ELSEVIER

Food Chemistry 84 (2004) 297–305

Food Chemistry

www.elsevier.com/locate/foodchem

Induced formation of dimethylamine and formaldehyde by lizardfish (*Saurida micropectoralis*) kidney trimethylamine-*N*-oxide demethylase

Soottawat Benjakul[a,*], Wonnop Visessanguan[b], Munehiko Tanaka[c]

[a]*Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90112, Thailand*
[b]*National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Phaholyothin Road., Klong 1, Klong Luang, Pathumthani, 12120, Thailand*
[c]*Department of Food Science and Technology, Tokyo University of Fisheries, Konan 4, Minato, Tokyo 108-8477, Japan*

Received 7 January 2003; received in revised form 6 May 2003; accepted 6 May 2003

Abstract

The distribution of trimethylamine-*N*-oxide demethylase (TMAOase) in various internal organs of lizardfish (*Saurida micropectoralis*) and its effect on the solubility of myofibrillar proteins were studied. Among the internal organs tested, kidney contained the highest TMAOase activity, followed by spleen, bile sac, intestine and liver, respectively. TMAOase from the kidney showed a higher activity at pH 7.0 than at pH 5.0. The molecular mass was estimated to be 128 kDa, based on activity staining. In the presence of trimethylamine-*N*-oxide (TMAO) and cofactors ($FeCl_2$, ascorbate and cysteine), addition of partially purified kidney TMAOase resulted in an increase of dimethylamine (DMA) and formaldehyde in washed mince from both red seabream (*Pagrus major*), white muscle fish, and skipjack (*Katsuwonus pelamis*), red muscle fish, during iced storage, especially in the first 6 days. TMAOase addition led to a loss in protein solubility, which is associated with increased cross-linking of protein. Therefore, contamination of muscle by TMAOase from internal organs, especially kidney, possibly causes the changes in fish texture.

Keywords: Lizardfish; Kidney; TMAOase; Formaldehyde; Dimethylamine; TMAO; Muscle protein; Solubility

1. Introduction

Formaldehyde and dimethylamine (DMA) can be produced enzymatically via the degradation of trimethylamine oxide (TMAO) in several fish species, especially gadoid fish such as cod, hake, pollack and whiting (Herbard, Flick, & Martin, 1982). The formaldehyde is believed to contribute to the toughening of fish muscle, caused by the aggregation of proteins (Ragnarsson & Regenstein, 1989). Formaldehyde also induces changes in protein molecules, as monitored by a decrease of ATPase activity, an increase of surface hydrophobicity and a loss of solubility (Ang & Hultin, 1989).

Trimethylamine-*N*-oxide demethylase (TMAOase) has been known to catalyze the conversion of TMAO to DMA and formaldehyde (Gill & Paulson, 1982). This enzyme is concentrated in the internal organs and red muscle (Rehbein & Schreiber, 1984; Gill & Paulson, 1982). Moreover, the muscle microsome of red hake was found to contain TMAOase (Parkin & Hultin, 1986). Recently, TMAOase associated with Alaska pollack myofibrils has been characterized (Kimura, Seki, & Kimura, 2000a,b). The enzyme requires co-factors for full activity. The system of NADH and FMN requires anaerobic conditions while the system of iron and cysteine and/or ascorbate functions in the presence or absence of oxygen (Parkin & Hultin, 1986). TMAOase from red hake muscle is activated by cysteine and Fe^{2+} or by methylene blue and ascorbic acid (Lundstrom, Correia, & Wilhelm, 1982a,b). In general, reducing conditions enhance, whereas oxidizing conditions inhibit the enzymatic degradation of TMAO to DMA and

* Corresponding author. Tel.: +66-74-286334; fax: +66-74-212889.
E-mail address: bsoottaw@ratree.psu.ac.th (S. Benjakul).

0308-8146/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0308-8146(03)00214-0

FA (Harada, 1975). As a consequence, packaging conditions directly influence the formation of dimethylamine. Red hake packaged in an atmosphere without oxygen, produced DMA at a rapid rate (Lundstrom, Correia, & Wilhelm, 1982a). Additionally, soaking of red hake fillet to remove TMAO before freezing significantly reduces DMA formation (Landolt & Hultin, 1982).

Lizardfish is an important commercial species in Thailand and is used mostly for surimi production. This is due to its high gel-forming ability and appreciable whiteness (Benjakul, Visessanguan, & Tueksuban, 2003). However, the gel-forming ability of this fish decreases rapidly during post-harvest handling. This is believed to be associated with proteolysis as well as with the formation of formaldehyde (Benjakul et al., 2003). Storage in ice is a common practical approach to maintain the prime quality of lizardfish. However, many reactions, especially enzymatic reactions induced by both proteinases and TMAOase localized in both muscle and viscera, may contribute to the quality deterioration of this species. Also, the contamination of TMAOase from the internal organs during processing, particularly mincing, possibly induces the changes in protein aggregation caused by formaldehyde formed. So far, no information on TMAOase from lizardfish caught in Thailand has been reported. Also, changes in muscle proteins induced by TMAOase can be different among species and muscle types, possibly caused by the differences in reactivity of muscle toward formaldehyde formed. Our objectives were to determine TMAOase activity in different internal organs of lizardfish and to study the formation of dimethylamine and formaldehyde in washed mince from red seabream and skipjack to which lizardfish kidney TMAOase was added in a simulated system.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan), except trimethylamine-*N*-oxide, which was obtained from Aldrich Chemical Company, Inc (Milwaukee, WI, USA). High-molecular-weight standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Fish species and raw materials

