CHAPTER 5

EFFECT OF TRIMETHYLAMINE-*N*-OXIDE DEMETHYLASE FROM LIZARDFISH KIDNEY ON BIOCHEMICAL CHANGES OF HADDOCK NATURAL ACTOMYOSIN STORED AT 4 AND -10°C

5.1 Abstract

Trimethylamine-*N*-oxide demethylase (TMAOase) was partially purified from lizardfish (*Saurida tumbil*) kidney by heat-treatment, ammonium sulfate precipitation and a series of chromatographies including Sephacryl S-300 and DEAE-cellulose to 1276-fold with a yield of 15%. The addition of partially purified trimethylamine-*N*-oxide demethylase (TMAOase) from lizardfish kidney to haddock natural actomyosin (NAM) in the presence of cofactors ($FeCl_2$, ascorbate, and cysteine) accelerated formaldehyde formation throughout the storage either at 4°C or -10°C ($P < 0.05$). ^{1}H NMR spectroscopic study revealed that dimethylamine (DMA) was enhanced with a concomitant decrease in trimethylamine oxide (TMAO) content with the addition of TMAOase, particularly at higher concentration. The loss of protein solubility increased as the result of FA formation, which was associated with the increased denaturation/aggregation of proteins. Lipid oxidation determined as hexanal content occurred during extended storage at different degrees. Generally, simulated systems without TMAOase and TMAO contained the highest hexanal content. Differential scanning calorimetry (DSC) of NAM after storage at 4 and -10°C for 15 days and for 8 weeks, respectively, showed the lower T_m and enthalpy of endothermic peaks corresponding to myosin and actin, suggesting the conformational changes induced by FA formed. Therefore, TMAOase exhibited the detrimental impact on haddock NAM, mainly caused by FA formation.

5.2 Introduction

Low-temperature storage is one of the primary methods to maintain fish freshness, based on the reduction in the rates of microbiological, chemical, and biochemical changes at decreased temperature (Wang *et al*., 2003). However, an essentially irreversible phenomenon known as denaturation of the muscle proteins, mainly the myofibrillar proteins, still take place (Ando *et al*., 1991; Hatae, 1994). Additionally, cold storage of some fish species may not be sufficient to retard the alterations or deterioration caused by microorganism and enzymes. Among various enzymes, trimethylamine-*N*-oxide demethylase (TMAOase) is capable of catalyzing the conversion of trimethylamine oxide (TMAO) to dimethylamine (DMA) and formaldehyde (FA) (Gill and Paulson, 1982; Howell *et al*., 1996). This enzyme is concentrated in the internal organs and red muscle (Gill and Paulson, 1982; Rehbein and Schreiber, 1984). Benjakul *et al*. (2004) reported that lizardfish kidney contained the highest TMAOase activity, followed by spleen, bile sac, intestine, and liver, respectively. The high reactivity and diffusivity of produced FA mainly caused the textural deterioration of muscular tissue (Gill *et al*., 1979; Haard, 1992). FA can react with a number of chemical groups including amino groups, and various low-molecular-weight compounds, leading to denaturation and possibly cross-linking of proteins (Sikorski and Kolakowska, 1990; Hultin, 1992). Those changes were dependent on species and storage conditions (Careche *et al*., 1998a, 1998b). Generally, proteins susceptible to aggregation induced by FA are myosin and actin.

In Thailand, lizardfish have been used to produce the low-grade surimi, which is mainly served for low-priced fishcake products (Benjakul *et al*., 2003b). Storage in ice is a common practical approach to maintain the quality of lizardfish. The gel-forming ability of this fish decreased rapidly during post-harvested handling (Yasui and Lim, 1987). This was associated with proteolysis as well as the formation of FA (Benjakul *et al*., 2003b). Additionally, FA production is also considered as one of the major causes of the liability of lizardfish in the frozen state (Benjakul *et al*., 2005). The contamination of TMAOase from the internal organs during fish processing, particularly mincing, possibly induced protein aggregation caused by FA formed (Benjakul *et al*., 2004). Apart from FA, lipid oxidation has been reported to contribute to the quality changes of fish (Matsumoto, 1980). So far, the information regarding the impact of TMAOase from lizardfish kidney on the alteration of fish proteins has been scare. Furthermore, the effect of temperatures on

the changes of protein caused by TMAOase was little. The present work aimed to study the effect of partially purified lizardfish kidney TMAOase on biochemical changes of haddock actomyosin during storage at refrigerated (4°C) and frozen (-10°C) storage.

5.3 Materials and Methods

Chemicals

All chemicals for TMAOase activity assay were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan), except TMAO was obtained from Aldrich Chemical Company, Inc (Milwaukee, WI, USA). Acetylacetone, Triton X-100, and polyethylene glycol 20,000 were purchased from Fluka (Buchs, Switzerland). Tris (hydroxymethyl) aminomethane, sodium azide, and Folin-Ciocalteu's phenol reagent were obtained from Sigma chemical Co. (St. Louis, MO, USA).

Fish sample

Lizardfish (*Saurida tumbil*) of the average weight of 200-250 g/fish, caught off the Songkhla Coast along the Gulf of Thailand and off-loaded approximately 36-48 h after capture, were purchased from the dock in Songkhla. Fish kept in ice with fish/ice ratio of 1:2 (w/w) were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 1 h. Upon arrival, fish were eviscerated and kidney was removed. Kidney specimens were kept at -80°C until use.

Fresh fillets (230-335 g) of haddock (*Melanogrammus aeglefinus*) caught in North-east Atlantic ocean were purchased from M&J Seafood, Farham, UK and delivered in ice with the sample/ice ratio of 1:2 (w/w) to the School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey, UK.

Partial purification of TMAOase from lizardfish kidney

Frozen kidney specimens were thawed using running water (26-28°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces with a

thickness of 1-1.5 cm. Samples were ground into powder in the liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan).

Kidney powder was suspended in chilled extracting buffer (20 mM Tris-acetate buffer containing 0.1 M NaCl and 0.1% Triton X-100, pH 7.0) at a ratio of 1:3 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged for 1 h at 4°C at 19,400 × g using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and referred to as "TMAOase crude extract".

