was dissolved in 10 mM phosphate buffer, pH 6.0, containing 0.6 M NaCl to obtain the different concentrations, then mixed with the ANS. The fluorescence intensity of the 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 the NAM concentration was referred to as the S_0ANS.

Total sulfhydryl (SH) content

The total SH content was measured using DTNB according to the method of Ellman (1959) as modified by Benjakul *et al.* (1997). To 1.0 ml of the NAM solution

(0.4%), 9 ml of 0.2 M Tris-HCl buffer, pH 6.8, containing 8 M urea, 2% sodium dodecyl sulfate and 10 mM EDTA was added. To 4 ml of the mixture, 0.4 ml of 0.1% DTNB was added and incubated at 40°C for 25 min. The absorbance at 412 nm was then measured using a spectrophotometer (Shimadzu, Kyoto, Japan). A blank was conducted by replacing the sample with 0.6 M KCl, pH 7.0. The total SH content was calculated using a molar extinction coefficient of 13,600/M/cm.

Disulfide bond content

The disulfide bond content in the NAM was determined using 2-nitro-5-thiosulfobenzoate (NTSB) assay according to the method of Thannhauser *et al.* (1987). To 0.2 ml of the NAM sample (4 mg/ml), 3.0 ml of freshly prepared NTSB assay solution was added. The mixture was incubated in the dark at room temperature (25-27°C) for 25 min. The absorbance at 412 nm was measured. The disulfide bond content was calculated using the extinction coefficient of 13,900/M/cm.

Ca^{2+}-adenosine 5′-triphosphatase (ATPase) activity

The Ca^{2+}-ATPase activity was assayed according to the method of Benjakul *et al.* (1997). The NAM was diluted to obtain the concentration of 1-3 mg/ml with 0.6 M KCl, pH 7.0. One milliliter of the diluted solution was added into 0.6 ml of 0.5 M Tris-maleate, pH 7.0, and 1 ml of 0.1 M $CaCl_2$. Deionized water was added to make up a total volume of 9.5 ml. To the solution prepared, 0.5 ml of 20 mM ATP solution was added to initiate the reaction. The reaction was conducted at 25°C for 10 min and terminated by adding 5 ml of 15% (w/v) trichloroacetic acid. The reaction mixture was then centrifuged at 3,500 × g for 5 min at room temperature (25-27°C). The inorganic phosphate (Pi) liberated in the supernatant was measured according to the method of Benjakul *et al.* (1997). The specific activity was expressed as micromoles of inorganic phosphate (Pi) released per milligram of protein within 1 min for the reaction at 25°C. A blank solution was prepared by adding chilled trichloroacetic acid prior to the addition of ATP.

2,4,6-Trinitobenzenesulfonic acid (TNBS)-reactive amino groups

The TNBS-reactive amino groups were determined as described by Benjakul and Morrissey (1997). Properly diluted samples (125 μl) were thoroughly mixed 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 Na_2SO_3. The mixture was cooled at room temperature for 15 min. The absorbance was measured at 420 nm, and the TNBS-reactive amino groups were expressed in terms of L-leucine.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range Test (DMRT) (Steel and Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL).

2.4 Results and Discussion

Changes in TMAO, FA and DMA contents of muscle of lizardfish during frozen storage

Changes in free FA content are presented in Figure 7A. 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 (Figure 7B). At week 24, the DMA content increased 4.6- and 6.5-fold in samples kept in air and under vacuum, respectively. Careche *et al.* (1998b) 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 *et al*., 1997). 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 and Yamada, 1964). This enzyme is distributed in various organs (Rehbein and Schreiber, 1984; Benjakul *et al*., 2004) and muscle (Phillipy and Hultin, 1993; Kimura *et al*., 2000a, 2000b). The speed of TMAO degradation depends on many factors, including storage temperature, species, muscle integrity and reducing conditions (Parkin and Hultin, 1982a). The absence of oxygen has also been shown to accelerate the rate of DMA and FA production. Lundstrom *et al*. (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 and Hultin, 1982b). However, detectable free FA content was much lower than DMA content (Figures 7A and 7B). 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 *et al*., 1995b). 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 (Figure 7C). 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 and 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.