Lizardfish (*Saurida micropectoralis*) were purchased from the dock in Trang, Thailand. The fish, off-loaded approximately 36–48 h after catching, 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, Thailand within 3 h. Individual organs including kidney, liver, spleen, bile sac and intestine were carefully dissected. The same organs were pooled in order to avoid individual variation. Samples were stored at −20 °C until analyses were done.

Fresh red seabream (*Pagrus major*) and skipjack (*Katsuwonus pelamis*) were purchased from the local fish market in Tokyo, Japan. Washed mince was prepared from finely chopped muscle by treating with chilled 20 mM Tris–HCl, pH 7.0 at a ratio of 1:5 (w/v). The mixture was homogenized at a speed of 2 for 30 s, using a Hitachi homogenizer (Hitachi, Tokyo, Japan). The temperature was maintained below 5 °C throughout the preparation. The homogenate was then centrifuged at 3000×*g* for 20 min at 4 °C. The washing was repeated twice under the same conditions. The washed mince was kept in ice until used.

2.3. Preparation of TMAOase crude extract from different lizardfish internal organs

To prepare crude TMAOase extracts, the finely chopped organ was extracted with 3 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 1 min. The homogenate was centrifuged at 38,500×*g* for 30 min at 4 °C using a refrigerated centrifuge (Tomy CX250, Tokyo, Japan). The supernatant obtained was referred to as the crude TMAOase extract.

2.4. TMAOase assay

TMAOase activity was assayed using TMAO as a substrate in the presence of selected cofactors (Kimura et al., 2000b). To 2.5 ml of assay mixture (24 mM Tris–acetate, containing 24 mM TMAO, 2.4 mM cysteine, 2.4 mM ascorbate, 0.24 mM $FeCl_2$ and 0.12 M NaCl, pH 7.0), 0.5 ml of properly diluted enzyme solution was added to initiate the reaction. The reaction was performed at 25 °C for precisely 20 min and 1 ml of 10% trichloroacetic acid was added to terminate the reaction. The reaction mixture was then centrifuged at 8000×*g* for 15 min and the supernatant was subjected to dimethylamine (DMA) determination. One unit of TMAOase was defined as the activity, which released 1 μmol DMA per min.

2.5. Acidification of lizardfish kidney TMAOase

Crude TMAOase extract from lizardfish kidney was acidified to pH 4.5 by adding with 0.1 M Na–acetate buffer, pH 4.5 at the ratio of 1:1 (v/v). The mixture was centrifuged at 38,500×*g* for 30 min at 4 °C. The pH of supernatant was then adjusted to 7.0 by addition of 0.1 or 1.0 M NaOH. The neutralized supernatant was centrifuged at 38,500×*g* for 30 min at 4 °C to remove the

undissolved debris. The supernatant obtained was used as the partially purified TMAOase.

2.6. Characterization of partially purified TMAOase

Crude and acidified extracts were mixed with sample buffer containing no βME at a ratio of 2:1 (v/v). Then the fraction (20 μg) was loaded into the PAGEL®-precast gel (10%). Electrophoresis was run at a constant voltage of 100 V using a Compact-PAGE apparatus (Atto Corp., Tokyo, Japan). After separation, the gel was immersed in the TMAOase assay mixture for 30 min at 25 °C. The gel was rinsed with distilled water and transferred to acetylacetone reagent and incubated at 60 °C for 5 min for activity zone development. Crude and acidified extracts were also subjected to SDS-PAGE, followed by staining with 0.125% Coomassie-brilliant Blue R-250 and destained in 25% methanol and 10% acetic acid. High-molecular-weight standards were used for estimation of apparent molecular mass of TMAOase in the acidified fraction.

2.7. Effect of TMAOase on chemical changes in washed mince

To induce DMA and formaldehyde formation, partially purified TMAOase (500 μl) was added to these simulated systems (84.5 g), containing the washed mince from either red seabream or skipjack, 50 μmol TMAO/g, 1 μmol $FeCl_2$/g, 5 μmol ascorbate/g, 5 μmol cysteine/g and 0.1% Na-azide. The amount of enzyme added was equivalent to 2 and 10 mU/g of washed mince, as determined by the TMAOase assay. For the control, TMAOase and TMAO were excluded. After the addition of enzyme, the washed mince was mixed thoroughly. A portion of 10 g was placed in a glass vial and the lid was closed tightly. The samples were placed in ice and kept at 4 °C. The ice was changed every day to maintain the temperature of 0 °C. Samples were randomly taken for TMAO, DMA, formaldehyde and protein solubility determinations every three days for up to 12 days. For SDS-PAGE analyses, the samples were taken at 0, 6 and 12 days.