TMAOase crude extract was heated at 50°C for 5 min using a temperature-controlled water bath (Memmert, Schwabach, Germany) with continuous stirring, followed by immediate cooling in iced water. The supernatant was obtained after centrifugation at 13,800 × g for 15 min at 4°C and filtered through Whatman No. 4. The filtrate was subjected to ammonium sulfate precipitation at 60-80% saturation. The resulting precipitate was collected by centrifugation at 13,800 × g at 4°C for 15 min and dissolved in a minimal volume of cold 20 mM Tris-acetate buffer, pH 7.0, which was referred to as "starting buffer". The mixture was then dialyzed against 10 volumes of starting buffer for 10-12 h at 4°C. The dialysate was concentrated to 1.5 ml using polyethylene glycol 20,000. The concentrated fluid was applied onto a Sephacryl S-300 column (1.6 × 70 cm; Amersham Bioscience, Uppsala, Sweden) equilibrated with a starting buffer and the separation was carried out using the same buffer at the rate of 0.5 ml/min. Fractions of 3 ml were collected. The fractions rich in TMAOase activity were pooled and loaded on DEAE-cellulose (Wako Pure Chemical Co., Tokyo, Japan) column (1.6 × 20 cm) equilibrated with a starting buffer. The sample was loaded onto the column at a flow rate of 0.5 ml/min at room temperature. The column was washed with a starting buffer until the absorbance at 280 nm (A_{280}) was less than 0.05. The elution was performed using a linear gradient of 0 to 0.45 M NaCl in starting buffer at a flow rate of 1 ml/min. Fractions of 3 ml were collected and those with TMAOase activity were pooled and used for further studies.

TMAOase 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) pre-incubated at 45°C for 5 min, 0.5 ml of 500-fold diluted enzyme solution was added to initiate the reaction. The reaction was performed at 45°C for 10 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 as described by Benjakul *et al.* (2004). One unit of TMAOase was defined as the activity, which released 1 nmol DMA per min under the assayed condition.

Determination of protein

Protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Protein concentration in fractions obtained from DEAE-cellulose chromatography was determined according to the method of Bradford (1976).

Preparation of natural actomyosin (NAM)

NAM was extracted from haddock fillets in a walk-in cold room at 4 ± 2°C according to the method of Sultanbawa and Li-Chan (2001) and Benjakul *et al.* (1997) with some modifications as follows. The dark muscle was removed from the fillets. The ordinary muscle (50 g) was homogenized in an Omni mixer homogenizer (Waterbury, C.T, USA) for 1 min with 6 volumes of chilled 20 mM Tris-maleate buffer containing 0.05 M NaCl, pH 7.0. The homogenate was centrifuged at 5,000 × g for 30 min at 4°C. After the supernatant was decanted, the pellet was homogenized for 1 min with 6 volumes of chilled 20 mM Tris-maleate buffer containing 0.6 M NaCl, pH 7.0. The homogenate was filtered through a double layer of cotton gauze. Three volumes of chilled 20 mM Tris-maleate buffer, pH 7.0 were added to precipitate actomyosin and kept overnight. The

aqueous layer was decanted and the residue at the bottom was centrifuged at 5,000 × g for 30 min at 4°C. The pellets collected were then concentrated by centrifuging at 27,000 × g for 30 min at 4°C. The pellet from this final centrifugation step was referred to as "natural actomyosin; NAM".

Effect of partially purified TMAOase on biochemical changes of haddock NAM

Partially purified TMAOase at levels of 5 and 15 units/g NAM was added to the simulated system (30 g NAM containing 50 mM TMAO, 1 mM $FeCl_2$, 5 mM ascorbate, 5 mM cysteine and 0.1% sodium azide). For the control, TMAOase and TMAO were excluded and the distilled water and 20 mM Tris-acetate buffer, pH 7.0 were used instead. After the addition of enzyme, the system was mixed thoroughly. A portion of 30 g was placed in a plastic tube and the lid was closed tightly. The samples were kept either at 4°C or at -10°C for up to 15 days and 8 weeks, respectively. Samples were randomly taken for determination of FA and protein solubility at day 0, 3, 6, 9, 12 and 15 or week 0, 2, 4, 6 and 8 for samples stored at 4 and -10°C, respectively. DMA, DSC, and hexanal measurements were undertaken after storage for 15 days and 8 weeks for samples stored at 4 and -10°C, respectively.

Determination of formaldehyde (FA) content

FA in the sample, both free and bound forms, was extracted by acid distillation (Rehbein, 1987). Sample (5 g) was added with distilled water (50 ml) and 10% (v/v) phosphoric acid (2.5 ml). The mixture was heated using a heating mantle and the distillate (50 ml) was collected. FA content in the distillate was determined according to the method of Nash (1953). To the distillate (2 ml), 2 ml of acetylacetone reagent were added and mixed thoroughly. The reaction mixture was incubated at 60°C for 15 min and cooled in running water. The absorbance was measured at 412 nm. A blank was prepared by using distilled water instead of the distillate. FA was calculated from a standard curve with the concentrations ranging from 0 to 0.5 μM and expressed as μmol/g sample.

Determination of dimethylamine (DMA)

DMA was extracted and determined by ^{1}H NMR spectroscopy as described by Howell *et al.* (1996). Sample (1 g) was homogenized with 10 ml of distilled water using an Omni mixer homogenizer (Waterbury, C.T, USA) at a speed of 22,000 rpm for 1 min, followed by centrifugation at 3,000 × g for 20 min using a Sigma Centrifuge (6K 10, Osterode am Harz, Germany). The supernatant (1 ml) was mixed with 100 μl of D_2O thoroughly with a vortex mixture for 20 sec. Thereafter, it was subjected to NMR spectroscopy. Proton NMR spectra were run on a Bruker AC-300 or AC-400 Fourier Transform spectrometer at 400 MHz. Data points (16K) were collected with a 30 degree pulse, a 15 ppm spectral width and a repetition time of 3.4 s and data collection was conducted. The number of scans was set to 512. Where a large water signal was encountered, a presaturation sequence was employed, with low power decoupling for 3.4 s and data collection was carried out, without decoupling, for 1.6 s.

Determination of protein solubility

Sample (2.5 g) was mixed with 50 ml of 20 mM Tris-maleate buffer containing 0.6 M NaCl, pH 7.0. The mixture was homogenized with an Omni mixer homogenizer (Waterbury, C.T, USA) at a speed of 22,000 rpm for 1 min. The homogenate was then centrifuged at 27,000 × g for 30 min at 4°C. The supernatant was subjected to protein analysis using the Lowry's method (Lowry *et al.*, 1951). Bovine serum albumin was used as a standard.

