CHAPTER 3

PHYSICOCHEMICAL AND BIOCHEMICAL CHANGES IN WHOLE LIZARDFISH (*SAURIDA TUMBIL*) MUSCLES AND FILLETS DURING FROZEN STORAGE

3.1 Abstract

The physicochemical and biochemical changes in whole lizardfish (*Saurida tumbil*) muscles and its fillets kept in air and under vacuum during frozen storage at −20°C for 24 weeks were investigated. The 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 continuously decreased with a coincidental decrease in salt-soluble fraction. The disulfide bonds were increasingly formed throughout the storage ($P < 0.05$). The surface hydrophobicity increased and reached the maximum at week 12 with a subsequent decrease up to the end of storage. In general, the higher changes were observed in samples kept under vacuum than those kept in air. With the same atmosphere used, the whole fish showed slightly higher changes than the fillets. A marked increase in trimethylamine-*N*-oxide demethylase (TMAOase) activities was observed up to 12 weeks, followed by the continuous decrease up to 24 weeks of storage. The produced FA might play an important role in inducing protein denaturation and/or aggregation in lizardfish. The TMAOase activity as well as the FA formation could be reduced to some extent with the removal of internal organs and storage in the presence of oxygen. However, a detrimental effect of oxygen, especially on the promotion of lipid oxidation, would be an obstacle.

3.2 Introduction

Freezing is one of the best methods for fish preservation and has been increasingly employed both on shore and on board fishing vessels (Jiang *et al.*, 1987). However, fish muscle deteriorates during frozen storage mainly because of the denaturation and aggregation of myofibrillar proteins (Huidobro *et al.*, 1998). Different species exhibit different degrees of susceptibility to aggregation during frozen storage, depending on the intactness of the muscle or the storage temperature (Tejada *et al.*, 1996). The dehydration and increased salt concentration are induced by the formation of ice crystals. Additionally, the oxidation of lipids, the formation of free fatty acids as well as the formation of formaldehyde (FA) have been reported to be associated with the denaturation and/or aggregation of proteins, leading to the loss of functionality and texture (Shenouda, 1980; Careche *et al.*, 1998a). The FA can react with a number of amino acid residues in proteins to form covalent cross-links, resulting in the formation of inter- and intramolecular linkages between protein chains (Shenouda, 1980). The FA is produced in gadoids during frozen storage by the degradation of trimethylamine oxide (TMAO) induced by the trimethylamine-*N*-oxide demethylase (TMAOase) to form FA and dimethylamine (DMA) in equimolar amounts (Parkin and Hultin, 1982a). The rate of the reaction depends on many factors, e.g., storage temperature, species, muscle integrity and reducing conditions (Parkin and Hultin 1982b). The absence of oxygen has also been shown to accelerate the rate of DMA production (Lundstrom *et al.*, 1982). Generally, the TMAOase activity in the viscera was higher than in the muscle tissues (Rey-Mansilla *et al.*, 2002). Although TMAOase occurs in very high concentrations in the kidney tissue (Rehbein and Schreiber 1984), the deterioration observed during the frozen storage of gadoid fillets proves that muscle TMAOase also causes the production of significant amounts of FA during frozen storage (Benjakul *et al.*, 2003c, 2005; Nielsen and Jorgensen, 2004).

Lizardfish is an important commercial species in Thailand and is used mostly for surimi production. This is because of its high gel-forming ability and appreciable whiteness (Benjakul *et al.*, 2003b). However, the gel-forming ability of this fish rapidly decreases during postharvest handling. This is believed to be associated with proteolysis as well as with the formation of FA (Benjakul *et al.*, 2003b). Recently, Benjakul *et al.* (2003c) reported the increase in FA in whole lizardfish during frozen storage. Additionally, the TMAOase activity was found highest in the lizardfish kidney

compared to other internal organs (Benjakul *et al.*, 2004). The TMAOase localized in both muscle and viscera may contribute to the quality deterioration of this species. Also, the muscle can be contaminated by the TMAOase from the internal organs during processing, particularly during the mincing and thawing processes. Recently, Benjakul *et al.* (2005) reported that frozen lizardfish showed a poorer gel-forming ability compared with other fish species, especially with increasing freezing-storage time. This phenomenon was mainly caused by the formation of FA in this species. The lipid oxidation during frozen storage has been reported to contribute to the changes in the quality of the fish (Badii and Howell 2002a). Although lipid oxidation in lizardfish occurred at a lower extent compared with that of croaker, the decrease in gel formation was much greater, suggesting the predominant role of FA on the denaturation of muscle proteins in lizardfish (Benjakul *et al.*, 2005). Because packaging atmosphere with low oxygen or vacuum might show a detrimental effect, particularly the acceleration of FA formation, the storage of lizardfish in air might be more appropriated to retard the formation of FA in this species. However, no information regarding the changes in the whole lizardfish and its fillets under different packaging atmospheres has been reported. Therefore, the objective of this research was to compare the physicochemical and biochemical changes in the properties and TMAOase activity of whole lizardfish muscles and its fillets kept in air and under vacuum during prolonged frozen storage at -20°C.

3.3 Materials and Methods

Chemicals

All chemicals for the TMAOase-activity assay were purchased from WAKO Pure Chemical Industries (Tokyo, Japan). TMAO was obtained from Aldrich Chemical Company (Milwaukee, WI). 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). Acetylacetone and Triton X-100 were purchased from Fluka (Buchs, Switzerland). Tris(hydroxymethyl) aminomethane (Tris), urea, $NaBH_4$ and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany).

