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Raman spectroscopic analysis and rheological measurements on natural actomyosin from haddock (*Melanogrammus aeglefinus*) during refrigerated (4 °C) and frozen (−10 °C) storage in the presence of trimethylamine-*N*-oxide demethylase from kidney of lizardfish (*Saurida tumbil*)

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Abstract

Changes in viscoelasticity and structure of haddock natural actomyosin (NAM) treated with partially purified trimethylamine-*N*-oxide demethylase (TMAOase) in the presence of cofactors ($FeCl_2$, ascorbate and cysteine), after refrigerated storage (4 °C) for 15 days or after frozen storage (−10 °C) for eight weeks, were elucidated using FT-Raman spectroscopy and dynamic viscoelastic measurement. Greater increases in the final storage modulus (G') and loss modulus (G''), reflecting protein aggregation, were observed in the simulated NAM systems, containing NAM, trimethylamine oxide (TMAO) and cofactors, stored at −10 °C, compared to those stored at 4 °C, particularly in the system with a higher concentration of TMAOase ($p < 0.05$). Raman spectroscopy revealed that amide I and amide III bands of NAM were affected by TMAOase added as well as by storage temperature. The decrease in the CH_2 bending region near 1450 cm^{-1}, in the presence of TMAOase upon storage, suggested an increase in hydrophobic interactions of aliphatic residues. Changes in a doublet near 830 and 850 cm^{-1} indicated an involvement of tyrosine residues as hydrogen bond donors in the system containing TMAOase after storage at both temperatures. The systems stored at −10 °C generally showed greater structural alteration than those kept at 4 °C, especially in the presence of 15 units of TMAOase/g. Therefore, TMAOase played an important role in the structural alteration and aggregation of haddock muscle proteins, mainly by the induction of formaldehyde formation.

Keywords: Rheological property; Structure; Haddock; Actomyosin; TMAOase; Lizardfish

1. Introduction

Trimethylamine-*N*-oxide demethylase (TMAOase) is an enzyme found in the muscle and viscera of gadoids. It catalyzes the breakdown of trimethylamine oxide (TMAO) to equimolar quantities of formaldehyde (FA) and dimethylamine (DMA) (Hultin, 1992). TMAOase is present in very large quantities in the kidney and spleen of gadoid fish (Benjakul, Visessanguan, & Tanaka, 2004; Hultin, 1992). Differences in the rate of DMA and FA production depend on the amount of substrate, the amount of dark muscle, cofactors, temperature and the period of frozen storage (Shenouda, 1980). The activity of TMAOase has been considered to be associated with the loss in quality of some

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gadoid species during frozen storage. The interaction of produced FA with muscle proteins is a major cause of texture deterioration and decreased acceptability (Sotelo, Pineiro, & Pêrez-Martín, 1995). FA accelerated the formation of high-molecular weight aggregates in myosin isolated from cod (Ang & Hultin, 1989) and in hake natural actomyosin (NAM) (Del Mazo, Huidobro, Torrejon, Tejada, & Careche, 1994). Careche and Li-Chen (1997) reported that the addition of FA causes changes in the secondary structure of myosin, induces the exposure of the hydrophobic aliphatic groups, and eventually leads to the formation of covalent cross-links. Modification of amino acid side chains by FA is accompanied by alterations in solubility and ATPase activity (Ang & Hultin, 1989).

Lizardfish (*Saurida* spp.) has become a potential commercial species in Thailand and is used mostly for surimi production. This is because of its high gel-forming ability and whiteness (Benjakul, Visessanguan, & Tanaka, 2003). However, the gel-forming ability of this fish decreased rapidly during post-harvest handling. This is associated with proteolysis as well as the formation of FA (Benjakul et al., 2003). Additionally, FA production is also considered to be one of the major causes of the deterioration of lizardfish during the frozen storage (Benjakul et al., 2003; Leelapongwattana, Benjakul, Visessanguan, & Howell, 2005a, Leelapongwattana, Benjakul, Visessanguan, & Howell, 2005b). Benjakul et al. (2004) reported that lizardfish kidney contained the highest TMAOase activity, followed by spleen, bile sac, intestine, and liver, respectively. Therefore, 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 the formation of FA, lipid oxidation and storage temperature contributed to the quality changes of fish (Badii & Howell, 2002; Matsumoto, 1980; Shenouda, 1980). Though the effect of either naturally produced FA or added FA on protein aggregation and muscle toughening has previously been reported (Careche & Li-Chen, 1997; Leelapongwattana et al., 2005a, 2005b), no information regarding the structural changes of muscle proteins directly caused by TMAOase has been reported. Although haddock is a gadoid fish, it produces a negligible amount of FA in frozen fillets (Badii & Howell, 2001). Thus, haddock was used for this study in order to minimize the intrinsic factor involved in the formation of FA. Therefore, the purpose of this study was to examine whether partially purified lizardfish kidney TMAOase affected the rheological characteristics as well as the structure of NAM after refrigerated (4 °C) and frozen storage (−10 °C).

2. Materials and methods

2.1. Chemicals

All chemicals for TMAOase activity assay 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). 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 procured from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Partial purification of TMAOase from lizardfish kidney

2.2.1. General

Lizardfish (*Saurida tumbil*) of an average weight of 200–250 g, 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 a 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 the kidney was removed. Kidney specimens were kept at − 80 °C until used.

Frozen kidney specimens were thawed using running water (26–28 °C) until the core temperature reached −2 °C to 0 °C. The samples were cut into pieces with a thickness of 1–1.5 cm. Samples were ground into powder in 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 paper. 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 chromatographed on a 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 0–0.45 M NaCl in starting buffer at a flow rate of 1 ml/min. Fractions of 2 ml were collected and those with TMAOase activity were pooled and used for further studies. The partially purified TMAOase from lizardfish kidney was kept in dry ice and transported by aeroplane to the University of Surrey, UK within 12 h.

2.2.2. TMAOase assay

TMAOase activity was assayed using TMAO as a substrate in the presence of selected cofactors (Kimura, Seki, & Kimura, 2000). To 1.25 ml of the assay mixture (24 mM tris-acetate containing 24 mM TMAO, 2.4 mM cysteine, 2.4 mM ascorbate, and 0.24 mM $FeCl_2$, pH 7.0) pre-incubated at 45 °C for 5 min, 0.25 ml of 500-fold diluted enzyme solution was added to initiate the reaction. The reaction was performed at 45 °C for 10 min and 0.5 ml of 10% trichloroacetic acid was added to terminate the reaction. The reaction mixture was then centrifuged at 8000*g* for 15 min and the supernatant was subjected to DMA determination as described by Benjakul et al. (2004). One unit of TMAOase was defined as the activity, which released 1 nmole DMA per min under the assay condition.