Determination of hexanal

To the sample (10 g), 150 ml of the saturated sodium chloride solution, and 2 ml of the internal standard (2 mg/ml of isobutyl acetate in cyclohexane) were added and the mixture was transferred to a 500 ml-round bottomed flask. The mixture was refluxed for 15 min. Then, the obtained cyclohexane layer was transferred into vial and analyzed by gas chromatography. An Agilent Technologies 6890N gas chromatography equipped with a RTX-5 column (15 m, 0.25 mm I.D., and 0.25 μm film thickness; Restek Corp., Bellefonte, PA, USA). High-purity hydrogen was used as the carrier gas at

1 ml/min. Injector and detector temperatures were kept at 250 and 300°C, respectively. Working standard containing hexanal (2 mg/ml) and isobutyl acetate (2 mg/ml) in cyclohexane was run under the same condition.

Differential scanning calorimetry (DSC)

DSC was carried out using a Setaram Micro DSC VII calorimeter (Stetaram, Lyon, France). Samples (800 mg) were placed in a pre-weighed DSC cell. An equal weight of distilled water was also introduced into the reference cell to obtain a flat base line. The instrument was calibrated for temperature and enthalpy using naphthalene (T=80.23°C, Heat=147.6 J/g) and indium (T=156.6°C, Heat=28.51 J/g) as standards. Samples and reference (water) were heated at 0.5°C/min from 10°C to 80°C. Endothermic peaks were recorded. The total energy required to denature the protein expressed as the enthalpy changed (ΔH), was measured by integrating the area under the peak (Setaram DSC handbook and software; Stetaram, Lyon, France).

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 analysis was performed using the Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL).

5.4 Results and Discussion

Partially purification of TMAOase from lizardfish kidney

Purification of TMAOase from lizardfish kidney is summarized in Table 5. Approximately 84% of activities were remained after heat-treatment at 50°C for 5 min. Purification fold of 2.6 was obtained by heat-treatment, which was used to denature and remove heat-labile proteins. Heat-treated extract was subjected to ammonium sulfate fractionation and the precipitate obtained at 60-80% saturation was then further purified by

gel filtration using the Sephacryl S-300 column (Figure 24). Fractions having TMAOase activity (fractions 43-54) were pooled for further purification. Specific activity increased approximately 147 folds, compared to that of crude extract. Pooled Sephacryl S-300 fractions were chromatographed on a DEAE-cellulose column. After loading and washing, column was eluted by using a 0-0.45 M NaCl linear gradient. A peak of TMAOase activity (fractions 62-68) was eluted at approximately 0.23 M NaCl (Figure 25). Purification fold of 1276 with a yield of 15% was obtained by this final step. Even though high amount of TMAOase was lost during the purification, a large amount of contaminated proteins were removed, resulting in a substantial increase in purity of TMAOase obtained. Rey-Mansilla *et al*. (2004) purified TMAOase from kidney of European hake using chromatographic techniques including the weak anion exchanger ANX Sepharose 4 Fast Flow and gel filtration on a Superdex 200 HR column.

Table 5. Purification of TMAOase from lizardfish kidney

Purification steps	Total activity[a] (U)	Total protein[b] (mg protein)	Specific activity (U/mg)	Purify (fold)	Yield (%)
Crude extract	53801.6	5486.4	9.8	1	100
Heat treatment	45351.1	1798.9	25.2	2.6	84.3
Ammonium sulfate (60-80%)	12667.9	121.2	104.5	10.7	23.5
Sephacryl S-300	9375.2	6.5	1440.1	146.9	17.4
DEAE-cellulose	8195.8	0.66[c]	12508.8	1275.6	15.2

[a]Assay was carried out under standard conditions at pH 7.0, 45°C with an incubating time for 10 min. The unit of enzyme is expressed as nmol of DMA produced per min.

[b]Protein concentration was measured by Lowry method (Lowry *et al.*, 1951).

[c]Protein concentration was measured by Bradford method (Bradford, 1976).

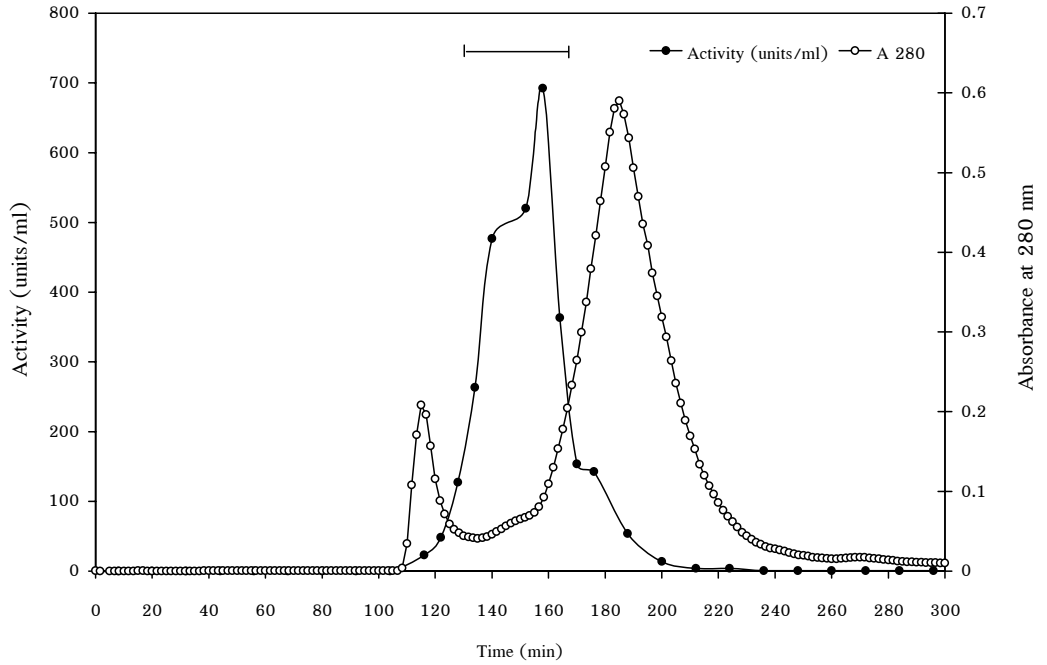

Figure 24. Elution profile of TMAOase on Sephacryl S-300 column (1.6 × 70 cm). Ammonium sulfate fraction (60-80%) were dialyzed against a starting buffer, concentrated with PEG 20,000 and applied onto a Sephacryl S-300 column. Elution was carried out with 20 mM Tris-acetate buffer, pH 7.0 at a flow rate of 0.5 ml/min.