2.8. *Trimethylamine*-N-*oxide determination*

To a simulated system (2 g), 8 ml of 5% trichloroacetic acid were added and the mixture was homogenized with a Hitachi homogenizer at a speed of 1 for 45 s. The homogenate was centrifuged at 3000×*g* for 15 min. The supernatant, containing TMAO, was used for analysis. TMAO was determined after reduction to TMA by the method of Yamagata, Horimoto, and Nagaoka (1969) with a slight modification. Supernatant (2 ml) was treated with 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 indigenous TMA content of the samples and expressed as μmoles/g.

2.9. Dimethylamine and formaldehyde determination

To a simulated system (2 g), 8 ml of 5% trichloroacetic acid were added and the mixture was homogenized using a Hitachi homogenizer at a speed of 1 for 30 s. The homogenate was centrifuged at 3000×*g* for 15 min. To the pellet, 4 ml of 5% trichloroacetic acid were added and homogenized as previously mentioned. The supernatants were combined and neutralized to pH 6.0–6.5 and the final volume was made up to 20 ml using distilled water. The supernatant was then used for dimethylamine (DMA) and formaldehyde determination.

DMA was determined by the copper-dithiocarbamate method, as described by Dyer and Mounsey (1945) with a slight modification. To 2 ml of neutralized supernatant in screw-capped test tube, 1 ml of copper–ammonia reagent was added and mixed thoroughly, followed by the addition of 4 ml of 5% CS_2-toluene solution. The tubes were then closed tightly and the mixture was incubated at 50 °C for 2 min. The mixture was mixed with a vortex mixer for 20 s, followed by the addition of 400 μl of 30% acetic acid. The mixture was then mixed thoroughly using a vortex mixer for 15 s. After leaving for 10 min at room temperature, the toluene layer was transferred to a tube containing 0.5–1.0 g of anhydrous Na_2SO_4 to remove the water. The absorbance was measured at 440 nm. The blank was prepared by using 3% trichloroacetic acid instead of sample supernatant. DMA content was calculated and expressed as μmoles/g sample.

Formaldehyde was determined using acetylacetone reagent according to the method of Nash (1953) with a slight modification. To the neutralized supernatant (3 ml), 3 ml of acetylacetone reagent was 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 3% trichloroacetic acid instead of sample supernatant. Formaldehyde was calculated and expressed as μmoles/g sample.

2.10. Solubility determination

Washed mince (1.0 g) was treated with 20 ml of 10 mM Na–phosphate buffer containing 0.6 M NaCl, pH 7.0. The mixture was homogenized with a Hitachi homogenizer at speed of 1 for 20 s. The homogenate was then centrifuged at 37,500×*g* for 30 min at 4 °C. The supernatant was subjected to protein analysis using the Bradford method (Bradford, 1976).

2.11. Electrophoretic analysis

Washed mince (1 g) was homogenized with 9 ml of 20 mM Tris–HCl, pH 7.0, containing 2% SDS, 8 M urea and 2% βME for 30 s. The mixture was then heated in boiling water for 3 min. The dissolved protein (10 μg) was loaded onto the PAGEL®-precast gel (10%). Electrophoresis was carried out at a constant voltage of 100 V using a Compact-PAGE apparatus (Atto Corp., Tokyo, Japan). After separation, proteins were stained with 0.125% (w/v) Coomassie-brilliant Blue R-250 in 25% (v/v) methanol and 10% acetic acid and destained with 25% (v/v) methanol and 10% (v/v) acetic acid. High-molecular-weight markers were used to estimate the molecular weights of proteins.

2.12. Protein determination

Protein concentration was determined using the Coomassie Blue staining method, as described by Bradford (1976). Bovine serum albumin was used as a standard.

2.13. Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test (Steel & Torrie, 1980).

3. Results and discussion

3.1. Distribution of TMAOase in various lizardfish internal organs

TMAOase activities in different internal organs are shown in Fig. 1. Kidney contained the highest activity, followed by spleen, intestine, bile sac and liver, respectively. The distribution of TMAOase in different organs varied, depending on species. For *Gadus morrhua* (L.) and *Melanogrammus aeglefinus* (L.), TMAOase in kidney was higher than spleen, whereas spleen contained higher activity than kidney in *Molva molva* (L.) and *Molva dypterygia* (Rehbein & Schreiber, 1984). When comparing the activity between pH 5.0 and 7.0, it was found that activity at pH 7.0 was much higher than that observed at pH 5.0 in all organs tested. Especially for kidney, the activity assayed at pH 7.0 was 75% higher than that at pH. 5.0. Our results were in contrast to the TMAOase previously reported in other species. Rehbein and Schreiber (1984) and Gill and Paulson (1982) reported that TMAOase from cod internal organs, including kidney, spleen and pyloric caeca, had the optimum pH at 4.5–5.0. Moreover, Harada (1975) found that TMAOase from liver of lizardfish (*Saurida tumbil*) exhibited the maximum activity at pH 5.0. It is postulated that TMAOase from lizardfish (*S. micropectoralis*) used in this study might have distinctive molecular and enzymatic properties. Since TMAOase in lizardfish internal organs was likely to be more active at neutral pH than at acidic pH, it might exhibit a high activity in the muscle, which generally has a post mortem pH ranging from 6.5 to 7.0 and is implicated in muscle hardening during storage and processing.