2.2.3. Protein determination

Protein concentration was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard. Protein concentration in fractions obtained from ion exchange chromatography was determined according to the method of Bradford (1976).

2.3. Preparation of natural actomyosin

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

Natural actomyosin (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, Seymour, Morrissey, and An (1997) with some modifications. The ordinary muscle (50 g) was homogenized in an Omni mixer homogenizer (Waterbury, CT, USA) for 1 min with six volumes of chilled 20 mM tris-maleate buffer containing 0.05 M NaCl, pH 7.0. The homogenate was centrifuged at 5000*g* for 30 min at 4 °C. After the supernatant was decanted, the pellet was homogenized for 1 min with six 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 the mixture was kept overnight. The aqueous layer was decanted and the residue at the bottom was centrifuged at 5000*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'.

2.4. Effect of partially purified TMAOase on rheological and structural changes of haddock NAM

Partially purified TMAOase, at levels of 5 and 15 units/g NAM, was added to the simulated NAM 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 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 15 days and eight weeks, respectively. After the designated storage time, the samples were subjected to dynamic viscoelastic measurement and FT-Raman spectroscopic analysis.

2.5. Dynamic viscoelastic measurement

Small deformation rheological analysis was undertaken using a Rheometrics Constant Stress 200 rheometer (Rheometrics, Leatherhead, Surrey, UK), using a temperature sweep from 20 °C to 90 °C and cooling back to 20 °C at a rate of 2 °C/min. The applied stress was 1 Pa to keep the oscillatory strain at about 1%, sufficiently low to ensure that the measurements taken were within the linear viscoelastic region (Hamann, 1991). The frequency of oscillation of 1 rad was chosen, in which sufficient data were obtained without compromising the measurement of entanglements. A 40 mm parallel plate geometry with a gap of 1 mm was used and the sample was surrounded by silicone oil to prevent evaporation of solvent. The storage modulus (G') and loss modulus (G'') were recorded (Badii & Howell, 2002a). Samples were tested in triplicate.

2.6. FT-Raman spectroscopic analysis

Each sample was examined in 7 ml glass container (FBG-Anchor, Cricklewood, London) on a Perkin–Elmer System 2000 FT-Raman spectrophotometer (Beaconsfield, Bucks, England). An Nd:YAG laser, which emits at a wavelength of 1064 nm, was used as the excitation source. Frequency calibration of the instrument was performed using the sulfur line at 217 cm^{-1}. Triplicate sets of samples were prepared and analyzed on three different occasions using laser power of 1785 mW. The spectra were an average of 64 scans, which were baseline corrected, smoothed, and normalized to the intensity of the band at

$1004 \pm 1\ cm^{-1}$, which was reported to arise from vibrational motions of phenylalanine residues and to be insensitive to micro-environment (Li-Chan, Nakai, & Hirotsuka, 1994). This stability enabled its use as an internal standard for normalization of the relative Raman scattering intensity for comparison of different samples. The recorded spectra were analyzed using Gram 32 (Galactic Industries Corp., Salem, NH, USA). Assignments of the bands in the spectra to protein vibrational modes were made based on the literature (Badii & Howell, 2002b; Careche, Herrero, Rodriguez-Casado, Del Mazo, & Carmona, 1999; Howell & Li-Chan, 1996; Li-Chan et al., 1994).

2.7. Statistical analysis

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

3. Results and discussion

3.1. Rheological behaviour of haddock NAM

Dynamic viscoelastic properties as a function of temperature of fresh haddock NAM are depicted in Fig. 1. A decrease in storage modulus (G') of NAM was observed when heated up to 36 °C. Thereafter, G' markedly increased and reached a maximum at 45 °C, indicating denaturation of myosin. Gel network formation of haddock NAM was initiated at 36 °C and three-dimensional structure was fully developed at 45 °C. Heating of NAM at temperatures below 40 °C generally resulted in dissociation of some myofibrillar components. For example, tropomyosin was dissociated from the F-actin in its super helix structure (Ziegler & Acton, 1984). Although myosin is present in a complex form with actin and other proteins, it is responsible for the gel elasticity development of

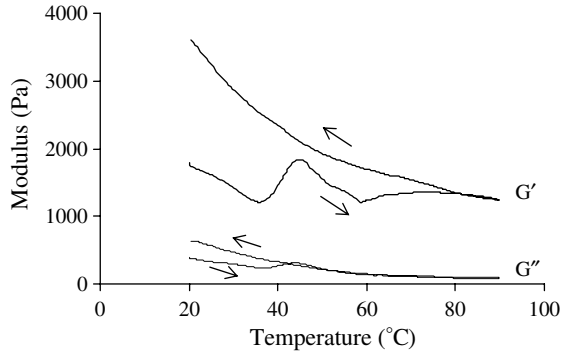

Fig. 1. Rheogram of fresh haddock NAM heated linearly from 20 °C to 90 °C at the rate of 2 °C/min, followed by cooling to 20 °C. Arrow sign indicates the direction of heating or cooling.

NAM during thermal gelation (Sano, Noguchi, Tsuchiya, & Matsumoto, 1988). It is postulated that partial unfolding of the protein structure initiated by the dissociation of myosin light chain subunits from the heavy chains may lead to an interfilamental association of myosin to form a three-dimensional structure (Benjakul, Visessanguan, Ishizaki, & Tanaka, 2001). Upon further heating, G' decreased rapidly up to 58.9 °C, followed by a gradual increase until 90 °C. The decrease in G' was possibly caused by dissociation of actin-myosin complex and the denaturation of myosin tail (Egelandsdal, Fretheim, & Samejima, 1986). It is assumed that helix-coil transformation can lead to a large increase in fluidity of the semi-gel and may disrupt some of protein network already formed (Xiong, 1997). Furthermore, the increase in G' with heating from 58.9 °C to 90 °C was probably attributable to the formation of irreversible gel networks (Egelandsdal et al., 1986). This final step was referred to as 'gel strengthening' and it was ascribed to both an increase in the numbers of cross-links between protein aggregates and a deposition of additional denatured proteins in the existing protein networks to strengthen the gel matrix (Xiong, 1997). For the loss modulus (G''), continuous decrease was observed when NAM was heated between 20 °C and 35.8 °C. Thereafter, a slight increase was obtained up to 43.7 °C, followed by a gradual decrease up to 90 °C.