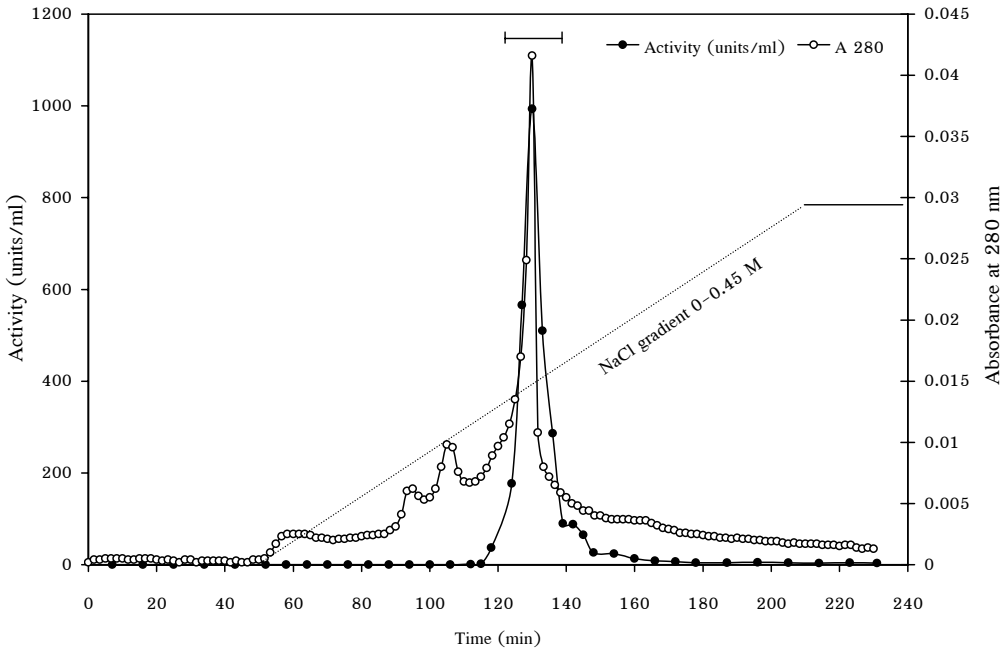

Figure 25. Elution profile of TMAOase on DEAE-cellulose column (1.6 × 20 cm). Pooled fractions from Sephacryl S-300 column were applied onto a DEAE-cellulose column with a flow rate of 0.5 ml/min. After washing, elution was carried out with a linear gradient of 0-0.45 M NaCl in 20 mM Tris-acetate buffer, pH 7.0.

Effect of TMAOase on FA, DMA, and TMAO contents of simulated system during refrigerated and frozen storage

FA content of the system in the presence of TMAOase increased significantly during storage at 4 and -10°C ($P < 0.05$) (Figure 26). The greatest changes were observed in the system with higher level of TMAOase added at both temperatures. At 4°C, a sharp increase in FA was observed in samples containing TMAOase within the first 6 days of storage. Thereafter a slight increase was noticeable up to 15 days of storage (Figure 26A). The highest FA formation was observed in the system containing 15 units TMAOase/g, which increased from 0.12 to 30.32 μmol/g sample at the end of storage. Similar changes in FA formation, but at a slower rate, were also found in the system stored at -10°C for 8 weeks (Figure 26B). A marked increase in FA

content was observed in the first 2 weeks of storage and reached the maximum values of 36.52 and 50.99 μmol/g sample at week 8 for systems having TMAOase at 5 units/g and 15 units/g, respectively. It was noted that a slight increase in FA was also observed in the system without TMAOase addition at both temperatures. Spinelli and Koury (1981) suggested that the TMAO decomposition can be catalyzed nonenzymatically by iron and various reductants. As a result, it was postulated that FA formation could be catalyzed nonenzymatically by $FeCl_2$, cysteine, and ascorbate, which were added as cofactors in the system. For the control (without TMAO and TMAOase), no significant increase was found at both temperatures ($P > 0.05$). It was suggested that TMAO in NAM was leached out during its preparation. Landolt and Hultin (1982) reported the removal of TMAO in red hake fillet after soaking, leading to a significant decrease in DMA and FA formation.

DMA and TMAO contents in the simulated systems after storage at 4°C for 15 days and -10°C for 8 weeks were evaluated by NMR spectroscopy as depicted in Figures 27 and 28. The NMR spectra peaks at 2.7 and 3.3 ppm represent DMA and TMAO, respectively (Howell *et al*., 1996). In the presence of TMAOase, the formation of DMA was more pronounced. The decrease in TMAO peak intensity was coincidental with the increase in DMA peak intensity. DMA and FA formations are the results of the TMAO decomposition catalyzed by TMAOase (Howell *et al*., 1996). The greater changes of peak intensity of DMA and TMAO were observed in the systems added TMAOase, compared to those without TMAOase. Additionally, no differences were found among samples without TMAOase stored at both temperatures. TMAO peak with high intensity with a very small DMA peak were observed in the systems without TMAOase. For the control, the DMA and TMAO peaks were not detected at both storage temperatures. This was most likely that TMAO was totally removed during the NAM preparation. Similar patterns of NMR profiles were obtained in samples kept at both temperatures. Nielsen and Jorgensen (2004) found the decrease in TMAO concentration and the accumulation of free FA caused by TMAOase during frozen storage of muscles from nine gadiform species. Although haddock is a gadoid fish, it produces a small amount of FA in frozen fillets (Badii and Howell, 2001). From the results, it was suggested that the addition of TMAOase to the system containing TMAO and cofactors could induce the formation of DMA and FA even in the frozen state. This was possibly due to a low activation energy needed for its activity (Parkin and Hultin, 1982b). Benjakul *et al*. (2003a) reported that the activation energy of TMAOase from

lizardfish kidney was 30.5 $kJmol^{-1}K^{-1}$. Thus, FA formation was induced by TMAOase in a concentration dependent manner.