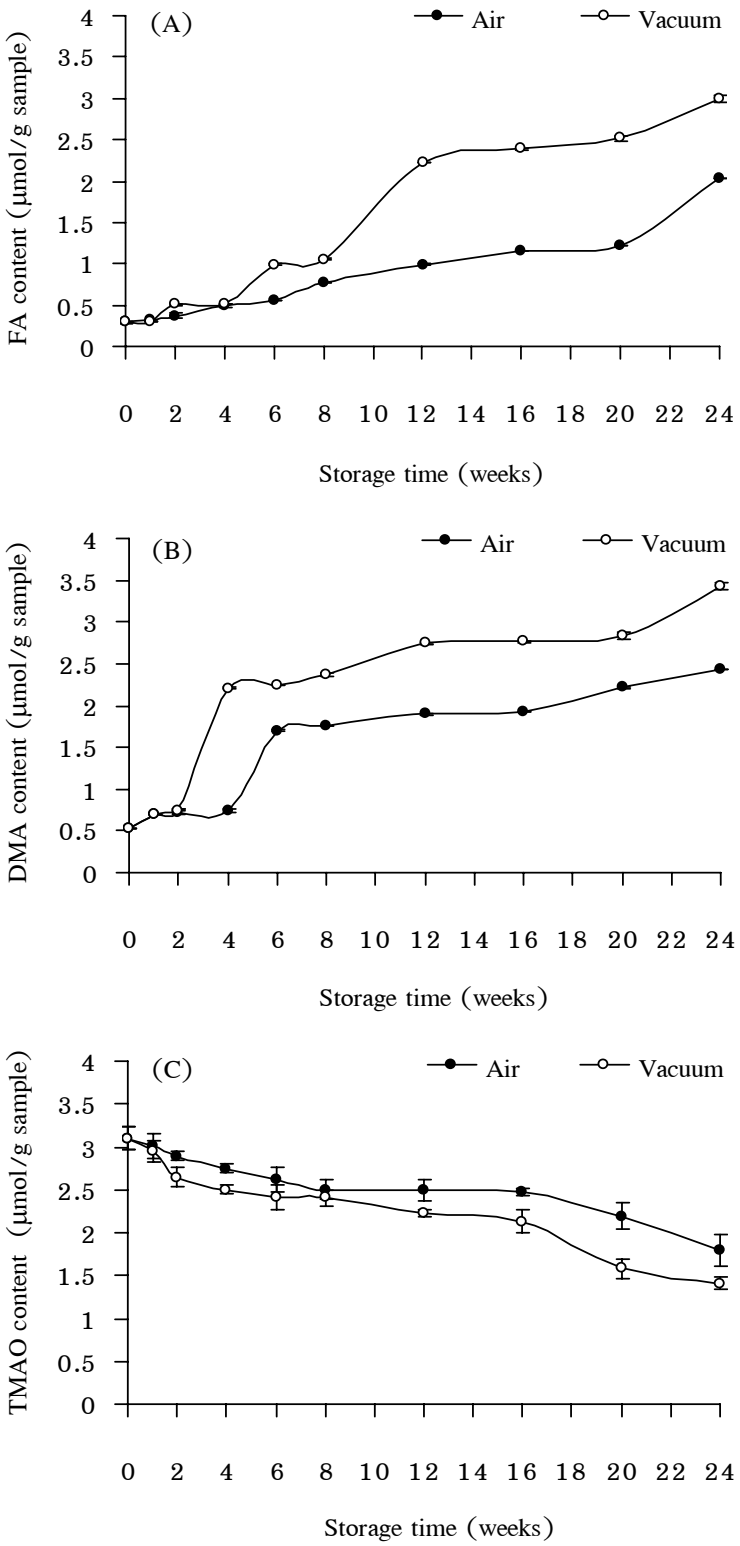

Figure 7. Changes in FA (A), DMA (B) and TMAO (C) contents in lizardfish mince kept in air and under vacuum during frozen storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