Subsequent cooling from 90 °C to 20 °C resulted in a much higher increase in both final G' and G'' (Fig. 1). The marked increase in both G' and G'' during the cooling period was an indication of the development of rigidity of the gels. Increase in gel rigidity during cooling was attributed to the reorientation and rearrangement of denatured polypeptides (Howell & Lawrie, 1985). Montejano, Hamann, and Lanier (1983) proposed a formation of stable network by protein aggregation through hydrophobic interactions after cooling. Changes in G' have been used to monitor gelation of muscle proteins (Benjakul et al., 2001; Venugopal et al., 2002). G' is a measure of the energy recovered per cycle of sinusoidal shear deformation and its increase indicates rigidity of the sample associated with the formation of elastic gel structure (Dileep, Shamasundar, Binsi, Badii, & Howell, 2005). G'' is a measure of the energy dissipated or lost as heat per cycle of sinusoidal strain when different systems are compared at the same strain amplitude (Dileep et al., 2005). G'' indicates the extent of viscous element in the sample. In the present study, G'' values were lower than G' values, indicating the viscoelastic nature of the gel (Dileep et al., 2005; Saeed & Howell, 2004).

3.2. Effect of TMAOase on rheological behaviour of haddock NAM after refrigerated and frozen storage

Changes in G' and G'' of the control sample (without TMAO and TMAOase) stored at 4 °C for 15 days (Fig. 2) were similar to those observed in NAM extracted from fresh haddock (Fig. 1). A peak was obtained at the temperature range of 36–56 °C. However, the greater final G' and G'' after

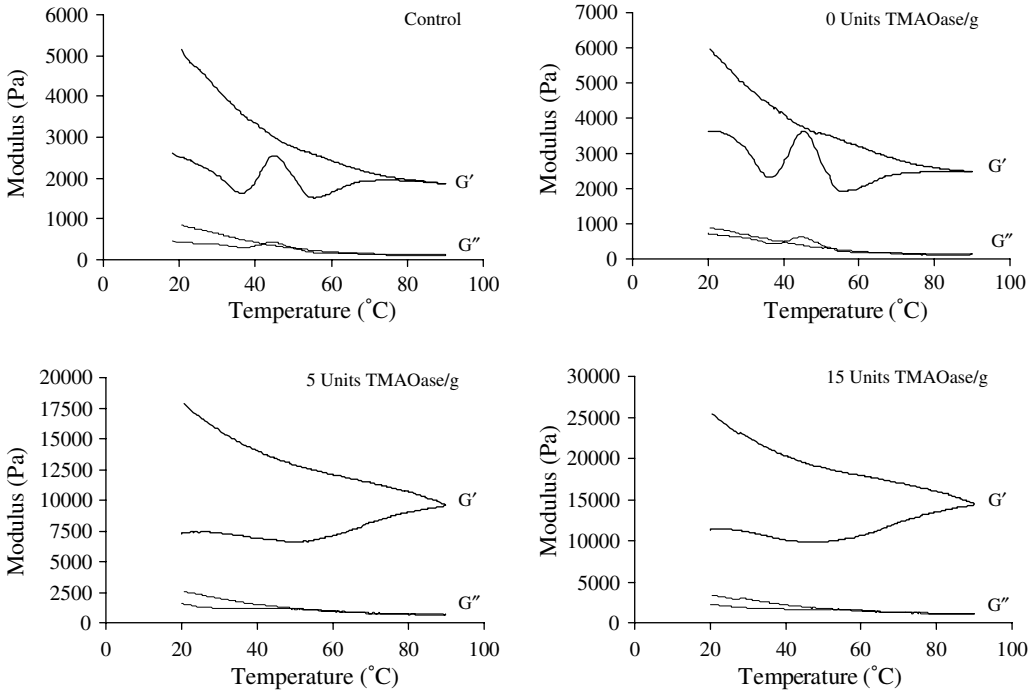

Fig. 2. Changes in storage modulus (G') and loss modulus (G'') of simulated haddock NAM system containing different levels of TMAOase from lizardfish kidney after storage at 4 °C for 15 days. System contained 30 g NAM, 50 mM TMAO, and cofactors (1 mM $FeCl_2$, 5 mM cysteine and 5 mM ascorbate).

cooling were found in the sample stored at 4 °C for 15 days ($G' = 5176$ Pa, $G'' = 901$ Pa), compared to NAM prepared from fresh haddock ($G' = 3114$ Pa, $G'' = 589$ Pa) (Table 1). For the simulated NAM system containing no TMAOase, similar changes in G' and G'' were also observed (Fig. 2), but the magnitude was higher than the control and NAM from fresh haddock (Fig. 1; Table 1). On the other hand, no peak was observed in the system containing TMAOase (at both levels). The greater magnitudes of G' and G'' was found in the simulated NAM system having the higher level of TMAOase. Leelapongwattana, Benjakul, Visessanguan, and Howell (in press) found that the addition of partially purified TMAOase from lizardfish kidney to haddock NAM in the presence of cofactors accelerated formation of FA throughout storage, either at 4 °C for 15 days or −10 °C for eight weeks. Therefore, added TMAOase most likely contributed to the alteration of the native structure of myosin and actin as a result of FA formed. For the systems containing TMAOase, a slight decrease in G' values was observed during heating up to 50–51 °C, followed by a gradual increase up to 90 °C. For the simulated NAM system stored at −10 °C for eight weeks (Fig. 3), a similar pattern of G' and G'' development was noticeable, in comparison with those kept at 4 °C for 15 days (Fig. 2). However, a smaller peak was found at 36–56 °C. In addition, higher initial G' values (at 20 °C) were observed in the systems stored at −10 °C, compared to those stored at 4 °C. The result suggests substantial formation of aggregates at −10 °C. With a longer time, even at lower temperature, FA formation induced by added TMAOase took place. Benjakul et al. (2003) reported that TMAOase from lizardfish kidney showed very low activation energy.

The system containing no TMAOase had low G' and G'' values in the temperature range, where the peak was observed, when compared with the control. This might be due to the presence of TMAOase associated with

Table 1
Storage modulus (G') and loss modulus (G'') after heating to 90 °C and cooling from 90 °C to 20 °C haddock NAM and simulated NAM system containing different levels of TMAOase from lizardfish kidney after storage at 4 °C and −10 °C[a,b]

Samples	4 °C, 15 days		−10 °C, eight weeks	
	G' (Pa)	G'' (Pa)	G' (Pa)	G'' (Pa)
Fresh NAM	3114.2 ± 418 d	588.5 ± 46 e	3114.2 ± 418 e	588.5 ± 46c
Control	5175.6 ± 591c	901.0 ± 121d	37,426.3 ± 2226d	5927.1 ± 247b
0 Units TMAOase/g	5900.0 ± 130c	1182.5 ± 4c	42,965.3 ± 1674c	6328.0 ± 515b
5 Units TMAOase/g	17,829.3 ± 243b	2579.3 ± 33b	53,601.3 ± 2384b	8286.7 ± 1000a
15 Units TMAOase/g	25,043.3 ± 1574a	3361.7 ± 182a	58,083.3 ± 601a	8828.3 ± 160a

[a] Values are given as means ± SD from triplicate determinations.
[b] Different letters in the same column indicate significant differences ($p < 0.05$).