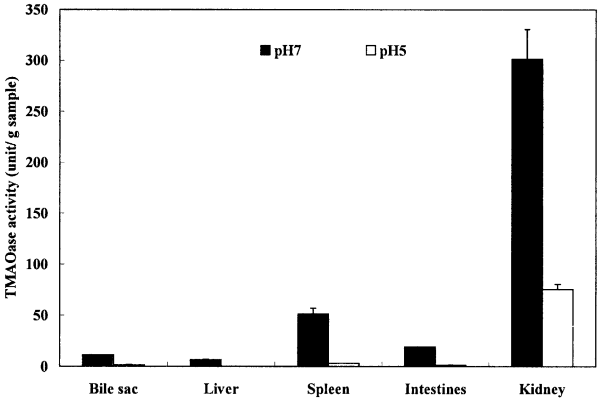

Fig. 1. TMAOase activities in different organs of lizardfish assayed at pH 5.0 and 7.0. One unit of TMAOase was defined as the activity which released 1 μmol DMA per min. Bars indicate the standard deviation from triplicate determinations.

3.2. Characteristics of partially purified lizardfish kidney TMAOase

Crude kidney TMAOase was partially purified by acidification to pH 4.5 and subsequent neutralization to pH 7.0. From our preliminary study, acid treatment effectively removed approximately 70% of total protein while retaining about 70% of total activity, leading to an increase in purity of 2.8-fold compared to crude extract. In order to purify TMAOase, acidification was used to remove other proteins in the Alaska pollock myofibrillar fraction (Kimura et al., 2000b). SDS-PAGE revealed that proteins with high molecular weight were removed in the acidification process (Fig. 2a). Also, proteins with the molecular weights of 35–38 kDa were also removed. From activity staining, one activity zone with a bright yellow colour was observed in both crude and acidified extracts (Fig. 2b). The molecular weight of lizardfish kidney TMAOase from both crude and acidified extract was estimated to be 128 kDa based on activity staining. A wider zone was observed with the acidified extract. This suggested that this acidified extract contained higher specific TMAOase activity. The separated TMAOase band on the polyacrylamide gel was able to convert TMAO into formaldehyde and DMA. Formaldehyde reacted with ammonium acetate and acetylacetone in acetyl acetone reagent, resulting in the formation of diacetyldihydrolutidine (DDL), a yellow compound. TMAOase from Alaska pollock muscle had a molecular weight of

400 kDa, as demonstrated by Sephacryl S-300 chromatography (Kimura et al., 2000b).

3.3. Effect of lizardfish kidney TMAOase on DMA and formaldehyde formation

The addition of TMAOase increased DMA in washed mince from both red seabream and skipjack during iced storage ($P<0.05$) (Fig. 3). The increase was proportional to the amount of enzyme added. For the mince with TMAOase added at the level of 10 mU/g, DMA was formed rapidly in the first 3 days of storage ($P<0.05$). After 3 days of storage, DMA gradually increased in skipjack washed mince up to day 12, while no changes were found in red seabream washed mince ($P>0.05$). For the washed mince with TMAOase added at a level of 0 and 2 mU/g, a gradual increase was generally observed in the first 6 days of storage. No marked changes occurred after 6 days of storage for both washed mince ($P>0.05$). Interestingly, an increase in DMA was also observed in the samples without TMAOase addition. It was postulated that TMAOase associated with myofibrils played an important role in converting TMAO to DMA. Though a thorough washing of mince was performed, TMAOase bound tightly to muscle or membrane could not be entirely removed. As a result, DMA was possibly produced by the myofibril-bound TMAOase. TMAOase was found in the microsome fraction of red hake muscle (Parkin & Hultin, 1986) and in the myofibrillar fraction of Alaska pollack (Kimura et al., 2000a). For the control (without TMAO and TMAOase), no significant increase was found in either washed mince (from red seabream or skipjack) ($P>0.05$). This was because TMAO, a water-soluble compound, was removed by water washing or soaking. Landolt and Hultin (1982) reported that significant TMAO removal occurred in red hake fillet due to soaking treatment, leading to a significant decrease in DMA and formaldehyde formation. Therefore, the washing process can be a promising means to remove TMAO, which is a substrate for TMAOase. As a consequence, DMA, as well as formaldehyde formation, can be prevented.