Fish sample preparation

Lizardfish (*Saurida tumbil*) with a size of 200-250 g were purchased from the dock in Trang, Thailand. The fish after harvest off the Trang Coast along the Indian Ocean were stored in ice and off-loaded within 10-12 h after capture. The fish were transported in ice with a fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai within 3 h. Upon arrival, the fish were dressed and filleted by hand. The fish used in this study were either whole fish without viscera containing kidney or fillet. Three whole fish or three matched pairs of fillets were packaged in a polyethylene bag and sealed either in air or under vacuum. The samples were frozen at -20°C in an air-blast freezer and stored at the same temperature during the study. At definite time intervals (0, 1, 2, 4, 6, 8, 12, 16, 20 and 24 weeks), three bags of whole fish or matched fillets were randomly removed and thawed under running water (26-28°C) to obtain the core temperature of 0-2°C. For the whole fish, the samples were filleted. Then the fillets were chopped finely and mixed well prior to analyses.

Determination of trimethylamine oxide (TMAO)

A sample (2.5 g) was added to 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 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 micromoles per gram.

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

A sample (2.5 g) was added to 10 ml of 5% (w/v) trichloroacetic acid and homogenized with a homogenizer at a speed of 12,000 rpm for 2 min. The homogenate was centrifuged at 3,000 × g for 15 min and the supernatant was removed.

To the pellet, a further 5 ml of 5% (w/v) trichloroacetic acid were added and the mixture was homogenized as previously described. The supernatants were 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 determinations 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 the method of Benjakul *et al.* (2004) with some modifications. After thawing, the whole fish were filleted. The frozen fillets were also thawed and used for TMAOase extraction. To prepare crude TMAOase extracts, a finely chopped fillet was extracted with two 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 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). The supernatant obtained was referred to as the TMAOase crude extract.

TMAOase activity assay

TMAOase activity was assayed using TMAO as a substrate in the presence of selected cofactors (Benjakul *et al*., 2004). 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% (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 is defined as the activity, which released 1 nmol DMA per min.

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

Natural actomyosin (NAM) was prepared by the method of Benjakul *et al.* (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 milligram of protein within 1 min for the reaction at 25°C.

Total sulfhydryl (SH) and disulfide bond (SS) 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 *et al.* (1987).

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 S_0ANS.

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

The TNBS-reactive amino groups were 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 TNBS-reactive amino groups were expressed in terms of L-leucine.

Protein solubility

Solubility was determined according to the method of Hamada *et al.* (1977) with some modifications. A sample was thawed with running water until the core temperature reached to 0-2°C. To 2 g sample, 20 ml of 0.6 M KCl were added and the mixture was homogenized for 3 min at 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 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 *et al.*, 1951) using bovine serum albumin as 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%).

Statistical analysis

The data were subjected to analysis of variance, and the mean comparison was carried out using Duncan's multiple-range test (Steel and Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 10.0 for Windows, SPSS, Chicago, IL).

3.4 Results and Discussion

Changes in TMAO, FA and DMA contents of whole lizardfish and its fillets during frozen storage

The FA and DMA contents of whole lizardfish and its fillets significantly increased with increasing storage time ($P < 0.05$) (Figures 14A and 14B). The drastic increase in the FA content was observed after 8 weeks of storage. The highest FA content was obtained in whole lizardfish kept under vacuum ($P < 0.05$). When comparing the FA content in the samples kept under different atmospheres, the samples kept under vacuum had greater FA content than those kept in air ($P < 0.05$). Under the same atmosphere, higher FA contents were obtained in whole lizardfish, compared with those of fillets. Similarly, the DMA content significantly increased during frozen storage in all the samples ($P < 0.05$). The DMA content found in all samples rapidly increased during the first 8 weeks of storage, followed by the gradual increase except for the whole lizardfish kept under vacuum, which had the continuous increase in FA content up to 24 weeks. In general, the samples kept under vacuum had larger amounts of DMA than those kept in air ($P < 0.05$). Under the same atmosphere used, higher DMA formation was found in whole lizardfish than those found in fillet ($P < 0.05$). Among all samples, the fillets kept in air showed the lowest DMA content ($P < 0.05$). Benjakul *et al.* (2003b) reported that FA formation in whole lizardfish was approximately two-fold higher than that of head/eviscerate samples during ice storage. The increase in FA content was also found in whole lizardfish during frozen storage (Benjakul *et al.*, 2003c). The TMAOase is capable of catalyzing the conversion of TMAO to DMA and FA (Gill and Paulson, 1982). This enzyme is found in the muscle and viscera of gadoids (Gill and Paulson, 1982; Rehbein and Schreiber, 1984). The TMAOase activity in the viscera is higher than in muscle tissues (Rey-Mansilla *et al.*, 2002). Benjakul *et al.* (2004) reported that the highest TMAOase activity was found in lizardfish kidney, followed by its spleen, bile sac, intestine and liver, respectively. From the result, the higher DMA and FA formed were observed in the whole sample, possibly because of the result of greater TMAOase activity in kidney that could be released into the muscle during processing as well as thawing. Rey-Mansilla *et al.* (2001) reported that the white muscle located right over the kidneys produced more DMA