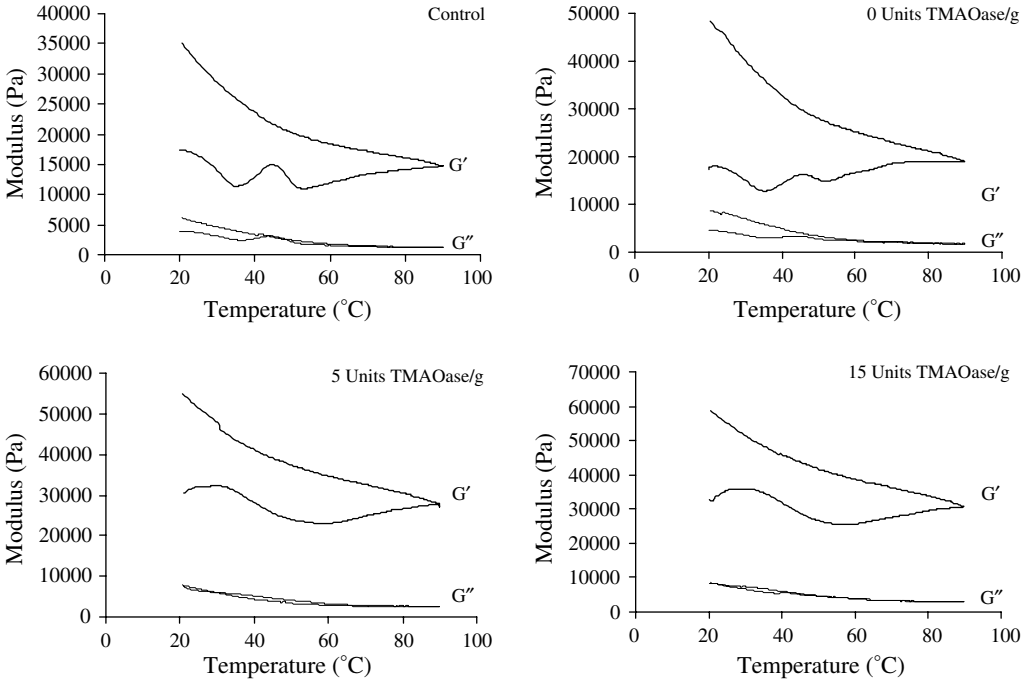

Fig. 3. Changes in storage modulus (G') and loss modulus (G'') of simulated haddock NAM system containing different levels of TMAOase from lizardfish kidney after storage at −10 °C for eight weeks. System contained 30 g NAM, 50 mM TMAO, and cofactors (1 mM $FeCl_2$, 5 mM cysteine and 5 mM ascorbate).

myofibrils, as reported for walleye Pollack muscle by Kimura et al. (2000). As a result, enough FA may be formed to cross-link with nucleophilic amino acids and alter the denaturation properties of myosin and actin. This was indicated by the disappearance of the peak at 36–56 °C.

It was noted that final G' and G'' values of simulated NAM systems containing increasing concentrations of TMAOase, which were stored at −10 °C, were higher than those of systems without TMAOase ($p < 0.05$) (Table 1). At the same level of TMAOase added, the systems kept at −10 °C showed higher final G' and G'', than did those stored at 4 °C. Apart from the aggregation via covalent bonds mediated by FA, other bonds, involving disulfide bonds, hydrogen bonds, hydrophobic and electrostatic interactions, also played an important role in cross-linking of protein molecules during extended frozen storage. This might result in the enhanced aggregation of proteins, as indicated by the higher final G' and G'' values. An increase in G' values of raw materials, such as fish fillets, is related to protein–protein and protein–lipid interactions, causing undesirable tough products (Badii & Howell, 2002; Dileep et al., 2005).

3.3. Raman spectra of haddock NAM before and after refrigerated or frozen storage

Raman spectra in the range of 800–1800 cm^{-1} of fresh haddock NAM, haddock NAM stored at 4 °C for 15 days, or stored at −10 °C for eight weeks are shown in Fig. 4a. The most prominent band, centred near 1654 cm^{-1}, corresponding to the amide I region was found in all samples. Amide I vibrational mode involves mainly C=O stretching and, to lesser degrees, C—N stretching, C_α—C—N bending, and N—H in-plane bending of peptide groups (Careche et al., 1999). Generally, there is a correlation between the frequencies of the amide I band and the amount as well as types of protein backbone conformation (Tu, 1982). Amide I band consists of overlapped band components falling in the 1650–1658, 1665–1680, and 1660–1665 cm^{-1} ranges, which are attributable to α-helices, β-sheets, and random coil structures, respectively (Ngarize, Herman, Adams, & Howell, 2004; Tu, 1982). In this study, the amide I band was centred at 1654 cm^{-1}, indicating a predominance of α-helical structure in haddock NAM. After storage at 4 °C and −10 °C, the peak maximum of the amide I region of haddock NAM shifted slightly to higher frequencies (Fig. 4a), indicating β-sheet formation. The amide III band results primarily from the C—N stretching and N—H in-plane bending vibrations of the peptide bond. Proteins with high contents of α-helical structure have a weak band, located in a broad region from 1260 to 1300 cm^{-1}. β-Sheet structure usually leads to a more intense band near 1238–1245 cm^{-1} while random coil structure appears near 1250 cm^{-1} (Bouraoui, Nakai, & Li-Chan, 1997). A broadening of the amide III band and the shift towards lower frequencies were found in stored NAM, particularly at −10 °C (Fig. 4a). This change was more pronounced with NAM treated with TMAOase. On the basis of the above structurally characteristic frequency ranges, those changes most likely suggested a decrease in α-helical structure, especially in the tail part of the myosin heavy chain.

