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Biochemical and physicochemical changes in catfish (*Silurus glanis* Linne) muscle as influenced by different freeze–thaw cycles

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Abstract

Freeze–thaw cycles affected the biochemical and physicochemical properties of catfish fillets. When the number of freeze–thaw cycles increased, the activities of α-glucosidase and β-*N*-acetyl-glucosaminidase increased ($P < 0.05$). No increase in non-heme iron content was observed ($P > 0.05$); however the heme iron content decreased ($P < 0.05$). This suggested that the freeze–thaw process caused damage of the cell and hemeprotein, resulting in the release of pro-oxidants. No differences in metmyoglobin and autolysis products ($P > 0.05$) were found as affected by freeze–thaw cycles. After 3 days of chilled storage (2–4°C), a small non-significant increase in non-heme iron content was observed with a concomitant decrease in heme iron. Thiobarbituric acid-release substance increased significantly ($P < 0.05$), especially for the fillets subjected to five freeze–thaw cycles. Components with spectral characteristics similar to hydrogen peroxide-activated hemoproteins were detected with a concomitant decrease in metmyoglobin content in five-cycle freeze–thawed samples after 3 days of storage. The freeze–thaw process therefore has a detrimental effect on the quality of catfish fillets, particularly after chilled storage.

Keywords: Freezing–thawing; Oxidation; Catalyst; Heme; Iron; Pigments; Autolysis

1. Introduction

Frozen storage is an important preservation method for fish and fish products. Quality deterioration is seen during freezing and frozen storage due to the osmotic removal of water, denaturation of protein and mechanical damage (Thyholt & Isaksson, 1997). Enzymes and other components are released (Nilsson & Ekstrand, 1993, 1995). Thawing plays an important role in membrane disintegration as well as affecting sensory attributes (Nilsson & Ekstrand, 1994, 1995). The freeze–thaw process is found to be detrimental to overall physicochemical and textural quality (Srinivasan, Xiong, Blanchard & Tidwell, 1997) and affects the thermal properties of freshwater prawn (Srinivasan, Xiong & Blanchard, 1997). The denaturation of muscle protein, induced by the freeze–thaw process mainly contributes to the detrimental textural changes of fish muscle (Ang & Hultin, 1989; Ragnarrsson & Regenstein, 1989).

Apart from textural changes, lipid oxidation is one of the major problems in frozen fish, since both fresh and saltwater fish contain significant levels of polyunsaturated fatty acids, especially eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Harris & Tall, 1994). Lipid oxidation in fish can be initiated and/or promoted by a number of mechanisms, including the production of singlet oxygen, enzymatic and non-enzymatic generation of partially reduced or free-radical oxygen species, active oxygen iron complexes and thermal- or iron-mediated homolytic cleavage of hydroperoxide (Kubow, 1992). The primary catalysts of lipid oxidation in skeletal muscle have been suggested to be hemoprotein and iron (Igene, King, Pearson & Gray, 1979). Two pools of iron in fish, heme and non-heme, are changed during frozen storage. Non-heme iron in cod and mackerel muscle increases while heme iron decreases during frozen storage (Gomez-Basauri & Regenstein, 1992a). The iron released from ferritin may be a lipid oxidation catalyst in muscle (Decker & Welch, 1990). The below 5-kDa fraction and above 5-kDa fraction from mackerel muscle catalyse lipid oxidation (Decker & Hultin, 1990a, b). Metmyoglobin plays a pro-oxidant role in fresh and

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cooked fish. However, inorganic metal shows a higher catalytic effect on oxidation (Tichivangana & Morrissey, 1985). A relation between pigment oxidation and lipid oxidation has been observed (Faustman, Specht, Malkus & Kineman, 1992). Andersen, Bertelsen & Skibsted (1990) provided evidence supporting the pigment oxidation process as an initiator of lipid oxidation. However, the mechanism of oxidation in fish muscle is still not fully understood.