Changes in TMAOase activity in lizardfish muscle during frozen storage

The changes in TMAOase activities of both samples were monitored throughout frozen storage (Figure 8). 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 ($P < 0.05$) (Figure 8). 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 in air, it is likely that TMAOase activity was inhibited to some extent. Thus, lower TMAOase activity was found in mince stored in 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 and Hultin, 1982b). Benjakul *et al.* (2003a) reported that the activation energy of TMAOase from lizardfish kidney was 30.5 $kJmol^{-1}K^{-1}$. Activation energy of TMAOase from Alaska pollack muscle was 38.4 $kJmol^{-1}K^{-1}$ (Kimura *et al.*, 2000b), while an activation energy of 5.2 $kJmol^{-1}K^{-1}$ was found in TMAOase from red hake muscle (Phillipy and 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.

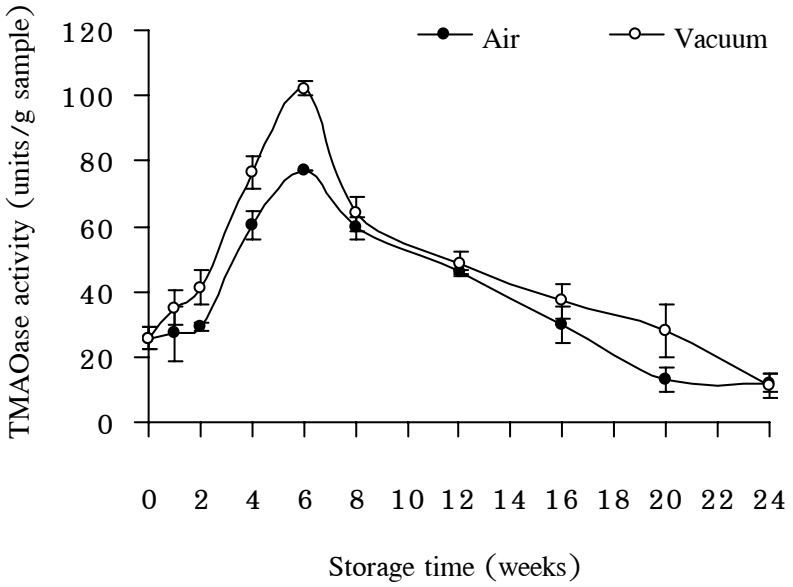

Figure 8. Changes in TMAOase acitivity in lizardfish mince kept in air and under vacuum during frozen storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

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 ($P < 0.05$) (Figure 9). 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 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.*, 2003c). Loss of Ca^{2+}-ATPase activity was associated with denaturation of myosin (Del Mazo *et al.*, 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 *et al.* (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 and Konno, 1998). Recently, Benjakul *et al.* (2003c) 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.* (2003c). 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 *et al.*, 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.