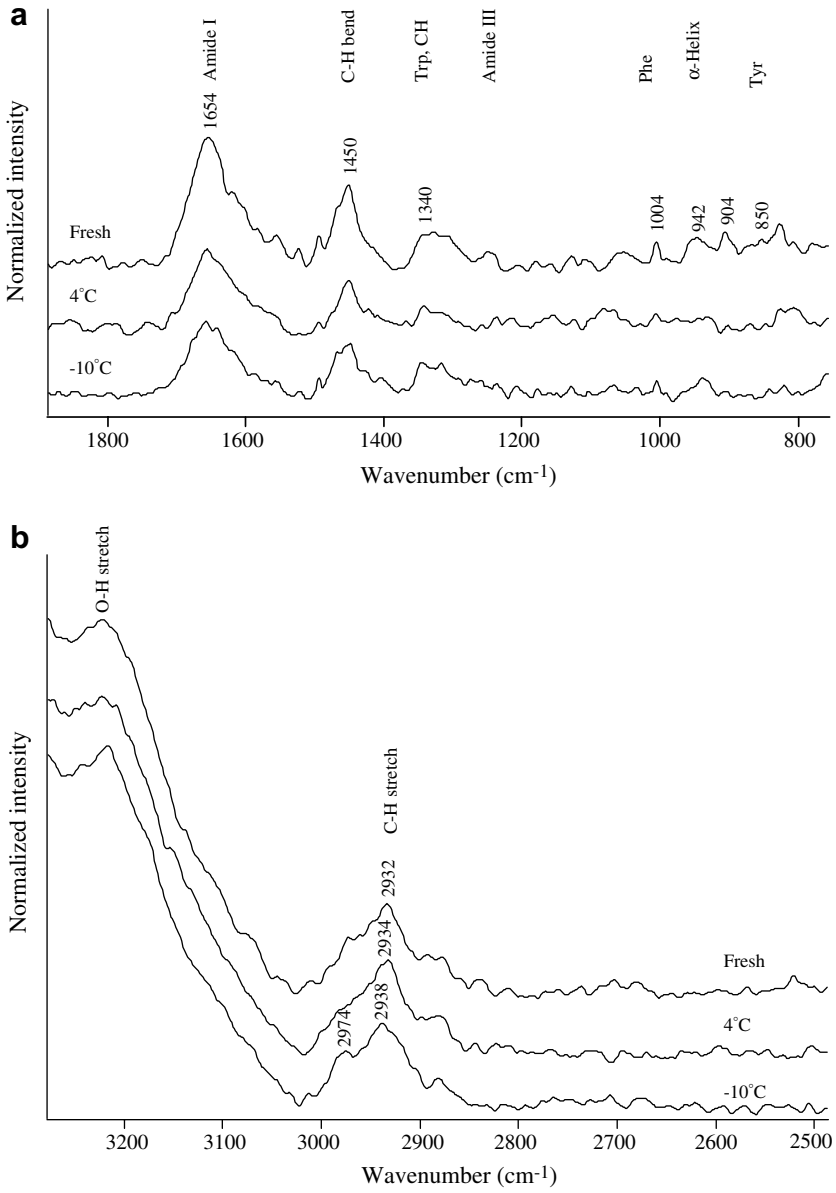

Fig. 4. Raman spectra in the 800–1800 cm^{-1} (a) and 2500–3400 cm^{-1}(b) wavenumber regions of haddock NAM, after storage at 4 °C for 15 days or at −10 °C for eight weeks.

The C—C stretching vibrations near 900 and 940 cm^{-1} are characteristic of α-helices and β-sheets, respectively. Generally, gradual loss of these structures leads to broadening and weakening in intensity (Tu, 1982). From the result, the marked decrease in 900 cm^{-1} band (Fresh = 0.95, 4 °C = 0.35, −10 °C = 0.15) and the broadening of the 940 cm^{-1} band were found in NAM stored at 4 °C and −10 °C ($p < 0.05$) (Fig. 4a). This suggested changes in both α-helix and β-sheet conformation. These changes could be due to an unfolding of helical structures, followed by the formation of sheet structures possibly through intermolecular interactions between exposed hydrophobic residues (Bouraoui et al., 1997). Generally, greater changes of these regions were observed in samples stored at −10 °C, than in those stored at 4 °C ($p < 0.05$).

The doublet bands located near 830 and 850 cm^{-1} can be useful in monitoring the microenvironment around tyrosyl residues (Li-Chan et al., 1994). This doublet band is assigned to vibrations of the para-substituted benzene ring of tyrosine residues which are affected by the environment and the involvement of the phenolic hydroxyl group in hydrogen bonding. In the case of tyrosine residues, which are exposed to the aqueous or polar environment or which act as simultaneous acceptor and donor of moderate to weak hydrogen bonds, the intensity ratio of the doublet band ($I_{850/830}$) usually ranges from 0.90 to 1.45, but can be as high as 2.5. On the other hand, $I_{850/830}$ values for tyrosine residues, which are buried in a hydrophobic environment and which tend to act as hydrogen donors, usually range between 0.7 and 1.0, and can be as low as

Table 2
Changes in the tyrosyl doublet ($I_{850/830}$) of haddock NAM and simulated NAM system containing 15 units of TMAOase from lizardfish kidney after storage at 4 °C and −10 °C[a,b]

Samples	Tyr doublet
Fresh	1.08 ± 0.09a
4 °C, 15 Days	
Control	0.82 ± 0.09b
15 Units TMAOase/g	0.56 ± 0.03d
−10 °C, Eight weeks	
Control	0.64 ± 0.09c
15 Units TMAOase/g	0.44 ± 0.05e

[a] Values are given as means ± SD from triplicate determinations.
[b] Different letters in the same column indicate significant differences ($p < 0.05$).

0.3 in the case of extremely strong hydrogen bonding to a negative acceptor (Li-Chan et al., 1994). The intensity ratio $I_{850/830}$ of fresh haddock NAM was 1.08 (Table 2), suggesting that the tyrosine residues of this samples were mainly exposed to a polar environment. The $I_{850/830}$ ratios decreased to 0.82 and 0.64 for NAM after storage at 4 °C and −10 °C, respectively ($p < 0.05$). This indicated that some tyrosine residues became buried in a more hydrophobic environment and may involve hydrogen bonding and cross-linking after storage, either at 4 °C or − 10 °C. The vibrations at 1340 and 1450 cm^{-1}, which were assigned to CH and CH_2 bending, show changes around aliphatic residues. A decrease in the intensity of these bands indicates exposure of the residues, whereas, an increase is a sign of buriedness (Howell & Li-Chan, 1996). There was a decrease in the intensity of the 1450 cm^{-1} band (Fig. 4a) which may have resulted from hydrophobic interactions of aliphatic residues (Lippert, Tyminski, & Desmeules, 1976). Greater changes were observed in NAM stored at −10 °C (Fig. 4a), than in that kept at 4 °C ($p < 0.05$). Leelapongwattana et al. (2005a) reported an increase in surface hydrophobicity of lizardfish mince kept at −20 °C within the first four weeks of storage.