and FA than other parts of the fish. Packaging and atmosphere were found to affect the DMA and FA production in red hake. The absence of oxygen was shown to accelerate the rate of DMA and FA formation (Lundstrom *et al.*, 1982). From the result, higher formations of DMA and FA were obtained in the samples kept under vacuum, compared with those kept in air. Thus, oxygen played an important role in FA formation as an inhibitor of TMAOase. The TMAOase catalyzes the breakdown of TMAO to DMA and FA in equimolar amounts (Parkin and Hultin, 1982a). However, detectable free FA was lower than DMA, indicating that the large amount of FA formed was tightly bound to muscle proteins in all the samples and could not be extracted. The TMAO content gradually decreased throughout the storage time (Figure 14C) with the concomitant increases in DMA and FA contents ($P < 0.05$). A marked decrease in the TMAO content was observed in the first 8 weeks of storage. The higher decreasing rate was found in the samples kept under vacuum, compared with those kept in air ($P < 0.05$). Under the same storage atmosphere, the TMAO content in whole lizardfish decreased to a higher extent, compared with those found in the fillets. The results suggested that TMAO was demethylated more greatly in the whole sample than in the fillets, probably because of the higher TMAOase in kidney. However, the decrease in TMAO content with the concomitant increase in FA and DMA contents was also found in the fillet. Kimura *et al.* (2000a) found TMAOase in walleye pollack myofibrillar fraction, which causes the reduction of TMAO to DMA and FA. The increases in DMA and FA formation in cod fillets were observed during frozen storage (Careche *et al.*, 1998b; Badii and Howell, 2001). Benjakul *et al.* (2004) also found that the addition of partially purified lizardfish kidney TMAOase resulted in an increase of DMA and FA in washed mince from both red seabream and skipjack muscle during iced storage. These led to a loss in protein solubility, which is associated with increased cross-linking of protein. The FA can react with a number of amino acid residues in proteins to form covalent cross-links that lead to inter- and intramolecular linkages between protein chains (Shenouda, 1980), which led to the textural deterioration of muscular tissue (Castell *et al.*, 1973). Therefore, the formation of FA in lizardfish during frozen storage can be reduced by the removal of viscera or by filleting. However, the FA was still formed at a lower rate in the muscle.

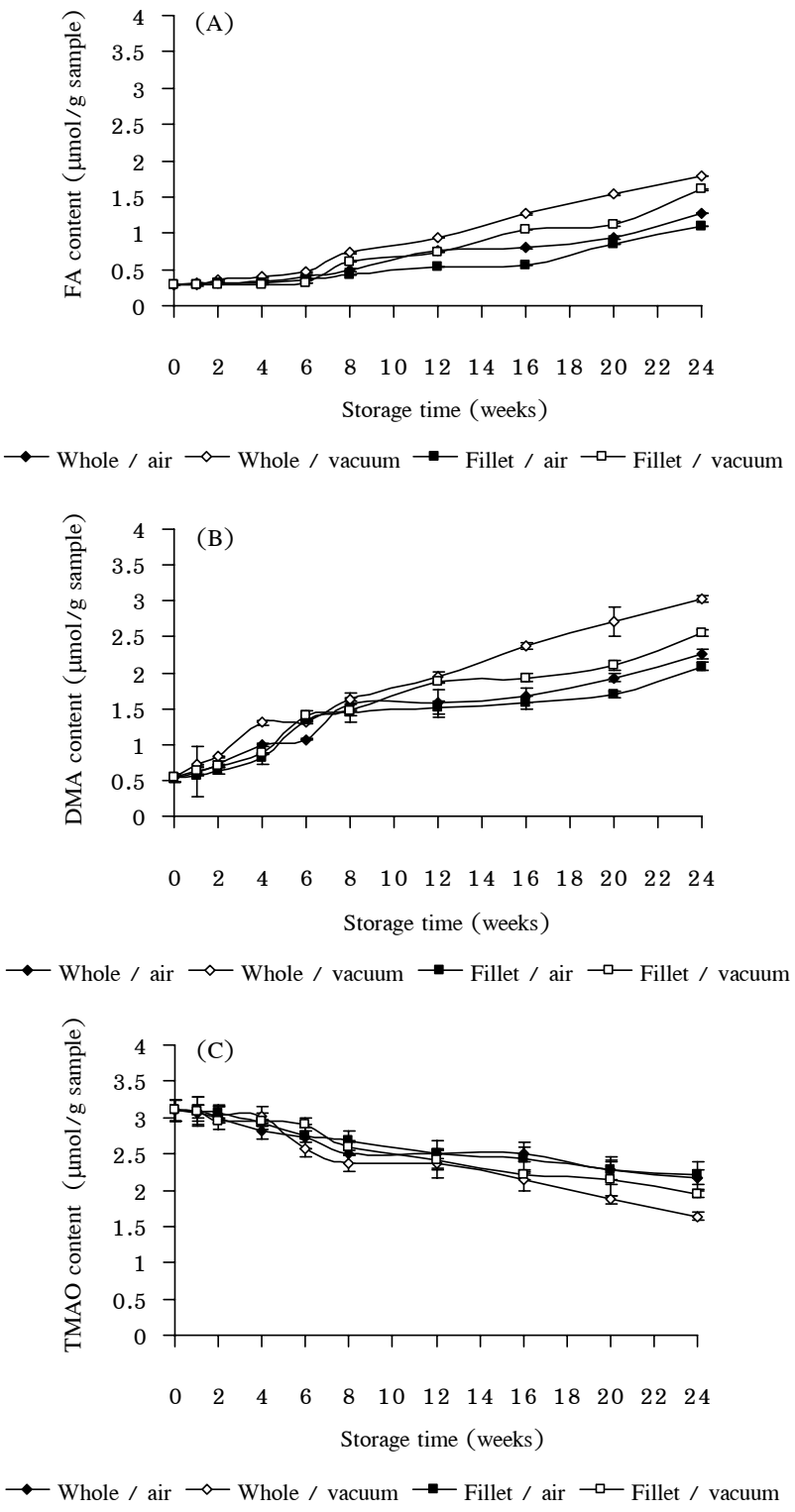

Figure 14. Changes in FA (A), DMA (B) and TMAO (C) contents in whole lizardfish and its fillets kept in air and under vacuum during frozen storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

Changes in TMAOase activity in whole lizardfish and its fillets during frozen storage