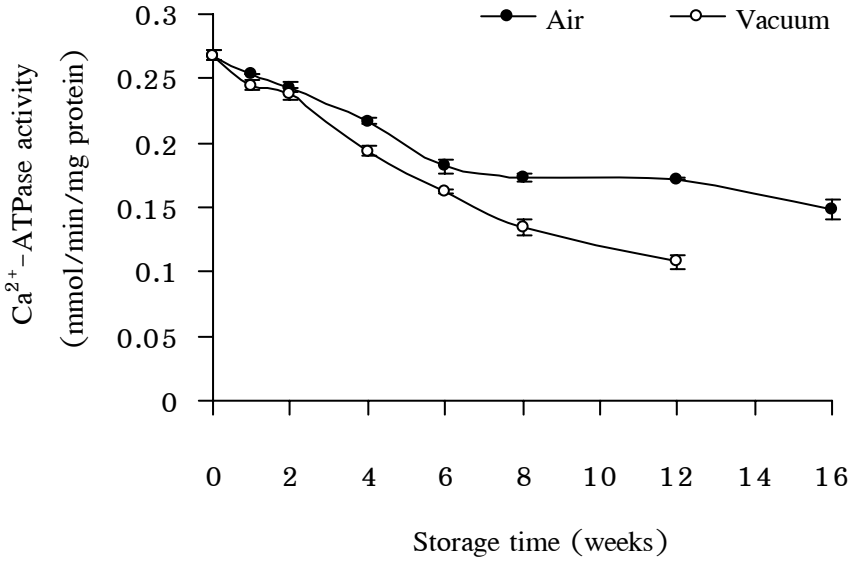

Figure 9. Changes in Ca^{2+}-ATPase activity of NAM extracted from lizardfish mince kept in air and under vacuum during storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

Changes in SH and disulfide bond contents of NAM extracted from lizardfish mince during frozen storage

Total SH content of both samples decreased gradually during frozen storage ($P < 0.05$) (Figure 10A). A sharp decrease in SH content was observed in the first 2 weeks of storage. Greater decrease in total SH content was observed in the samples kept under vacuum, than in those kept in air. At week 12, SH content in lizardfish mince kept in air and under vacuum decreased by 41.4% and 45.1%, compared to that found in fresh sample, respectively. The decrease in SH groups generally resulted from the formation of disulfide bonds through oxidation of SH groups or disulfide interchanges (Hayagawa and Nakai, 1985). SH groups on the myosin head portion, named SH_1 and SH_2, were reported to be involved in the ATPase activity of myosin (Kielley and Bradley, 1956). The reduction in the SH content might be explained by the following two reasons: the SH groups of inter- or intra-proteins formed cross-linkage (Huidobro *et al.*, 1998) or the exposed SH groups in protein interacted with additives or small molecular weight compounds (e.g., peptides) in the water-soluble protein fraction (Owusu-Ansah and Hultin, 1987). From the results, the decrease in SH groups was coincidental with the decrease in Ca^{2+}-ATPase activity (Figure 9). 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 SH groups of NAM extracted from hake fillets decreased with increasing storage time at -20 and -30°C. Ramirez *et al.* (2000) reported that reactive SH groups decreased to 55% of the initial value after 5 days of frozen storage. Additionally, Jiang *et al.* (1988b) reported that, during the first 2 weeks of frozen storage of milkfish actomyosin, the reactive SH 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 SH groups, masked in molecules, were gradually exposed and oxidized to disulfides. The rate of exposure and oxidation of reactive SH groups might reach an equilibrium state during storage when the amount of the reactive SH groups no longer changed (Jiang *et al.*, 1988a). From the result, a greater decrease in SH 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 SH groups ($P < 0.05$) (Figure 10B). In general, the amounts of disulfide bonds formed in samples kept under vacuum were slightly higher than in those kept in air (Figure 10B). 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 SH 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 SH groups with a concomitant disulfide bond formation (Figures 10A and 10B) was generally in accordance with the decreased Ca^{2+}-ATPase activity (Figure 9). Fish myosin molecule contains many SH groups (Buttkus, 1970). SH groups located in the head portion (SH_1 and SH_2) play an essential role in ATPase activity (Kielley and Bradley, 1956). Furthermore, SH groups localized in light meromyosin (SH_a) also contribute to oxidation (Sompongse *et al.*, 1996). The oxidation of SH 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 SH groups. Decrease in the total number of SH groups indicates that the loss of Ca^{2+}-ATPase activity might be due to the oxidation of SH groups on the active site of actomyosin (Jiang *et al.*, 1988b).