Raman spectra in the C—H stretching region (2500–3400 cm^{-1}) of NAM, before and after storage at 4 °C and −10 °C, are illustrated in Fig. 4b. A shift in peak maximum from 2932 to 2934 and 2938 cm^{-1} was noticeable

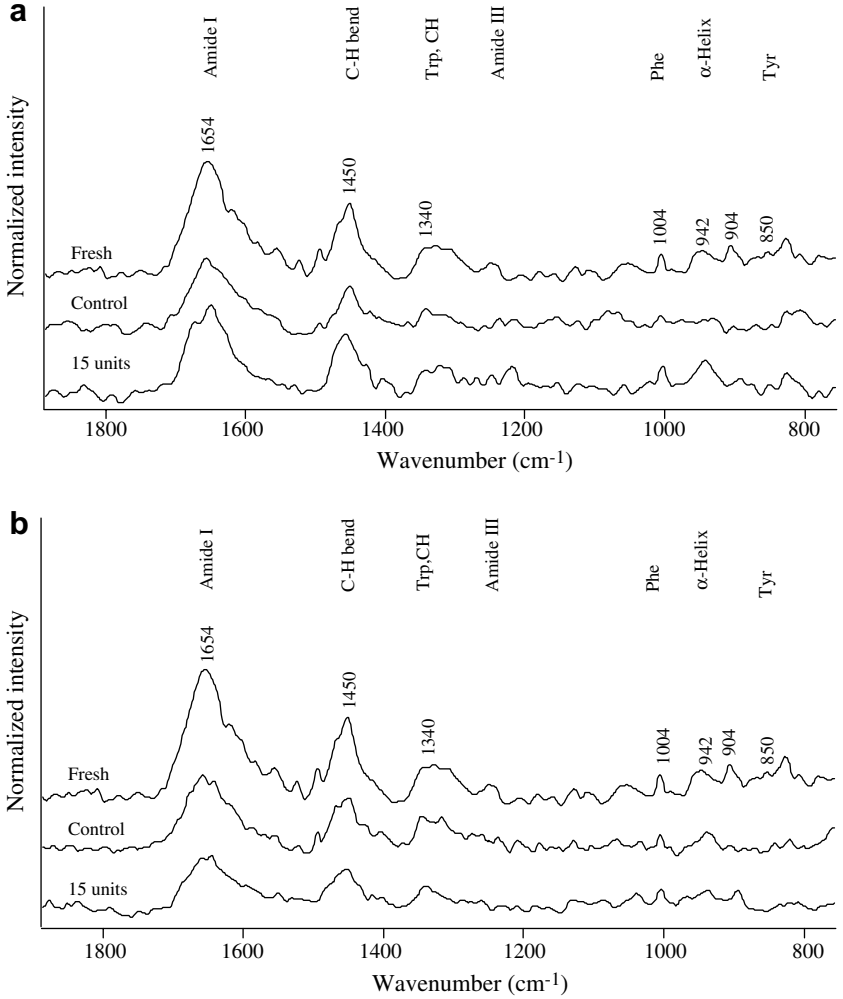

Fig. 5. Raman spectra in the 800–1800 cm^{-1} wavenumber region of haddock NAM and simulated NAM system before and after storage at 4 °C for 15 days (a) or at −10 °C for eight weeks (b) in the presence or the absence of 15 units TMAOase/g.

when NAM was stored at 4 and −10 °C, respectively. Additionally, a shoulder at 2974 cm^{-1} was found in the sample after frozen storage. A similar shift from 2931 cm^{-1} to a higher wavenumber (2938 cm^{-1}) was observed in cod NAM after frozen storage at −10 °C for 10 days (Sultanbawa & Li-Chan, 2001). This suggested the partial unfolding and increased exposure of the aliphatic residues of NAM to an aqueous environment after extended storage. The intensity of O—H stretching at 3200 cm^{-1} was lowered for NAM stored at −10 °C, in comparison with the fresh and sample stored at 4 °C. Such decreases may be related to dehydration as a result of formation of ice crystals during frozen storage. Careche et al. (1999) reported a decrease in the intensity ration of O—H/C—H stretch bands, which was correlated with increased hardness of hake fillet after frozen storage. From the above result, it was postulated that refrigerated and frozen storage resulted in structural alteration of haddock NAM. During frozen storage, the denaturation/aggregation of proteins was induced by the formation of ice crystals, resulting in dehydration, an increase in salt concentration and pH changes, followed by the removal of water through ice formation (LeBlanc, LeBlanc, & Blum, 1988). Additionally, during the extended refrigerated storage, autolysis