The TMAOase activity in the muscle was monitored during frozen storage. No changes in activity were observed in all the samples within the first 4 weeks ($P < 0.05$) (Figure 15). Thereafter, the TMAOase activity rapidly increased and reached the maximum value at week 12 of storage ($P < 0.05$), followed by the continuous decrease up to 24 weeks. Under the same storage atmosphere, greater activity was generally observed in the whole lizardfish, compared with those obtained in the fillets ($P < 0.05$). The higher TMAOase activity obtained in the whole lizardfish might be owing to the release of TMAOase from kidney, which could be contaminated or penetrated into the muscle during handling or processing. In addition, higher activity was generally observed in samples kept under vacuum, compared with those stored in air. This was probably because of the participation of oxygen in both acceleration of lipid oxidation, which could induce the conformation changes of enzyme structure and inhibition of TMAOase. An increase in TMAOase activity might result from the disruption of cell membrane during frozen storage. The formation and accretion of ice crystals, dehydration and increase in unfrozen phase led to the changes in muscle tissues as well as cell damage (Shenouda, 1980). Therefore, freezing can disrupt muscle cells, resulting in the release of mitochondrial and lysosomal enzymes into the sarcoplasm, especially with increasing frozen storage time (Hamm, 1979). The higher TMAOase could be extracted from the muscle, particularly in the first 12 weeks of storage ($P < 0.05$). The subsequent decrease in activities might be because of the denaturation of TMAOase. Because a lower TMAOase activity was observed in the samples kept in air, it was likely that TMAOase was inhibited to some extent in the presence of oxygen. Because of the low activation energy needed for TMAOase (Parkin and Hultin, 1982b), the TMAOase activity still maintained and played an important role in the textural deterioration of frozen whole lizardfish and its fillets. Benjakul *et al.* (2003a) reported that activation energy of TMAOase from lizardfish kidney was 30.5 $kJmol^{-1}K^{-1}$. The 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 (Phillippy and Hultin, 1993).

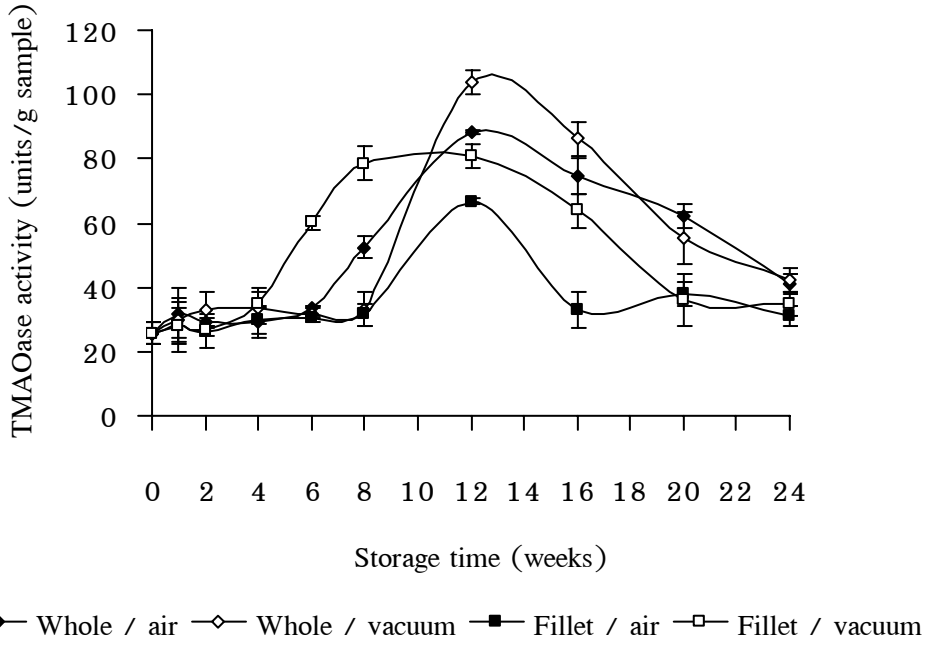

Figure 15. Changes in TMAOase activity in whole lizardfish and its fillets kept in air and under vacuum during frozen storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

Changes in protein solubility of whole lizardfish and its fillets during frozen storage

The solubility of lizardfish muscle in whole lizardfish and its fillets during frozen storage is shown in Figure 16. In general, the pattern of changes in protein solubility in all samples was similar. The changes were generally more pronounced in the samples kept under vacuum than those kept in air, especially for the whole fish ($P < 0.05$). The salt-soluble fraction (I) of both whole lizardfish and its fillets kept under vacuum (Figures 16B and 16D) decreased to a higher extent, compared with those kept in air (Figures 16A and 16C) ($P < 0.05$). For the whole lizardfish, those kept under vacuum had the greater decrease in salt-soluble fraction than those stored in air. This indicated that the salt-soluble protein underwent denaturation via aggregation, resulting in the loss of solubility. Conversely, the quantity of the urea-soluble fraction (II) of all the samples increased during the first 6-8 weeks of storage with a subsequent decrease during prolonged storage (Figure 16). The increase in the urea-soluble fraction indicated the formation of hydrogen and hydrophobic bonds during storage. However, after 8 weeks of storage, the decrease in

urea-soluble proteins might be because of the increased formation of the disulfides as well as covalent cross-links induced by the FA, which consequently caused the incomplete disruption of hydrogen and hydrophobic bonding by urea. The $NaBH_4$-soluble fraction (III) and insoluble fraction (IV) of all the samples gradually increased throughout the storage up to 24 weeks ($P < 0.05$), suggesting the increase in aggregation stabilized by disulfide bond and nondisulfide covalent bonds. The increase in $NaBH_4$-soluble proteins indicated an increased disulfide-bond formation, particularly when the time increased. For insoluble fraction, the FA formed during extended storage might contribute to the aggregate, which could not be solubilized by all denaturants. It was notable that higher insoluble-protein contents were obtained in samples which had higher FA content (Figure 14A). Aggregates with high molecular weight that were not extractable in 0.6 M NaCl were formed in frozen hake caused by the formation of nondisulfide covalent protein-protein bonds (Huidobro *et al.*, 1998). Careche *et al.* (2002) reported the formation of new methylene groups of aggregated protein pellets during frozen storage of hake myofibrils with added FA in accordance with the interaction of lysine or arginine side chains with FA.