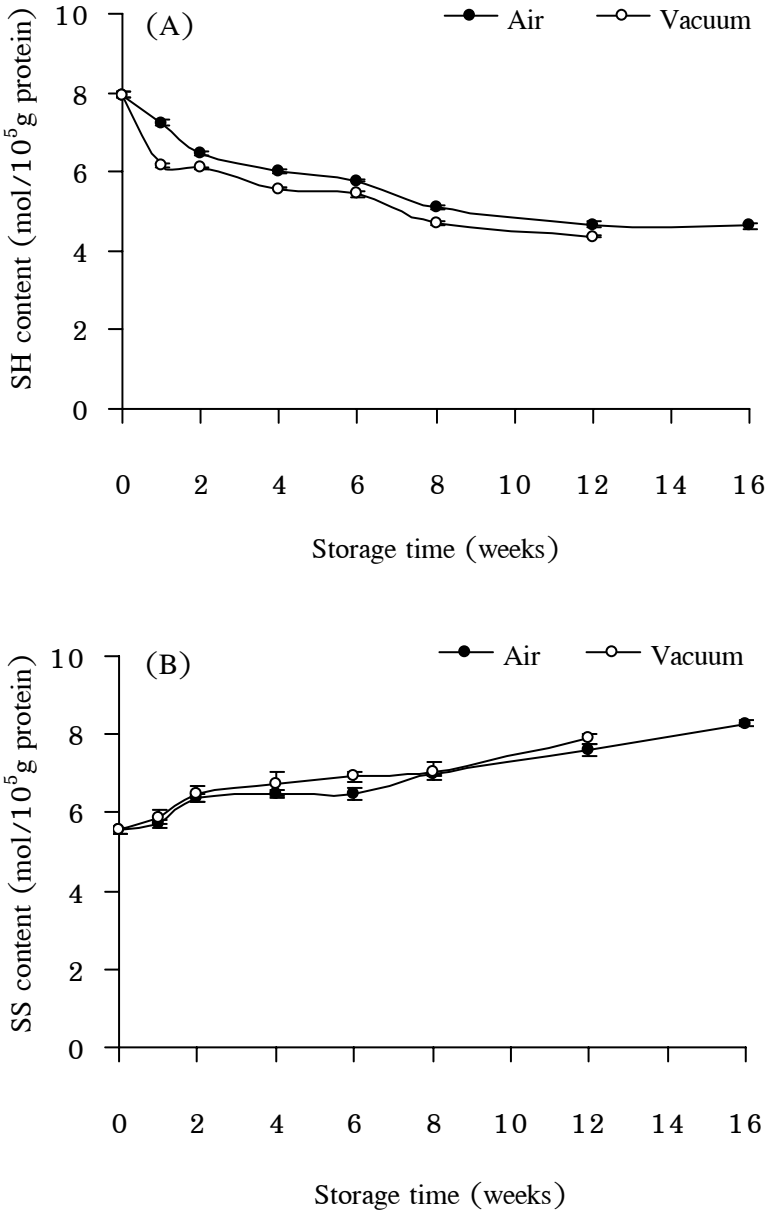

Figure 10. Changes in total sulfhydryl (SH) (A) and disulfide bond (SS) (B) contents of NAM extracted from lizardfish mince kept in air and under vacuum during storage at −20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

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

The surface hydrophobicity of NAM from both samples is shown in Figure 11. 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 (Badii and Howell, 2001). An initial increase in surface hydrophobicity was observed during frozen storage of isolated hake NAM (Del Mazo *et al*., 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 disulfide bonds, followed by a rearrangement of hydrophobic molecular 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.