The formation of free formaldehyde in washed mince from both fish species was also monitored during iced storage (Fig. 4). Free formaldehyde increased in all samples containing TMAO in the presence and absence of TMAOase as the storage time increased, especially in the first 6 days ($P<0.05$). However, no changes in free formaldehyde were found in the control (without TMAO and TMAOase) ($P>0.05$). From the results, the mince containing TMAOase at a higher level had a higher content of free formaldehyde throughout the storage up to 12 days. Free formaldehyde increased rapidly in the first 6 days. Thereafter, no additional free formaldehyde content was observed ($P>0.05$). It has been known that TMAOase catalyzes the breakdown of TMAO to DMA and formaldehyde in equimolar amounts. From the result, detectable free formaldehyde was much lower than DMA, indicating that the large

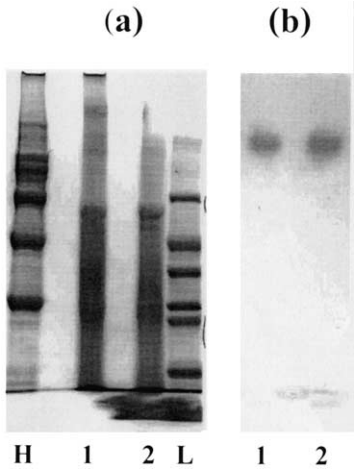

Fig. 2. Protein pattern (a) and activity staining (b) of crude (1) and acidified (2) TMAOase extracts from lizardfish kidney. H: high-molecular weight marker; L: low-molecular weight marker.

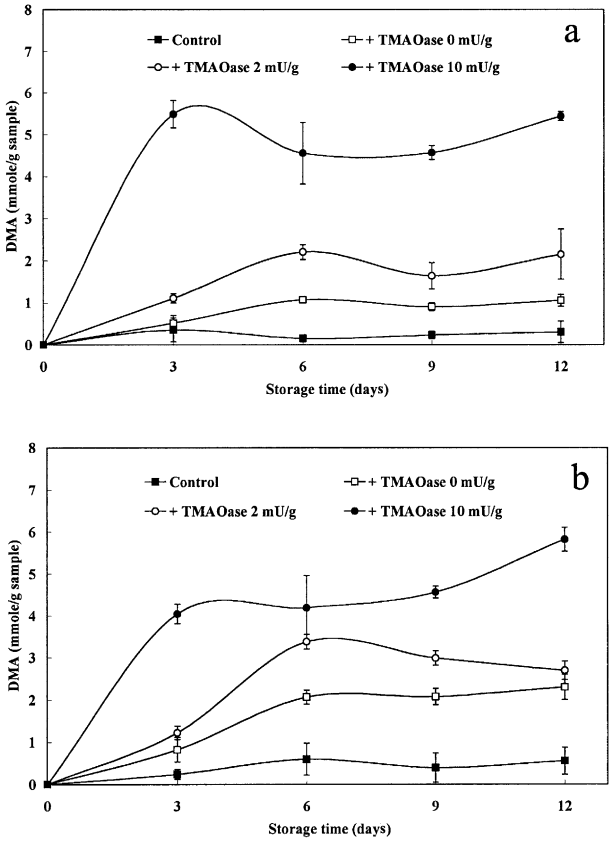

Fig. 3. Changes in DMA content in washed mince from red seabream (a) and skipjack (b) with different levels of lizardfish kidney TMAOase added during iced storage for 12 days. Washed mince contained TMAO and cofactors consisting of $FeCl_2$, cysteine and ascorbate. Bars indicate the standard deviation from triplicate determinations.

amount of formaldehyde formed was bound tightly to muscle proteins in both species and could not be extracted. Owusu-Ansah and Hultin (1987) suggested that the differences between the amount of DMA produced and free formaldehyde content represented bound formaldehyde. When comparing free formaldehyde content between two species, a higher amount was found in red seabream than in skipjack. This possibly suggests different reactivities between white and dark muscle fish in formaldehyde adduction.

From the results, the addition of TMAOase to washed mince, in the presence of TMAO and cofactors, effectively produced DMA and formaldehyde in a concentration dependent manner, even at 0 °C. The greater the amount of enzyme added, the higher were the amounts of products obtained. Parkin and Hultin (1982) found that TMAO demethylase of red hake muscle had a relatively low activation energy of slightly more than 5 kcal per degree-mol. As a result, TMAOase was still active, even in the frozen state.

The increases of DMA and free formaldehyde formed were coincidental with the decrease in TMAO (Fig. 5). A sharp decrease in TMAO was observed in the first 6 days. DMA and formaldehyde, in washed mince containing TMAO, $FeCl_2$ and ascorbic acid, were produced with a concomitant decrease of TMAO (Owusu-Ansah & Hultin, 1987). Though TMAO was available after 6 days of iced storage, no marked increases in DMA and formaldehyde were observed. It seems that TMAOase was possibly denatured to a high extent and could not catalyze the breakdown of TMAO to DMA and formaldehyde.