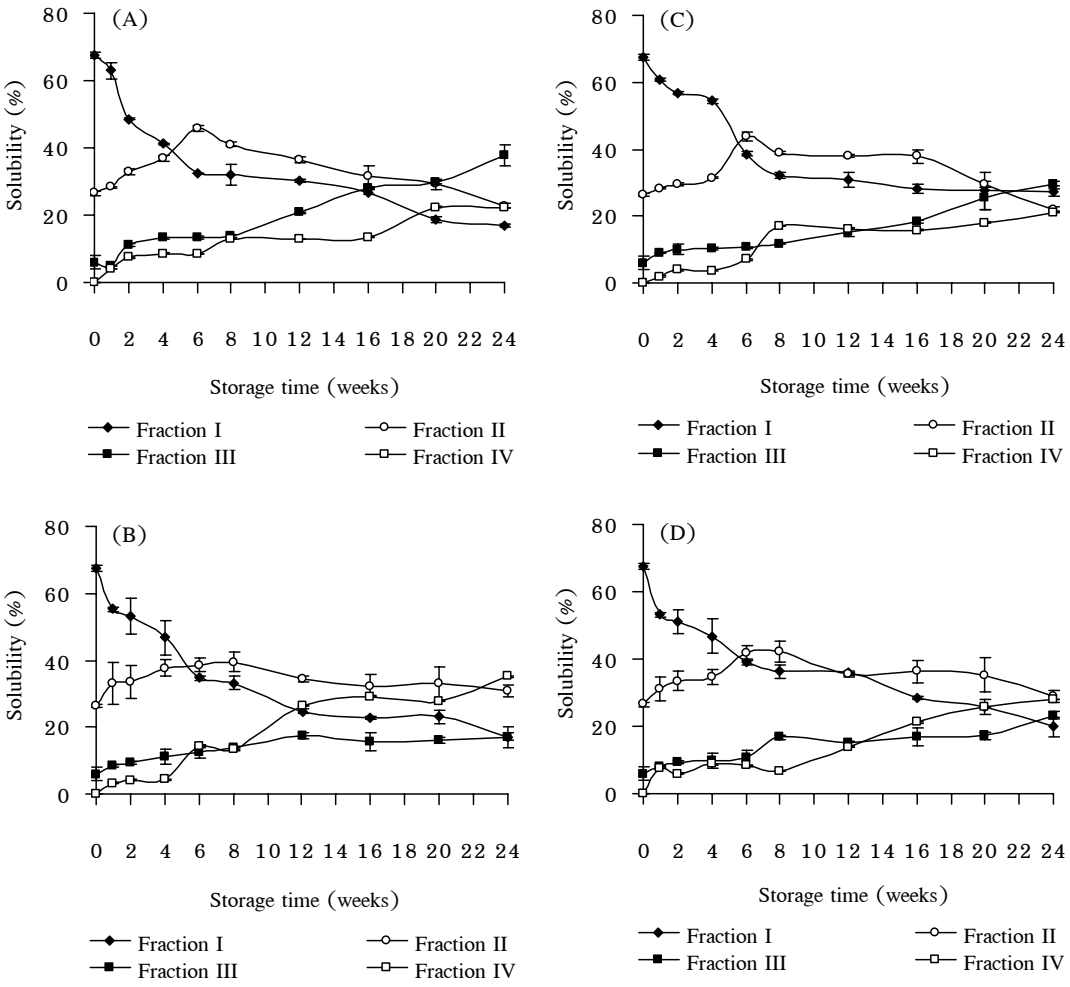

Figure 16. Changes in protein solubility of whole lizardfish kept in air (A), whole lizardfish kept under vacuum (B), fillets kept in air (C) and fillets kept under vacuum (D) during storage at −20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations. The solubility in each fraction was expressed as the percentage of soluble protein relative to total protein content in the sample.

Changes in surface hydrophobicity of NAM extracted from whole lizardfish and its fillets during frozen storage

During frozen storage of whole lizardfish and its fillets, the surface hydrophobicity of both samples increased as the storage time increased with a subsequent decrease during the extended frozen storage (Figure 17). At week 12, the surface hydrophobicity of NAM extracted from whole lizardfish stored in air, fillet stored in air and fillet stored under vacuum increased by 142.2, 143 and 154.2%, respectively, compared with that from the fresh sample. Thereafter, a slight decrease was observed up to 24 weeks of storage. For whole lizardfish kept under vacuum, the highest surface hydrophobicity was found in the first 12 weeks, compared with those of other samples ($P < 0.05$). A sharp decrease in surface hydrophobicity was observed after 12 weeks of storage, presumably induced by FA formed during extended frozen storage. The crosslink induced by the FA might lead to the considerable decrease in free hydrophobic groups. The FA caused the denaturation of cod myosin, resulting in more interaction between hydrophobic groups and formation of covalent bonds (Careche and Li-Chan, 1997). From the result, the denaturation of proteins presumably resulted in an exposure of the interior of the molecule, where the hydrophobic protein was located (Benjakul *et al.*, 2002). Exposed hydrophobic amino acids containing an aromatic ring, e.g., phenylalanine and tryptophan, were able to bind to ANS (Kato and Nakai 1980). Exposure of buried tryptophan residues was also observed during frozen storage (-10 and -30°C) of hake fillets (Careche *et al.*, 1999). Hydrophobic interaction between amino acids and oxidation of SH groups affected the surface hydrophobicity (Hill *et al.*, 1982). The subsequent decrease in hydrophobicity could be the consequence of a balance between proteins remaining in solution with more exposed hydrophobicity and aggregates with less exposed hydrophobicity (Careche and Li-Chan, 1997). Del Mazo *et al.* (1994) also reported an initial increase in surface hydrophobicity during frozen storage of isolated hake NAM in the presence of FA with a subsequent decrease with a longer storage time.