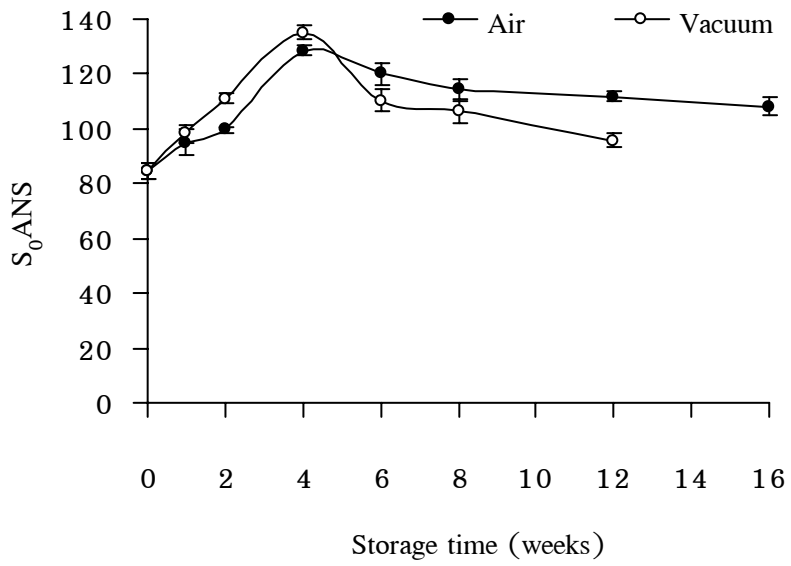

Figure 11. Changes in surface hydrophobicity of NAM extracted from lizardfish mince kept in air and under vacuum during storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

Changes in TNBS-reactive amino groups of NAM extracted from lizardfish mince during frozen storage

Figure 12 shows the changes in TNBS-reactive amino groups 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 TNBS-reactive amino groups was generally higher in samples kept under vacuum than in those kept in air. The decrease in the TNBS-reactive amino groups was possibly associated with the higher amount of FA produced by TMAOase during the storage (Figure 7A). FA is known to react with a wide spectrum of amino acyl side chains in proteins (Ang and Hultin, 1989). The decrease in TNBS-reactive amino groups was used as a measure of the reaction between FA and proteins (Ang and Hultin, 1989). Lysine is one of the more reactive side chain groups with FA (Tome *et al*., 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. From the result, the loss of TNBS-reactive amino groups might be due to the formation of aggregate induced by FA. Masking of TNBS-reactive amino groups, caused by the aggregate stabilized by other bonds, such as disulfide and hydrophobic interaction was also presumed.

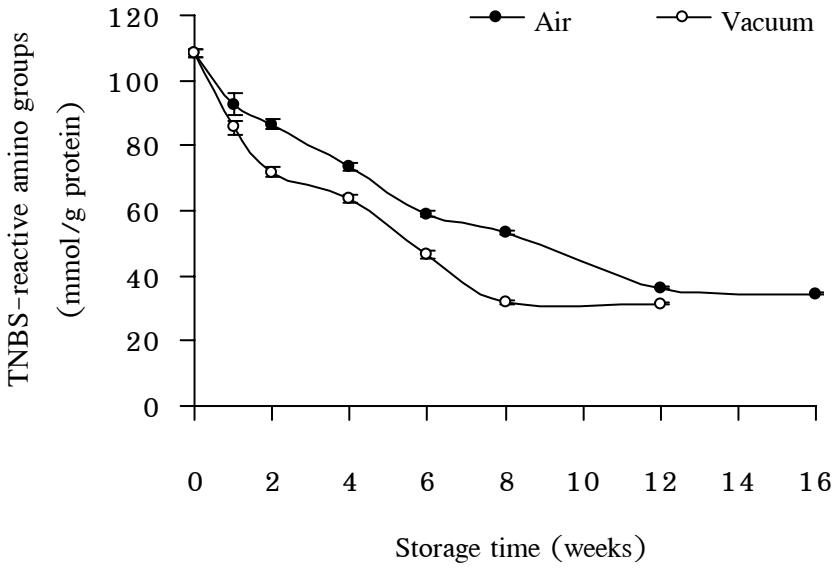

Figure 12. Changes in TNBS-reactive amino groups of NAM extracted from lizardfish mince kept in air and under vacuum during storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

Changes in protein solubility of lizardfish muscle during frozen storage

The solubility of lizardfish mince is shown in Figure 13. During extended storage, the salt-soluble fraction (I) of lizardfish mince kept under vacuum (Figure 13B) decreased to a greater extent, than in that kept in air (Figure 13A). 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 and 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 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 (Figure 10B). For the insoluble fraction, the FA formed during extended storage might contribute to the aggregate, which could not be solubilized 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 (Figures 13A and 13B). FA has been known as an effective cross-linker. Addition of FA to cod myosin causes loss of solubility (Careche and 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 $-20^{\circ}C$.