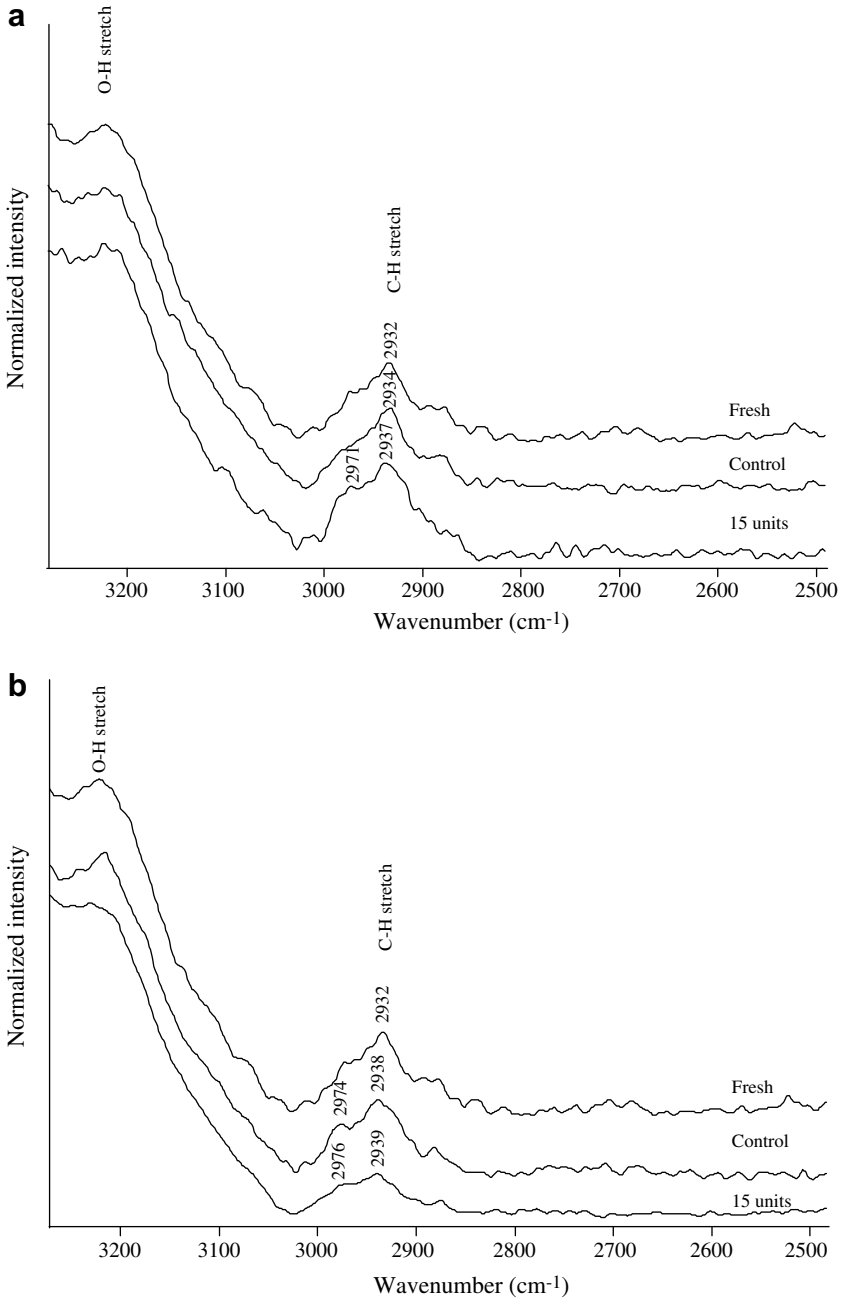

Fig. 6. Raman spectra in the 2500–3400 cm^{-1} wavenumber region of haddock NAM and simulated NAM system before and after storage at 4 °C for 15 days (a) or at −10 °C for eight weeks (b) in the presence or the absence of 15 units of TMAOase/g.

might take place, leading to the conformational changes of muscle proteins. Lipid oxidation also affected the hydrogen bonds, covalent bonds, and hydrophobic interactions in the proteins during storage (Saeed & Howell, 2002).

3.4. Effect of TMAOase on Raman spectra of NAM after refrigerated and frozen storage

Raman spectra obtained for the simulated NAM systems in the presence and the absence of TMAOase at the concentration of 15 units/g, after storage at 4 °C for 15 days and −10 °C for eight weeks, are shown in Figs. 5 and 6. The highest intensity in amide I band (1654 cm^{-1}) of fresh haddock NAM was attributed to high α-helix content of NAM (Fig. 5). The intensity maximum of this band in the systems containing TMAOase shifted from 1654 cm^{-1} to higher frequencies, 1656 and 1659 cm^{-1}, for the systems kept at 4 and −10 °C, respectively (Fig. 5a and b), probably associated with a decrease in α-helix content. The lowered α-helix content was confirmed by the lower band intensity of Raman band at 940 cm^{-1}, which originates from a coupled main chain and side chain C—C stretching mode common to α-helical proteins (Li-Chan et al., 1994; Tu, 1982). The intensity of this band decreased as a function of increased storage temperature and TMAOase concentration. A decrease and broadening of the amide III region were also found in NAM stored either at 4 °C or at −10 °C, which was more pronounced in the systems containing TMAOase. In addition to the changes in protein secondary structures, aromatic amino acid side chains also underwent changes. The intensity ratio of the tyrosine doublet decreased to a greater extent in the systems containing TMAOase compared to those without TMAOase ($p < 0.05$) (Table 2). The values were in the range of 0.4–0.6, suggesting that TMAOase increased buriedness into non-polar environment as a result of intermolecular hydrophobic interactions.

The C—H stretching region (2800–3100 cm^{-1}), corresponding to CH, CH_2, and CH_3 groups in the side-chains of amino acids, was found to be different between systems without and with added TMAOase at both temperatures of storage (Fig. 6). A slight shift to higher wavenumbers and broadening of the band, which was centred near 2932 cm^{-1}, was observed in the systems containing TMAOase (Fig. 6a and b). Arteaga (1994) reported a slight shift to higher wavenumbers of this band upon addition of aqueous solvents (water or deuterium oxide) to simple organic solvents or upon addition of urea to proteins in aqueous buffered solution. This resulted in partial unfolding and increased exposure of the aliphatic residues to an aqueous environment. Protein unfolding, with resulting exposure of methyl and methylene groups, exhibited spectral changes with a higher shift (Bouraoui et al., 1997). With TMAOase addition, the FA produced most likely induced the alteration of NAM. The greatest changes were observed in the system, containing TMAOase kept at −10 °C for eight weeks (Fig. 6b). The changes in location and the decrease in intensity of the vibration of the C—H stretching band revealed changes in the environment of aliphatic C—H groups, which might be related to hydrophobic interactions of NAM during extended storage. From the results, it can be inferred that the addition of TMAOase to haddock NAM resulted in structural changes of proteins caused by FA formation. As a consequence, extensive cross-links, induced by FA between the methyl groups of hydrophobic amino side chains, mainly contributed to protein denaturation and aggregation.

4. Conclusions

TMAOase from lizardfish kidney had a profound impact on denaturation and aggregation of haddock NAM during refrigerated and frozen storage. The interaction of hydrophobic groups and the exposure of other polar groups due to the unfolding of molecules, accompanied by changes in the secondary structure, were more pronounced in the presence of TMAOase. Such changes, presumably caused by FA formation induced by TMAOase, altered the rheological property of TMAOase-treated NAM as evidenced by the increased final G' and G'' after cooling and the disappearance of the typical peak found at 36–56 °C. Thus, the removal or inactivation of TMAOase in gadoid fish may help to maintain the muscle protein integrity and reduce toughness.

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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.

Arteaga, G. E. (1994). *Assessment of protein surface hydrophobicity by spectroscopic methods and its relation to emulsifying properties of proteins*. Ph.D. thesis, Department of Food Science, The University of British Columbia, Vancouver, B.C., Canada.

Badii, F., & Howell, N. K. (2001). A comparison of biochemical changes in cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) fillets during frozen storage. *Journal of the Science of Food and Agriculture, 82*, 87–97.