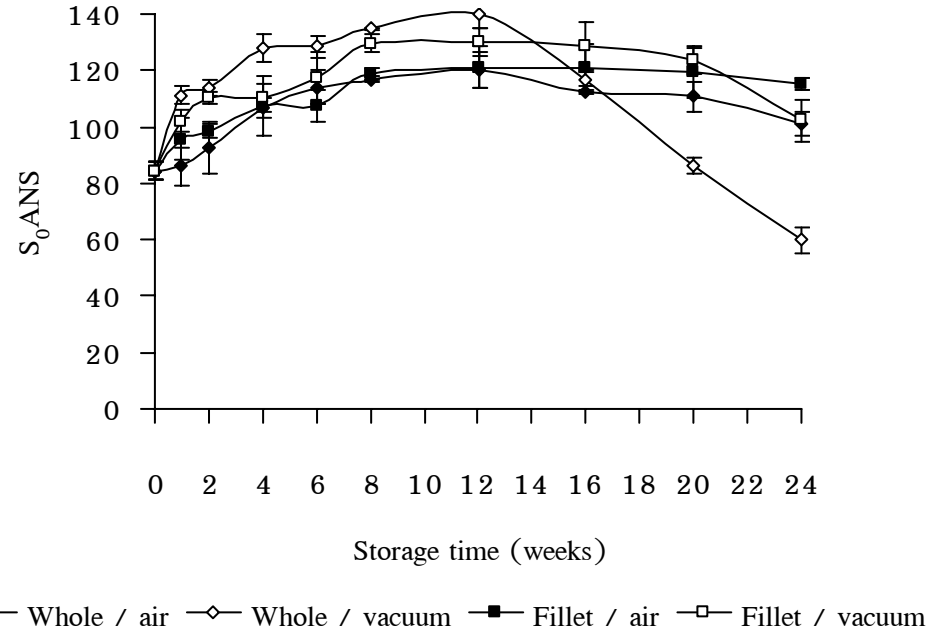

Figure 17. Changes in surface hydrophobicity of NAM extracted from whole lizardfish and its fillets kept in air and under vacuum during storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

Changes in SH and disulfide bond contents of NAM extracted from whole lizardfish and its fillets during frozen storage

The total SH content in all the samples slightly decreased over the 24 weeks of frozen-storage period (Figure 18A). Under vacuum, a greater decrease in total SH content was observed in the whole sample, compared to the fillets. A marked decrease was observed in the whole samples kept under vacuum, particularly after 16 weeks of storage ($P < 0.05$). However, no marked differences were found between the whole sample and fillets kept in air. The initial content of SH group in lizardfish was 7.95 mol/10^5g protein. This was in agreement with the value reported by Buttkus (1970). In freshly killed fish, 42-43 SH group/5 × 10^5g protein (~8 mol/10^5g protein) of myosin molecule was found (Buttkus, 1970). When stored at 0°C in air, 10 of these SH groups, which are very reactive, undergo oxidation to form intramolecular disulfide bonds (Buttkus, 1970). The decrease in SH groups generally resulted from the formation of disulfide bonds through oxidation of SH groups or disulfide interchanges (Hayakawa and Nakai, 1985).

From the result, the total SH content decreased to 61.7, 31.9, 62.6 and 61.2% of the initial value in whole samples stored in air, under vacuum and fillets stored in air and under vacuum, respectively. Because of the formation of hydrogen and hydrophobic bonds as well as covalent bonds induced by FA (Figures 16 and 17), structural changes might occur. The reactive SH groups masked in molecules were gradually exposed and oxidized to disulfides. The rate of exposure and oxidation of the SH group might reach an equilibrium state during storage when the amounts of SH groups were no longer changed (Jiang *et al.*, 1988b). From the result, whole lizardfish stored under vacuum showed the highest decrease in SH content throughout frozen storage, especially at week 24 ($P < 0.05$). High content of FA formed in this sample might induce the aggregation in the way that favored the oxidation of SH groups. Moreover, the masking of SH groups by protein aggregates was possibly associated with the decrease in free SH groups available for determination. The disulfide bond content of NAM from lizardfish during frozen storage is shown in Figure 18B. The disulfide-bond formation was observed throughout the frozen storage. Whole samples kept under vacuum contained the highest content of disulfide bonds ($P < 0.05$), followed by the fillets kept under vacuum, whole sample kept in air and fillets kept in air, 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. As a result, the FA formed during frozen storage was probably associated with disulfide-bond formation via inducing the conformational change in fashion, which oxidation of SH group could easily take place. The continuous decreases of total SH groups were observed during 15 days of frozen storage of fish myosin, confirming the importance of disulfides in frozen-induced aggregation of fish myosin solution (Ramirez *et al.*, 2000). The increase in disulfide bond was in agreement with the decrease in fraction I as well as the increase in fraction III. Thus, disulfide bond also contributed to the formation of aggregates in addition to the covalent bond induced by the FA.