3.4. Changes in protein solubility

A decrease in solubility of protein in all washed minces from two species was observed throughout the storage in ice for 12 days (Fig. 6). The samples with a higher amount of TMAOase added exhibited greater decrease in solubility ($P<0.05$) than the control or those without or with a lower amount of enzyme added. Presumably formaldehyde formed induced the aggregation of protein, resulting in the loss in solubility. Higher contents of formaldehyde, in samples treated with higher amounts of TMAOase, possibly caused the aggregation of protein to a larger extent, leading to a larger decrease in solubility. Formaldehyde was found to increase the denaturation of protein during frozen storage, as measured by the loss in solubility (Ang & Hultin, 1989). Careche and Li-Chan (1997) found

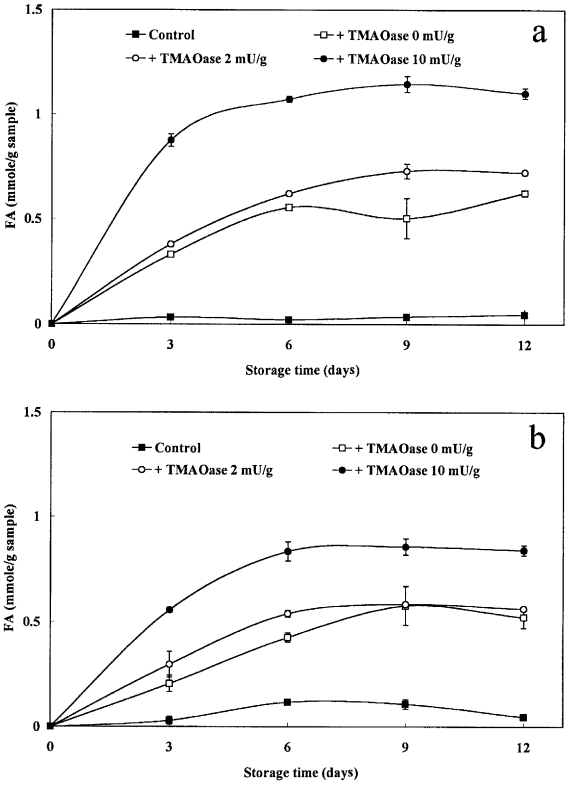

Fig. 4. Changes of formaldehyde content in washed mince from red seabream (a) and skipjack (b) with different levels of lizardfish kidney TMAOase added during iced storage for 12 days. Washed mince contained TMAO and cofactors consisting of $FeCl_2$, cysteine and ascorbate. Bars indicate the standard deviations from triplicate determinations.

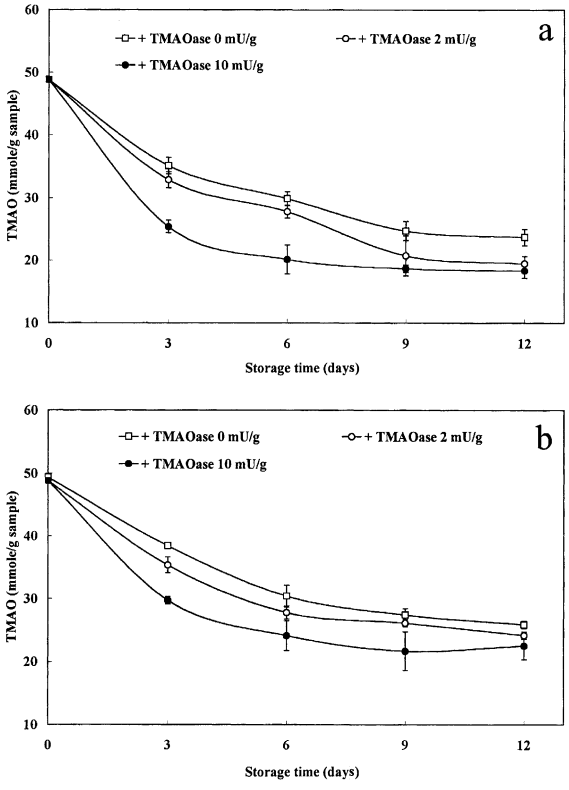

Fig. 5. Changes in TMAO content of washed mince from red seabream (a) and skipjack (b) with different levels of lizardfish kidney TMAOase added during iced storage for 12 days. Washed mince contained TMAO and cofactors, consisting of $FeCl_2$, cysteine and ascorbate. Bars indicate the standard deviations from triplicate determinations.

changes in cod myosin structure, especially exposure of the hydrophobic portion, after formaldehyde addition, leading to the formation of covalent cross-links responsible for insolubility of proteins. For the control, to which cofactors, including $FeCl_2$, cysteine and ascorbate were added, a continuous loss of solubility was observed during the storage. Denaturation (and aggregation) of heavy chain myosin, caused by the oxidation of sulfhydryl groups, occurs during iced storage of carp actomyosin (Sompongse, Itoh, & Obatake, 1996). In the presence of $FeCl_2$, lipid oxidation is possibly accelerated. Oxidation products cause decrease of ATPase activity and cross-linking of heavy chain myosin (Ooizumi & Kawasaki, 1997).