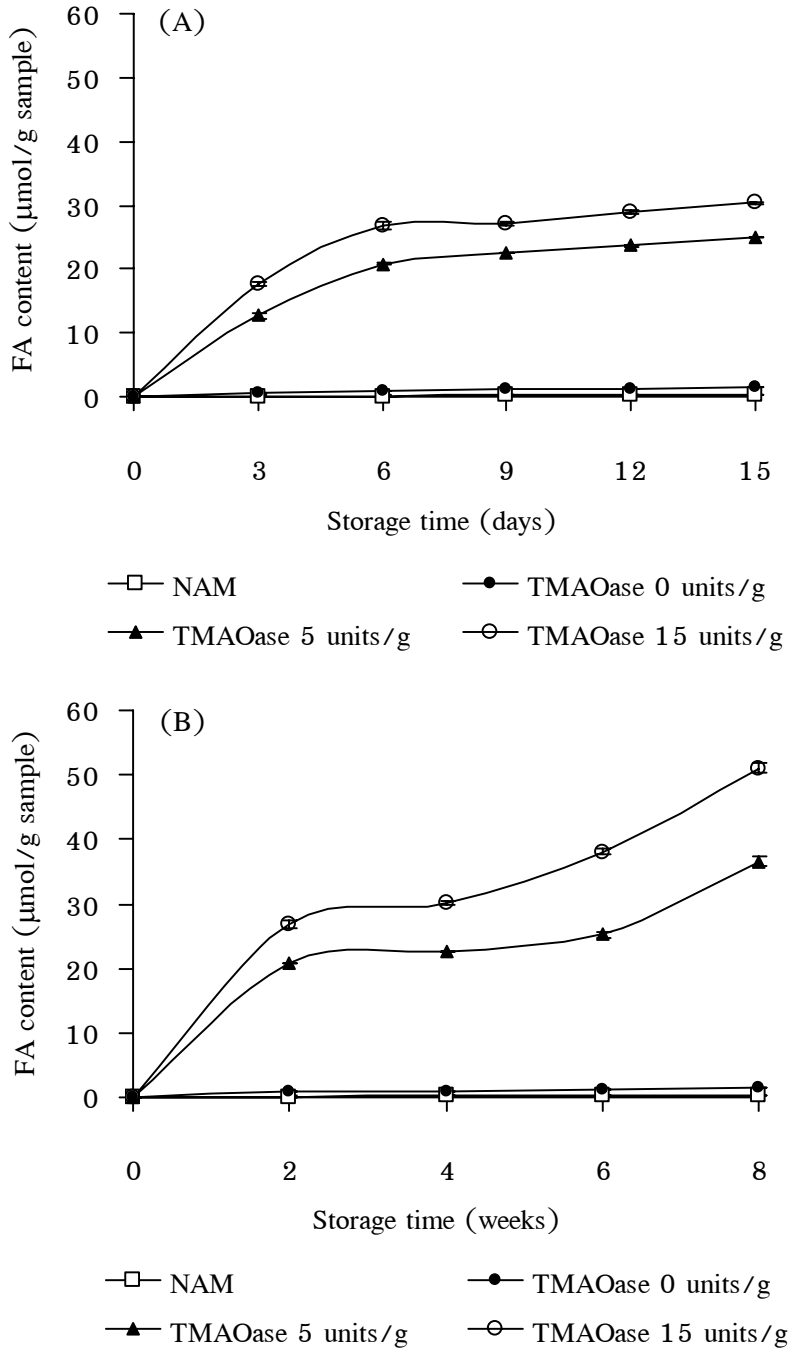

Figure 26. Effect of TMAOase from lizardfish kidney at different levels on FA content of simulated system during storage at 4°C for 15 days (A) and -10°C for 8 weeks (B). System contained 30 g NAM, 50 mM TMAO and cofactors consisting of 1 mM $FeCl_2$, 5 mM cysteine and 5 mM ascorbate. Bars indicate the standard deviations from triplicate determination.

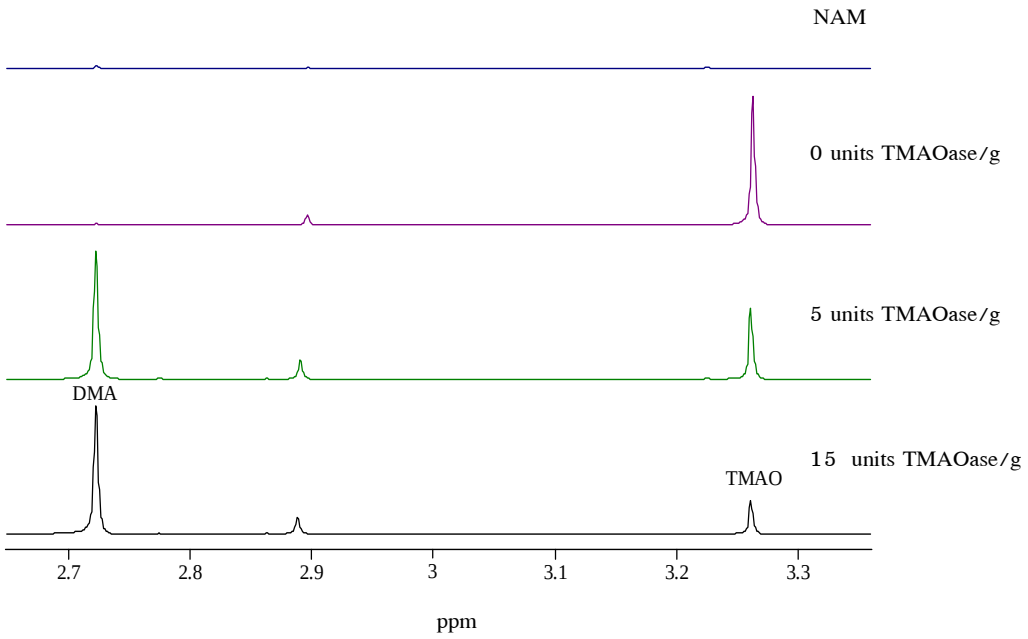

Figure 27. Effect of TMAOase from lizardfish kidney at different levels on proton ^{1}H NMR spectra (400 MHz) of simulated system after storage at 4°C for 15 days. System contained 30 g NAM, 50 mM TMAO and cofactors consisting of 1 mM $FeCl_2$, 5 mM cysteine and 5 mM ascorbate.