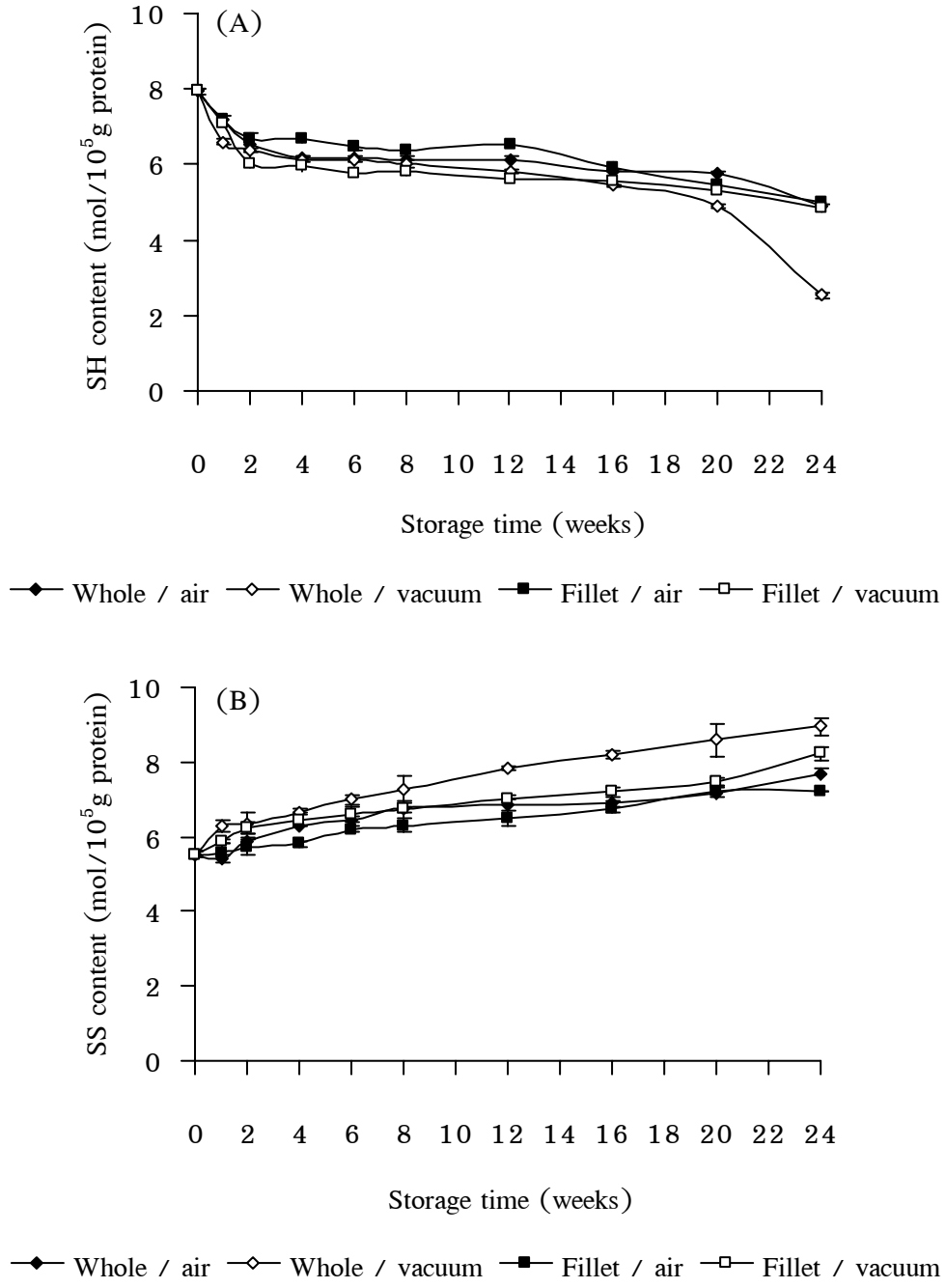

Figure 18. Changes in total sulfhydryl (SH) (A) and disulfide bond (SS) (B) contents of NAM extracted from whole lizardfish and its fillets kept in air and under vacuum during storage at −20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

Changes in Ca^{2+}-ATPase activity of NAM extracted from whole lizardfish and its fillets during frozen storage

A significant decrease in Ca^{2+}-ATPase activity was found in NAM from whole lizardfish and fillets during frozen storage up to 24 weeks of storage ($P < 0.05$) (Figure 19). Generally, NAM from the whole samples showed a higher decreasing rate of Ca^{2+}-ATPase activity than that from the fillets. The samples stored under vacuum showed a higher decreasing rate of Ca^{2+}-ATPase activity than those kept in air ($P < 0.05$). A slightly higher decreasing rate was observed in whole samples than in fillets under the same storage atmosphere. The Ca^{2+}-ATPase activity can be used as an indicator for the integrity of the myosin molecule (Benjakul *et al.*, 1997). At the end of storage, the Ca^{2+}-ATPase activity of whole samples kept in air, under vacuum and fillets kept in air and under vacuum decreased by 57.1, 45.5, 63.1 and 53%, compared with the initial value, respectively. The loss of activity was postulated to be because of the tertiary structural changes caused by the ice crystal and the increase in ionic strength of the system (Benjakul and Bauer, 2000). In addition, the rearrangement of protein via protein-protein interactions was also presumed to contribute to the loss of activity (Benjakul and Bauer 2000). Two types of SH groups on the myosin head portion (SH_1 and SH_2) have been reported to be involved in Ca^{2+}-ATPase activity of myosin (Kielley and Bradley, 1956). From the result, the decrease in Ca^{2+}-ATPase activity was concomitant with the decrease in the SH group content and the increase in disulfide bond formation (Figure 18). Hence, the loss of Ca^{2+}-ATPase activity might be because of the oxidation of SH groups on the head portion of the myosin heavy chain as well as the aggregation of myosin head induced by the FA formed. The decrease in Ca^{2+}-ATPase activity was more evident with increasing storage time. In model systems containing isolated NAM, the addition of FA reduced the solubility of NAM in saline solutions and Ca^{2+}-ATPase activity (Hill *et al.*, 1982). Ramirez *et al.* (2000) found that during 15 days of frozen storage, the Ca^{2+}-ATPase activity markedly decreased, losing 89% of its initial value, suggesting that myosin heads could be involved in myosin aggregation.