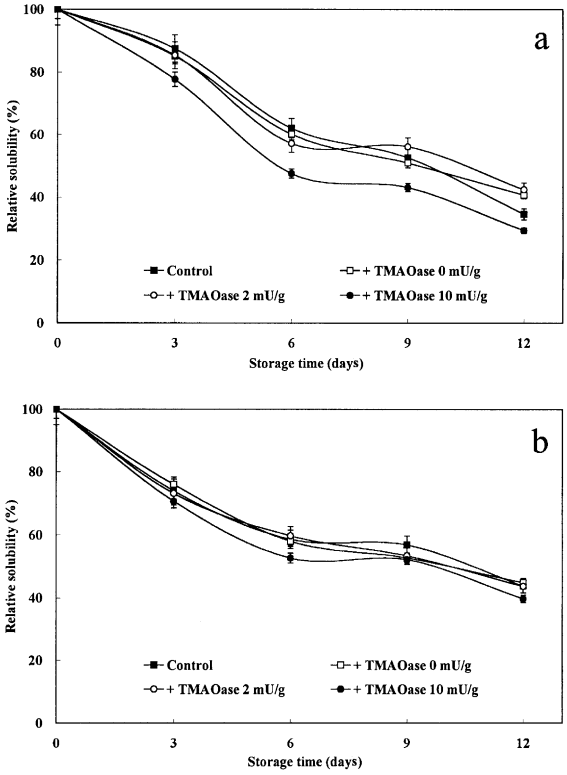

Fig. 6. Changes in protein solubility of washed mince from red seabream (a) and skipjack (b) with different levels of lizardfish kidney TMAOase added during iced storage for 12 days. Washed mince contained TMAO and cofactors, consisting of $FeCl_2$, cysteine and ascorbate. Bars indicate the standard deviation from triplicate determinations.

From the results, the decrease in solubility of all samples was lower in red seabream ($P<0.05$), than in skipjack in the first 3 days, although DMA, used to indicate total formaldehyde, was found to be higher in the former. This was possibly due to the differences in protein conformation as well as the amino acid composition. As a consequence, cross-linking induced by formaldehyde occurred differently. However, after 3 days of storage, red seabream washed mince, with TMAOase added at 10 mU/g, had a much lower solubility than skipjack with the same level of TMAOase added. This was possibly due to the conformational changes of the red seabream proteins in a fashion, which favoured the cross-linking induced by formaldehyde. Therefore, differences in the solubilities of proteins between the two species, in the presence of TMAOase, suggest that conformation of proteins is another factor determining the aggregation of proteins induced by formaldehyde, in addition to the amount of formaldehyde formed.

3.5. Changes in protein pattern

Similar protein patterns of the control and samples with different levels of TMAOase added were observed (Fig. 7). The occurrence of a high molecular weight protein band with a molecular mass of 245 kDa was noticeable as the storage time increased. Since this band was observed in both control and samples under reducing conditions, the protein cross-links were possibly formed through non-disulfide covalent bonding.

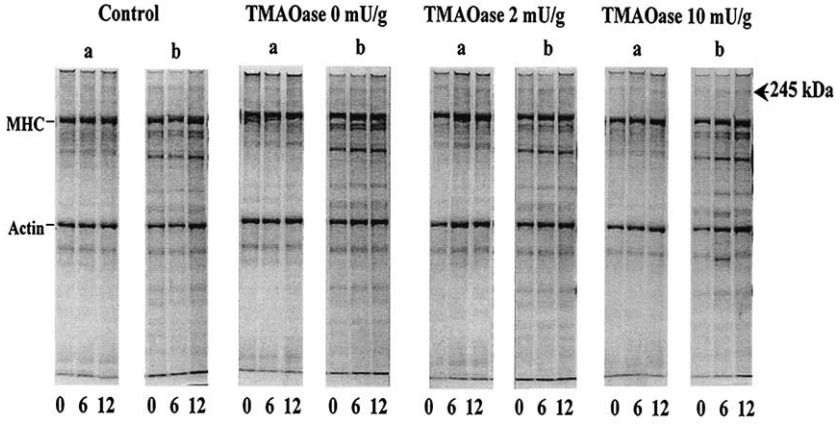

Fig. 7. Changes of protein patterns in washed mince from red seabream (a) and skipjack (b) with different levels of lizardfish kidney TMAOase added during iced storage for 12 days. Washed mince contained TMAO and cofactors, consisting of $FeCl_2$, cysteine and ascorbate. Numbers designate storage time in ice (days).

Although a pronounced effect of TMAOase added on the formation of this band was not evident, it was likely that samples, with 10 mU TMAOase/g added, showed the highest band intensity. Due to the observed loss in solubility of protein during iced storage, especially in those with higher amounts of TMAOase added (Fig. 6), it was presumed that formaldehyde induced the changes in protein conformation in a way, which favours the aggregation via non-covalent bonds as well as disulfide bonds, leading to lower solubility. Cross-linked protein with a molecular weight of 280 kDa was formed during frozen storage of gadoids (Ragnarsson & Regenstein, 1989). Lim and Haard (1984) also found non-disulfide covalently bonded polypeptides with molecular weight of about 500 and greater than 1000 kDa in frozen Greenland halibut mince. The cross-linked proteins resulted in a loss of solubility.