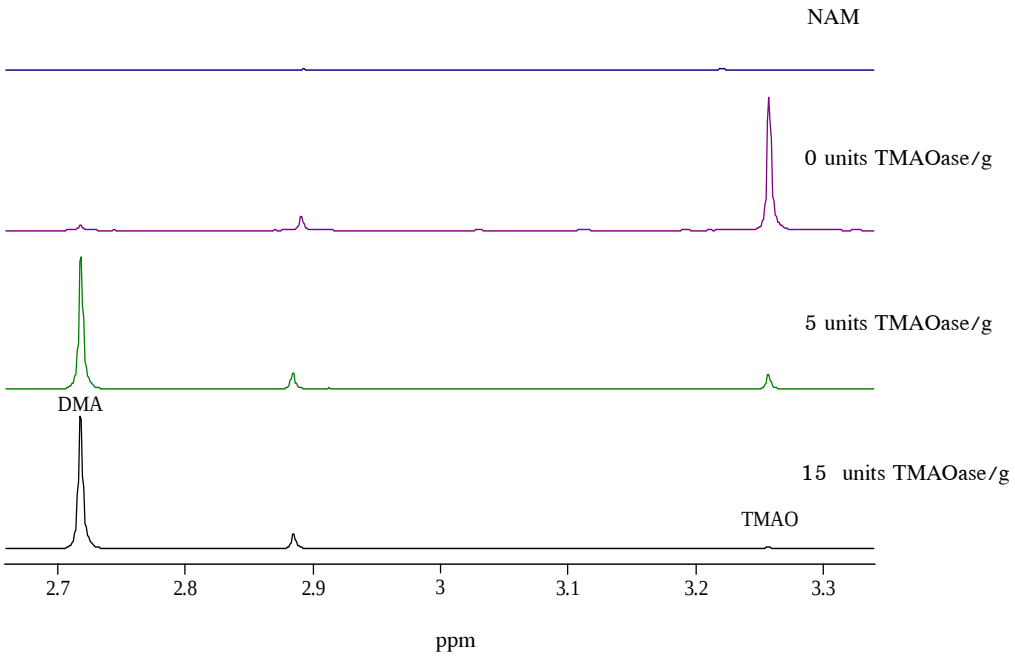

Figure 28. Effect of TMAOase from lizardfish kidney at different levels on proton ^{1}H NMR spectra (400 MHz) of simulated system after storage at −10°C for 8 weeks. System contained 30 g NAM, 50 mM TMAO and cofactors consisting of 1 mM $FeCl_2$, 5 mM cysteine and 5 mM ascorbate.

Effect of TMAOase on protein solubility of simulated system during refrigerated and frozen storage

The changes in protein solubility of the system containing different TMAOase levels as the function of storage time at 4 and -10°C are shown in Figure 29. In the first 3 days of storage at 4°C, the system containing TMAOase had the sharp decrease in protein solubility ($P < 0.05$). Thereafter, a slight decrease was found up to 15 days of storage (Figure 29A). TMAOase at a higher level caused the slightly higher loss in solubility. The system having TMAOase at a concentration of 15 units/g had the decrease in the protein solubility by 80% after storage for 15 days at 4°C. At -10°C, a sharp decrease in protein solubility was observed in the first 2 weeks of storage in all samples (Figure 29B). The greatest losses were also observed in the system with a higher level of TMAOase added. Higher loss of protein solubility was generally observed in systems containing TMAOase, compared with those without TMAOase added. From the result, less protein could be extracted with salt solutions when the greater amount of FA was produced (Figure 26). FA could function as a protein cross-linker and the large aggregate could be formed (Sikorski and Kolakowska, 1990; Hultin, 1992). Cross-linking of proteins could lower their solubility (Srinivasan and Hultin, 1997). As FA molecules bound to the proteins, unfolding and aggregation occurred, and the size of aggregates progressively increased, resulting in the greater loss in solubility. From the result, no FA was produced in the control. Nevertheless, a decrease in protein solubility still occurred throughout the storage at 4°C (Figure 29A). This was possibly due to the denaturation/aggregation of NAM during the extended storage. In the presence of $FeCl_2$, oxidation of lipids, especially membrane lipids, was possibly accelerated (Benjakul *et al*., 2004). Those oxidation products were reported to induce the cross-linking of proteins (Meynier *et al*., 2004; Tironi *et al*., 2004). Additionally, in the presence of TMAO, the disulfide bond might be formed. Brzezinski and Zundel (1993) suggested that when SH-groups containing amino acids, cysteine, or homocysteine, were reacted with TMAO, disulfides were formed.