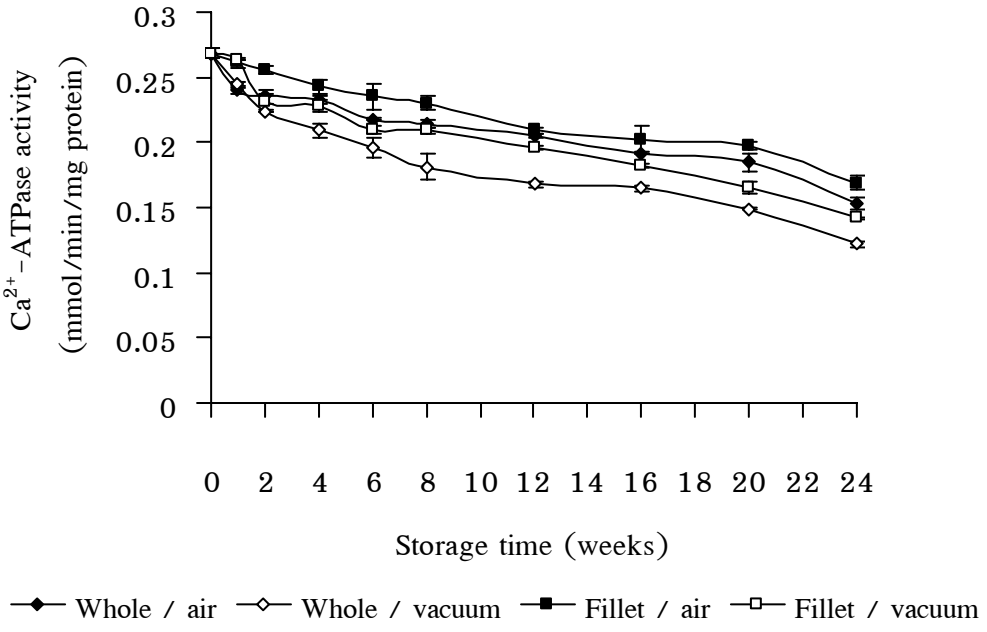

Figure 19. Changes in Ca^{2+}-ATPase activity of NAM extracted from whole lizardfish and its fillets kept in air and under vacuum during storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

Changes in TNBS-reactive amino groups of NAM extracted from whole lizardfish and its fillets during frozen storage

The changes in TNBS-reactive amino groups of all the samples are shown in Figure 20. Generally, the TNBS-reactive amino groups decreased as the storage time increased ($P < 0.05$). A sharp decrease was observed in the first 4 weeks of storage ($P < 0.05$). During 20-24 weeks of storage, lower TNBS-reactive amino groups were observed in the whole lizardfish kept under vacuum, compared with other samples ($P < 0.05$). The loss of TNBS-reactive groups of NAM was possibly caused by the FA, which could react with many functional groups of protein, especially the amino group of lysine. Lysine is one of the more reactive side chain groups with FA (Tome *et al.*, 1985). Ang and Hultin (1989) found the 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 because of the formation of aggregates induced by the FA. The masking of TNBS-reactive

amino groups caused by the aggregate stabilized by various bonds such as disulfide bond and hydrophobic interaction was also presumed.

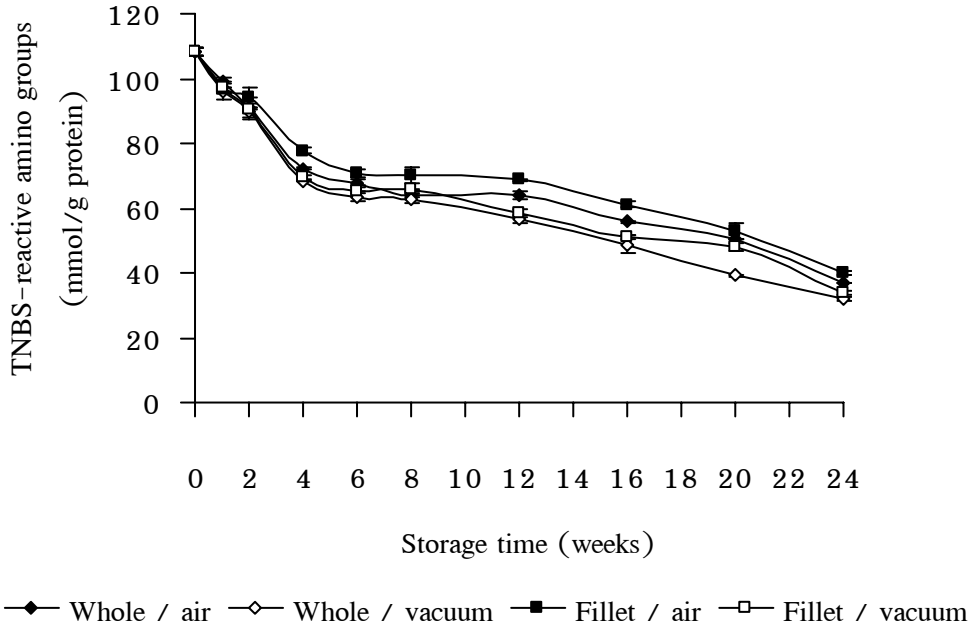

Figure 20. Changes in TNBS-reactive amino groups of NAM extracted from whole lizardfish and its fillets kept in air and under vacuum during storage at -20°C for 24 weeks. Bars indicate standard deviation from triplicate determinations.

3.5 Conclusion

The frozen storage of lizardfish resulted in protein denaturation and aggregation. Greater changes were observed in the whole lizardfish kept under vacuum than that stored in air. For the same atmosphere, the whole fish showed slightly greater changes than the fillets. Those changes were most likely influenced by the FA formed. The TMAOase activity in both whole fish and fillets continuously increased up to 12 weeks of frozen storage, followed by the decrease in activity. The TMAOase activity in the whole fish was generally greater than that in fillets, particularly after 6 weeks of storage. The absence of oxygen was shown to accelerate the formation of FA and DMA during frozen storage. Therefore, vacuum packaging for whole lizardfish and its fillets might induce quality loss during frozen storage.