Therefore, TMAOase from lizardfish kidney was shown to be an important factor inducing changes in protein functionality, especially a loss of solubility. Accordingly, the poorer functional properties, such as gelation, were attributed to fish flesh or mince contaminated with this enzyme. Further study of the effects of protein conformation and other factors on the changes of protein functionality, caused by TMAOase, needs to be under taken. Purification (and characterization) of TMAOase from lizardfish kidney is presently being done.

4. Conclusion

Lizardfish kidney was shown to be an important source of TMAOase. TMAOase from lizardfish kidney effectively induced the formation of DMA and formaldehyde in the presence of TMAO and cofactors. This led to increased protein cross-linking with a concomitant loss in solubility. Changes in red seabream and skipjack muscle protein, induced by lizardfish TMAOase, varied, indicating different susceptibilities to formaldehyde, produced by TMAOase, among fish species.

Acknowledgements

Authors would like to express their sincere thanks to the Japanese Society for the Promotion of Science (JSPS) for the financial support.

References

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

Benjakul, S., Visessanguan, W., & Tueksuban, J. (2003). Changes in physico-chemical properties and gel-forming ability of lizardfish (*Saurida tumbil*) during iced storage. *Food Chemistry*, *80*, 535–544.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microorganism quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, *72*, 248–254.

Careche, M., & Li-Chan, E. C. Y. (1997). Structural changes in cod myosin after modification with formaldehyde or frozen storage. *Journal of Food Science*, *62*, 717–723.

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

Dyer, W. J., & Mounsey, Y. A. (1945). Amines in fish muscle. II. Development of trimethylamine and other amines. *Journal of the Fisheries Research Board of Canada*, *6*, 359–367.

Gill, T. A., & Paulson, A. T. (1982). Localization, characterization and partial purification of TMAO-ase. *Comparative Biochemistry Physiology*, *71B*, 49–56.

Harada, K. (1975). Studies on enzymes forming formaldehyde and dimethylamine in fish and shellfish. *Journal of Shimonoseki University of Fisheries*, *23*, 163–241.

Herbard, C. E., Flick, G. J., & Martin, R. E. (1982). Occurrence and significance of trimethylamine and its derivatives in fish and shellfish. In R. W. Martin, G. J. Flick, C. E. Herbard, & D. R. Ward (Eds.), *Chemistry and biochemistry of marine food products* (pp. 149–304). Westport, CT: AVI Publishing.

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

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

Landolt, L. A., & Hultin, H. O. (1982). Inhibition of dimethylamine formation in frozen red hake muscle after removal of trimethylamine oxide and soluble proteins. *Journal of Food Biochemistry*, *6*, 111–125.

Lim, K. H., & Haard, N. F. (1984). Protein insolubilzation in frozen Greenland halibut (*Reinhardtius hippoglossoides*). *Journal of Food Biochemistry*, *8*, 163–188.

Lundstrom, R. C., Correia, F. F., & Wilhelm, K. A. (1982a). Dimethylamine production in fresh red hake (*Urophycis chuss*): the effect of packaging material oxygen permeability and cellular damage. *Journal of Food Biochemistry*, *6*, 229–241.

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

Nash, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochemical Journal*, *55*, 416–421.

Ooizumi, T., & Kawasaki, K. (1997). Comparison of reactivity of several fish myofibrils with peroxides lipid. *Fisheries Science*, *63*, 615–618.

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

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

Parkin, K. L., & Hultin, H. O. (1986). Characterization of trimethylamine-N-oxide (TMAO) demethylase activity from fish muscle microsomes. *Journal of Biochemistry*, *100*, 77–86.

Ragnarsson, K., & Regenstein, J. M. (1989). Changes in electrophoretic patterns of gadoid and non-gadoid fish muscle during frozen storage. *Journal of Food Science*, *54*, 819–823.

Rehbein, H., & Schreiber, W. (1984). TMAO-ase activity in tissue of

fish species from the Northeast Atlantic. *Comparative Biochemistry Physiology*, *79B*, 447–452.

Sompongse, W., Itoh, Y., & Obatake, A. (1996). Role of SH_a in the polymerization of myosin heavy chain during ice storage of carp actomyosin. *Fisheries Science*, *62*, 110–113.

Steel, R. G. J., & Torrie, J. H. (1980). *Principles and procedures of statistics; a biometrical approach* (2nd ed.). New York: McGraw-Hill.

Yamagata, M., Horimoto, K., & Nagaoka, C. (1969). Assessment of green tuna: determining trimethylamine oxide and its distribution in tuna muscle. *Journal of Food Science*, *34*, 156–159.