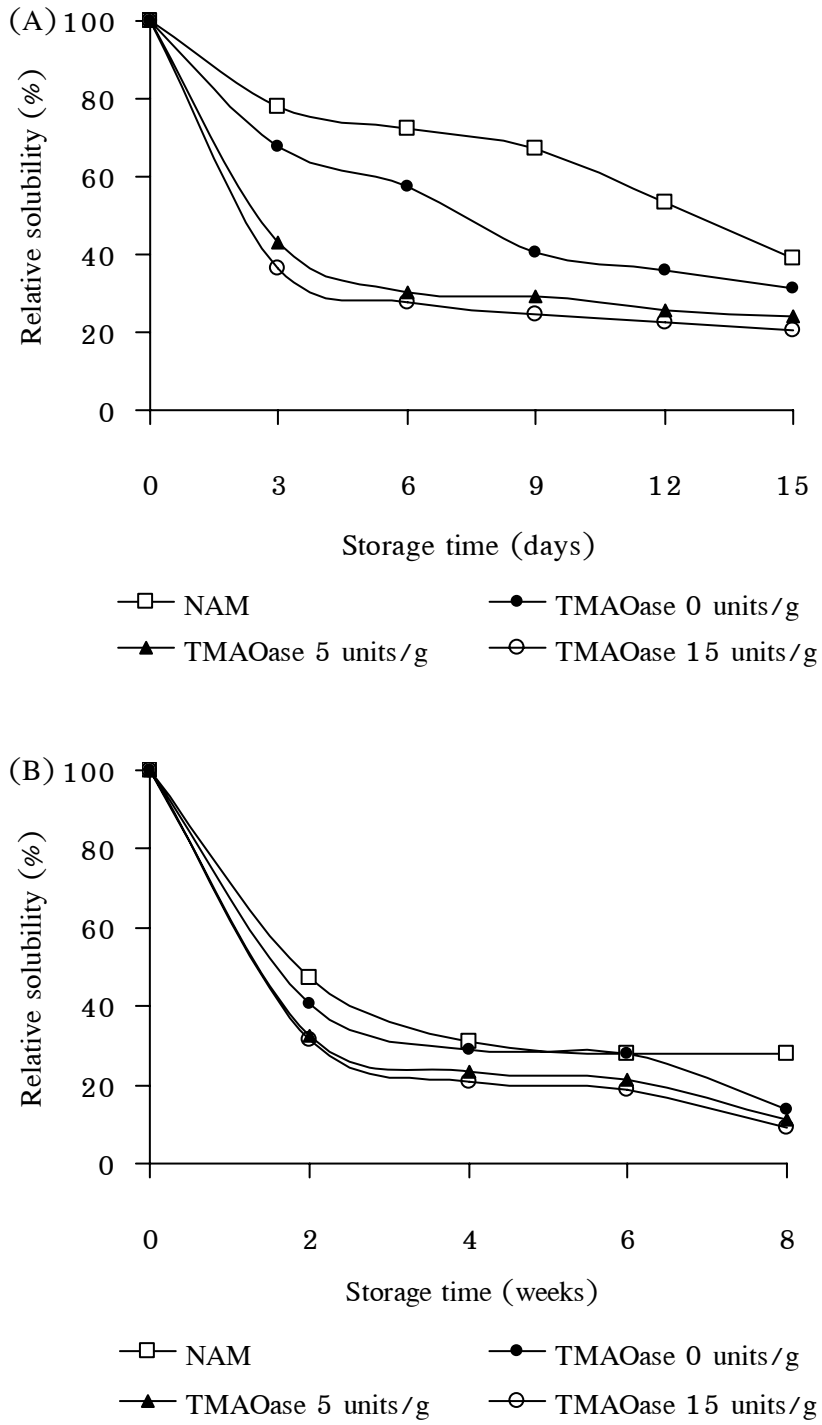

Figure 29. Effect of TMAOase from lizardfish kidney at different levels on protein solubility of simulated system during storage at 4°C for 15 days (A) and -10°C for 8 weeks (B). System contained 30 g NAM, 50 mM TMAO and cofactors consisting of 1 mM $FeCl_2$, 5 mM cysteine and 5 mM ascorbate.

From the result, the greater loss of protein solubility was observed in all systems and the control stored at -10°C, compared to those stored at 4°C, particularly with higher TMAOase levels. The decrease in solubility was generally in agreement with the greater FA formation (Figure 26). This might be due to the denaturation/aggregation of proteins during frozen storage caused by the formation and accretion of ice crystals, resulting in the dehydration, an increase in salt concentration and pH changes, following the removal water through ice formation (LeBlanc *et al*., 1988). Lipid hydrolysis/or oxidation as well as the formation of FA also contribute to denaturation of muscle proteins during frozen storage (Matsumoto, 1980). Careche *et al*. (1998a) reported that the addition of FA to hake NAM resulted in the decrease in salt soluble protein content during frozen storage at -12°C. Thus, in addition to the amount of produced FA, the storage temperature also had the influence on the protein solubility.

Effect of TMAOase on hexanal content of simulated system during refrigerated and frozen storage

Hexanal as a marker of lipid peroxidation was determined in the systems containing different levels of TMAOase after storage at 4 and -10°C for 15 days and 8 weeks, respectively. There was a difference in hexanal content between systems without and with TMAOase addition at both storage temperatures (Table 6). At the beginning of storage, no hexanal was detectable in all systems (data not shown). For the same storage temperature, the control had the highest hexanal contents, 0.5 and 0.47 mg/kg sample for the system stored at 4 and -10°C, respectively. In general, *trans*-2-hexanal is formed by fish lipoxygenases (Josephson and Lindsay, 1986) and also via autoxidation of fatty acids (Belitz and Grosch, 1999). In the presence of iron and ascorbate, lipid oxidation might be accelerated. Iron and ascorbate were used to induce lipid peroxidation in *Biceps femoris* muscle (Cava *et al*., 2000). Free iron can behave as a free radical and take part in electron transfer reactions with molecular oxygen, leading to the generation of a superoxide anion (Harris and Tall, 1994). Aro *et al*. (2003) found an increase in hexanal peak area of Baltic herrings stored for 8 days at refrigerated temperature, indicating the oxidation of long chain unsaturated fatty acids. On the other hand, no hexanal was found in the systems containing TMAOase at both temperatures. This was possibly due to the cross-linking of protein induced by FA. As a result, the cross-linked proteins might prevent the interaction

between catalysts and lipids, particularly membrane lipids, leading to the minimized lipid oxidation. Furthermore, iron and ascorbate functioned as cofactors for the activity of TMAOase. As a result, their availability of those compounds to accelerate the lipid oxidation in the system containing TMAOase was presumed. Aubourg and Medina (1999) found that haddock (non-FA-forming species) showed higher lipid oxidation and hydrolysis than in cod (FA-forming species) during storage at -10°C. From the result, the higher decrease in protein solubility was observed in the system possessing the higher FA along with a lower extent of lipid oxidation. This was most likely that lipid oxidation possibly contributed to protein denaturation to a lower extent in the system studied, compared to FA formation. However, both lipid oxidation and FA formation had the impact on the induced protein denaturation of systems stored at both temperatures.

Table 6. Hexanal content in simulated system containing different levels of TMAOase from lizardfish kidney after storage at 4 and -10°C[a,b]

Samples	Hexanal content (mg/kg sample)	
	4°C, 15 days	-10°C, 8 weeks
NAM	0.5±0.01b	0.47±0.02b
0 units TMAOase/g	0.16±0.02a	0.14±0.01a
5 units TMAOase/g	ND	ND
15 units TMAOase/g	ND	ND

[a]Values are given as mean ± SD from triplicate determinations.

[b]Different letters in the same column indicate significant differences ($P < 0.05$).