Badii, F., & Howell, N. K. (2002a). Changes in the texture and structure of cod and haddock fillets during frozen storage. *Food Hydrocolloids, 16*, 313–319.

Badii, F., & Howell, N. K. (2002b). Effect of antioxidants, citrate and cryoprotectants on protein denaturation and texture of frozen cod (*Gadus morhua*). *Journal of Agricultural and Food Chemistry, 50*, 2053–2061.

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

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

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

Benjakul, S., Visessanguan, W., Ishizaki, S., & Tanaka, M. (2001). Differences in gelation characteristics of natural actomyosin from two species of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus*. *Journal of Food Science, 66*, 1311–1318.

Bouraoui, M., Nakai, S., & Li-Chan, E. Y. C. (1997). In situ investigation of protein structure in Pacific whiting surimi and gels using Raman spectroscopy. *Food Research International, 30*, 65–72.

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

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

Careche, M., Herrero, A. M., Rodriguez-Casado, A., Del Mazo, M. L., & Carmona, P. (1999). Structural changes of hake (*Merluccius merluccius* L.) fillets: Effects of freezing and frozen storage. *Journal of Agricultural and Food Chemistry, 47*, 952–959.

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

Dileep, A. O., Shamasundar, B. A., Binsi, P. K., Badii, F., & Howell, N. K. (2005). Effect of ice storage on the physicochemical and dynamic viscoelastic properties of ribbonfish (*Trichiurus* spp.) meat. *Journal of Food Science, 70*, 537–545.

Egelandsdal, B., Fretheim, K., & Samejima, K. (1986). Dynamic rheological measurement on heat-induced myosin gels: Effect of ionic strength, protein concentration and addition of adenosine tripolyphosphate or pyrophosphate. *Journal of the Science of Food and Agriculture, 37*, 915–926.

Hamann, D. D. (1991). Rheology. A tool for understanding thermally induced protein gelation. In N. Pariss & R. Barford (Eds.), *Interactions of food proteins* (pp. 212–227). Washington, DC: American Chemical Society.

Howell, N. K., & Lawrie, R. A. (1985). Functional aspects of blood plasma proteins 4. Elucidation of the mechanism of gelation of plasma and egg albumen protein. *Journal of Food Technology, 20*, 489–504.

Howell, N. K., & Li-Chan, E. C. Y. (1996). Elucidation of interactions of lysozyme with whey proteins by Raman spectroscopy. *International Journal of Food Science and Technology, 31*, 439–451.

Hultin, H. (1992). Trimethylamine-*N*-oxide (TMAO) demethylation and protein denaturation in fish muscle. In G. Flick & R. Martin (Eds.), *Advances in seafood biochemistry* (pp. 25–42). Technomic Publisher: Lancaster.

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

LeBlanc, E. L., LeBlanc, R. J., & Blum, I. E. (1988). Prediction of quality in frozen cod (*Gadus morhua*) fillets. *Journal of Food Science, 53*, 328–340.

Leelapongwattana, K., Benjakul, S., Visessanguan, W., & Howell, N. K. (2005a). Physicochemical and biochemical changes during frozen storage of minced flesh of lizardfish (*Saurida micropectoralis*). *Food Chemistry, 90*, 141–150.

Leelapongwattana, K., Benjakul, S., Visessanguan, W., & Howell, N. K. (2005b). Physicochemical and biochemical changes in whole lizardfish (*Saurida micropectoralis*) muscles and fillets during frozen storage. *Journal of Food Biochemistry, 29*, 547–569.

Leelapongwattana, K., Benjakul, S., Visessanguan, W., & Howell, N. K. (in press). Effect of trimethylamine-*N*-oxide demethylase from lizardfish kidney on biochemical changes of haddock natural actomyosin stored at 4 and −10 °C. *European Food Research and Technology*.

Li-Chan, E. Y. C., Nakai, S., & Hirotsuka, M. (1994). Raman spectroscopy as a probe of protein structure in food systems. In R. Y. Yada, R. L. Jackman, & J. L. Smith (Eds.), *Protein structure-function relationships in foods* (pp. 547–569). London: Blackie Academic and Professional.

Lippert, J. L., Tyminski, D., & Desmeules, P. J. (1976). Determination of the secondary structure of proteins by laser Raman spectroscopy. *Journal of the American Chemical Society, 98*, 7075–7080.

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

Matsumoto, J. J. (1980). Chemical deterioration of muscle proteins during frozen storage. In J. R. Whitaker & M. Fujimaki (Eds.), *Chemical deterioration of proteins* (pp. 95–124). Washington, DC: American Chemical Society [ACS Symposium Series 123].

Montejano, J. G., Hamann, D. D., & Lanier, T. C. (1983). Final strengths and rheological changes during processing of thermally induced fish muscle gels. *Journal of Rheology, 27*, 557–579.

Ngarize, S., Herman, H., Adams, A., & Howell, N. K. (2004). Comparison of changes in the secondary structure of unheated, heated and high-pressure treated β-lactoglobulin and ovalbumin proteins using Fourier Transform Raman spectroscopy and self deconvolution. *Journal of Agricultural and Food Chemistry, 52*, 6470–6477.

Sano, T., Noguchi, S. F., Tsuchiya, T., & Matsumoto, J. J. (1988). Dynamic visocelastic behavior of natural actomyosin and myosin during thermal gelation. *Journal of Food Science, 53*, 924–928.

Saeed, S., & Howell, N. K. (2002). Effect of lipid oxidation and frozen storage on muscle proteins of Atlantic mackerel (*Scomber scombrus*). *Journal of the Science of Food and Agriculture, 82*, 579–586.

Saeed, S., & Howell, N. K. (2004). Rheological and differential scanning calorimetry studies on structural and textural changes in frozen Atlantic mackerel (*Scomber scombrus*). *Journal of the Science of Food and Agriculture, 84*, 1216–1222.

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

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

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

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

Tu, A. T. (1982). *Raman spectroscopy in biology: Principles and applications*. New York: Wiley.

Venugopal, V., Kakatkar, A., Bongirwar, D. R., Karthikeyan, M., Mathew, S., & Shamasundar, B. A. (2002). Gelation of shark meat under mild acidic conditions: Physicochemical and rheological characterization of the gel. *Journal of Food Science, 67*, 2681–2686.

Xiong, X. L. (1997). Structure–function relationships of muscle proteins. In S. Damodaran & A. Paraf (Eds.), *Food proteins and their applications* (pp. 341–392). New York: Marcel Dekker.

Ziegler, G. R., & Acton, J. C. (1984). Mechanism of gel formation by proteins of muscle tissues. *Food Technology, 38*, 77–82.