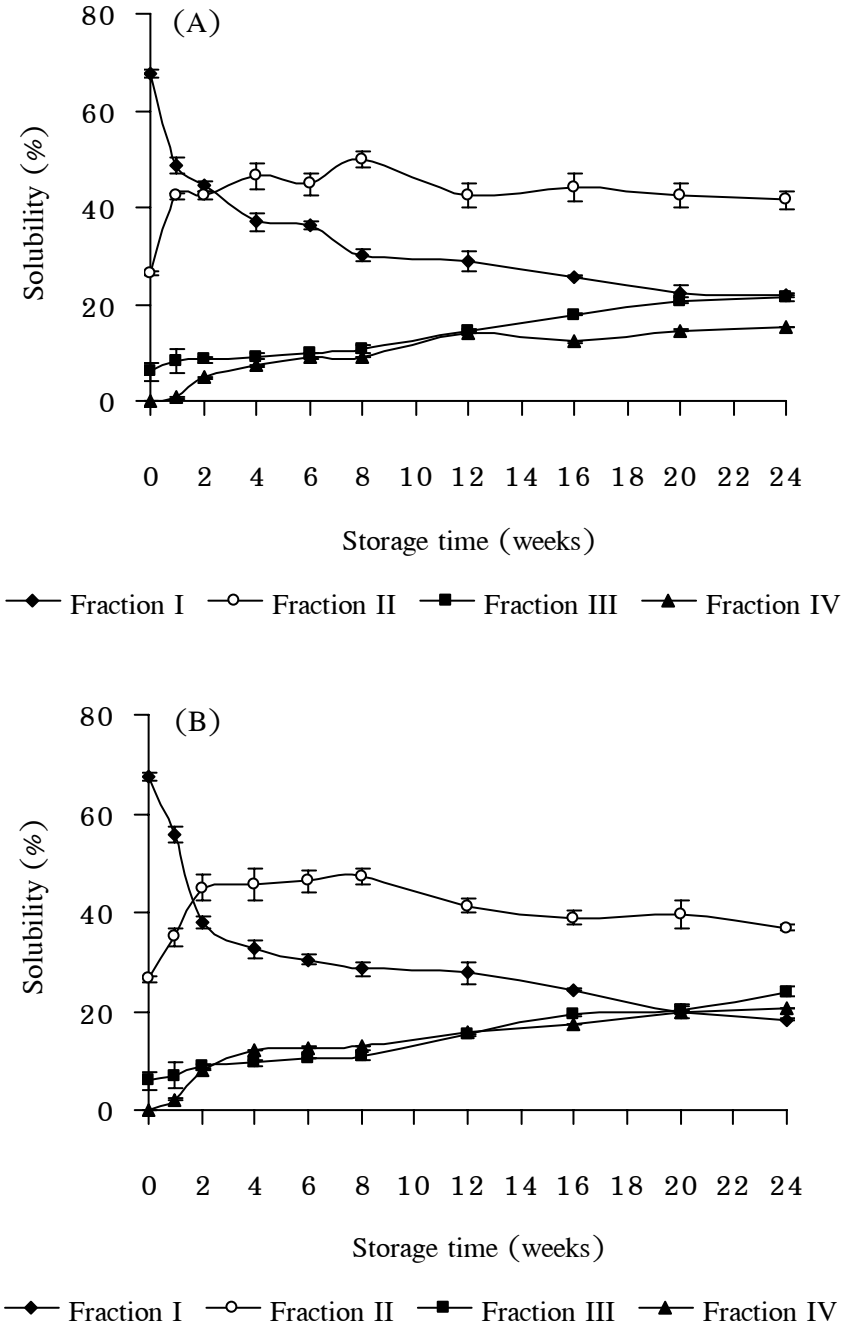

Figure 13. Changes in protein solubility of lizardfish mince kept in air (A) and under vacuum (B) during storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations. The solubility in each fraction was expressed as the percentage of soluble protein relative to total protein content in the sample.

2.5 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.