ND: non detectable

Effect of TMAOase on DSC thermogram of simulated system during refrigerated and frozen storage

Figure 30 shows a typical DSC thermogram of fresh haddock NAM. Fresh NAM exhibited two major peaks with the maximum transition temperatures (T_m) at 32.16°C (ΔH=0.53 J/g) which could be attributed to the denaturation of myosin, and 62.39°C (ΔH=0.25 J/g), which was due to the denaturation of the thin filament proteins, mainly actin (Stabursvik and Martens, 1980). The observed T_m of myosin was within the temperature range (25-46°C) reported in various fish species (Ogawa *et al.*, 1993). T_m of myosin and actin of haddock NAM were in accordance with Dileep *et al.* (2005) who reported that T_m of myosin and actin in minced Alaska pollock were found at 33.17 and 60.96°C, respectively. The peaks on the DSC thermogram indicated unfolding of protein molecules and transition of native form to a denatured conformation due to heating. Protein denaturation usually indicates an endothermic peak in the DSC thermogram. The changes in heat energy during thermal denaturation of protein mainly result from the rupture of inter-molecular hydrogen bonds (Privalov and Khechinashvili, 1974). The area under the transition peak shows the enthalpy value (ΔH) which is correlated with the content of ordered secondary structure of a protein (Koshiyama *et al.*, 1981).

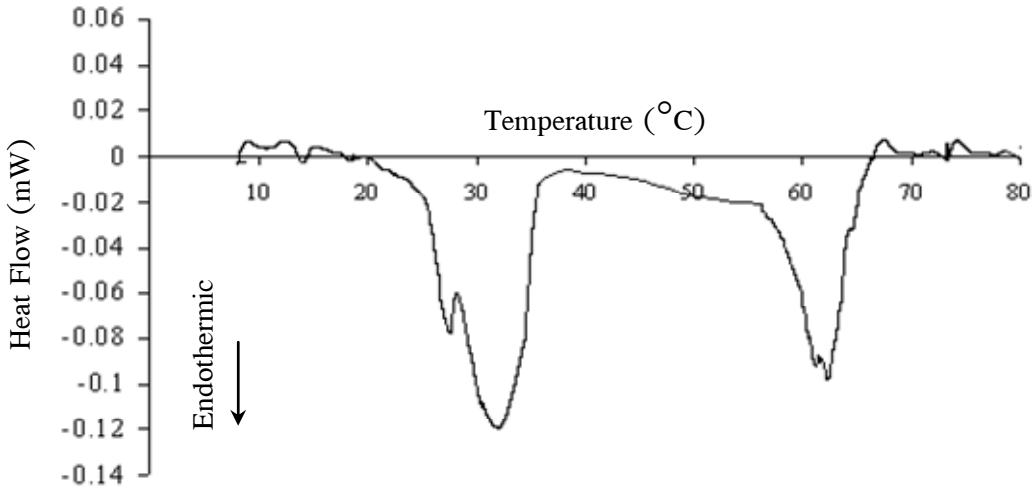

Figure 30. Differential scanning calorimetry (DSC) thermogram of fresh haddock NAM.

T_m and enthalpy for the myosin and actin transitions of haddock NAM in the presence and the absence of TMAOase at different levels stored at 4 and -10°C for 15 days and 8 weeks, respectively, are given in Table 7. In general, a non-significant decrease in T_m of myosin and actin was observed in all samples after storage at both temperatures. A marked decrease in ΔH of both transition peaks was found in all samples after storage at both temperatures ($P < 0.05$). The greater decrease was observed in the system with TMAOase addition, compared with those without TMAOase. For the control stored at 4°C, T_m of myosin denaturation decreased from 32.16°C (fresh NAM) to 31.69°C after the storage at 4°C for 15 days. ΔH also decreased from 0.53 to 0.098 J/g at 4°C. T_m of actin decreased from 62.39°C (fresh NAM) to 60.02°C and ΔH also decreased from 0.25 to 0.093 J/g. The decreases in both T_m and ΔH of NAM after storage indicated the protein denaturation. During the extended storage, autolysis might take place, leading to the losses in protein integrity. A decrease in both T_m and ΔH was noticeable in the control simulated system stored at -10°C for 8 weeks, where T_m for myosin and actin of the control were 30.52 (ΔH=0.093 J/g) and 60.63°C (ΔH=0.046 J/g), respectively. No transition peaks were observed in samples containing TMAOase at levels of 5 and 15 units/g after storage at -10°C. This might be indicative of destabilization of myosin and actin or the reduced cooperative native of the major segment of the protein (Srinivasan *et al.*, 1997b). From the result, it was suggested that the reduction in enthalpy energy was higher for the systems with TMAOase. During extended storage, an alteration of the native structure of myosin and actin due to FA formation, lipid oxidation products as well as the formation of ice crystals, resulted in the lowered thermal energy for denaturation (Martone *et al.*, 1986). Herrera *et al.* (2001) found that the transition of the myosin peak of minced blue whiting muscle became less clearly defined and gradually flattened over the storage period at -10°C. The denaturation enthalpy of myosin peak transition of this fish decreased more or less continuously in the samples stored at -10°C during the whole storage period. Jittinandana *et al.* (2003) reported a greater susceptibility of rainbow trout myosin to thermal denaturation after 2 weeks of storage at -10°C.

Table 7. Transition temperature (T_m) and enthalpy (ΔH) of myosin and actin in simulated system containing different levels of TMAOase from lizardfish kidney after storage at 4 and −10°C[a,b]

Samples	Myosin		Actin	
	T_m (°C)	ΔH (J/g)	T_m (°C)	ΔH (J/g)
4°C, 15 days				
NAM	31.69±1.3a	0.098±0.002e	60.02±1.1ab	0.093±0.003f
0 units TMAOase/g	30.8±1.1a	0.089±0.005d	59.5±1.6ab	0.054±0.002e
5 units TMAOase/g	30.67±1.4a	0.043±0.001bc	58.79±1.7ab	0.040±0.002d
15 units TMAOase/g	29.99±1.0a	0.035±0.007ab	57.84±1.3a	0.031±0.004c
−10°C, 8 weeks				
NAM	30.52±1.2a	0.046±0.002c	60.63±1.1b	0.013±0.003b
0 units TMAOase/g	29.84±1.3a	0.033±0.003a	59.42±1.6ab	0.008±0.001a
5 units TMAOase/g	−	−	−	−
15 units TMAOase/g	−	−	−	−

[a]Values are given as mean ± SD from duplicate determinations.

[b]Different letters in the same column indicate significant differences ($P < 0.05$).

5.5 Conclusion

TMAOase from lizardfish kidney induced the formation of FA and DMA in the simulated system. This led to the losses in protein solubility and lowered thermal stability of haddock NAM during storage. Therefore, TMAOase played an essential role in fish muscle protein denaturation via induction of FA formation, a potential protein cross-linker. However, lipid oxidation also detrimentally affected the biochemical property of muscle proteins even to a lower degree, compared with FA.