Temperature fluctuation or abuse generally occur during transportation, storage or consumption. This directly contributes to the biochemical and physicochemical changes of the muscle system. Although there are a large number of publications on the effects of frozen storage on fish quality, little has been reported about the effect of freeze–thaw cycles on the physicochemical properties, especially the distribution of pro-oxidants and oxidation stability. The purpose of this investigation was to determine the effects of multiple freeze–thaw cycles on the biochemical and physicochemical changes in catfish muscle.

2. Materials and methods

2.1. Chemicals

Bathophenanthroline disulfonic acid, 2-thiobarbituric acid, thioglycolic acid and iron standard were purchased from Sigma-Aldrich (Vienna, Austria). Malondialdehyde tetraethylacetal was obtained form Merck (Darmstadt, Germany). Bovine serum albumin was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Trichloroacetic acid was obtained from Riedel-dehaeun (Seelze, Germany).

2.2. Preparation of fish samples

Freshwater catfish with a length of 30–40 cm were caught in The Netherlands, kept on ice and transported to the market in Vienna within 48–60 h. The fish were subsequently filleted in the market. The fillets were purchased and kept on ice and transported to the Institute of Meat Hygiene, Meat Science and Food Science, Vienna. The fillets were sliced into pieces with a thickness of 0.4–0.7 cm and packaged in polyethylene bags.

2.3. Freezing and thawing of fish sample

Five to seven slices were randomly chosen and packaged in a polyethylene bag, sealed and subjected to air-blast freezing at −20°C for 6 h. The frozen samples in the sealed bag were thawed by placing the bag in the tray with running tap water (20±2°C) for 20 min. The flow rate of water was maintained low to avoid agitation. The temperature at the centre of thawed samples measured by the thermocouple was 18±2°C. The samples were subjected to zero, one, three and five freeze–thaw cycles, respectively. All frozen or freeze–thawed samples were kept at −20°C for 2 weeks before analysis or chilled storage. The thawed samples were placed on ice during handling or analysis. Prior to analysis, the fillets were chopped to homogeneity.

2.4. Chilled storage of catfish fillets

Packaged catfish fillets were placed in the chiller at 2–4°C for 3 days. Samples were illuminated continuously with cool white fluorescent light (1600±100 lux). The package exposed to the light were moved periodically to ensure even exposure.

2.5. Preparation of fish muscle exudate

Fish exudate was used as the source of enzyme. The fish slices (25 g) were chopped into small pieces, followed by centrifuging at 34,500×***g*** for 40 min at 4°C in a Sorvall RC 26 Plus (Sorvall, Norwalk, CT, USA). The exudate from the fish muscle was collected using a Pasteur pipette and the volume obtained was measured. The exudate was brought to 25 ml with distilled water before enzyme assay. The protein content in exudate was determined by the Lowry method (Lowry, Rosebrough, Farr & Randell, 1951).

2.6. α-glucosidase (AG) and β-N-acetyl-glucosaminidase (NAG) activity assay

The AG (E.C. 3.2.1.20) and NAG (E.C. 3.2.1.30) activities were assayed according to the method of Nilsson and Ekstrand (1993) with a slight modification. For AG activity assay, the activity was measured spectrophotometrically using ρ-nitrophenyl-α-glucopyranoside as a substrate. The reaction mixture contained 0.3 ml of 0.1 M Na citrate buffer (pH 4.0), 0.2 ml of 1.0 M NaCl, and 1 ml of diluted fish muscle fluid. The reaction mixture was pre-incubated at 37°C for 10 min. The reaction was initiated by adding 1 ml of 4.2 mM ρ-nitrophenyl-α-glucopyranoside. After 60 min, the reaction was terminated by adding 1 ml of 0.3 M KOH. The absorbance was measured at 405 nm. The blank was performed using distilled water instead of fish muscle fluid. The negative control was carried out by adding the stopping reagent prior to the addition of substrate.

NAG activity was determined using ρ-nitrophenyl-*N*-acetyl-β-*D*-glucose amide as a substrate. The reaction mixture consisted of 0.3 ml of 0.1 M Na-citrate buffer (pH 4.5), 0.2 ml of 0.6 M KCl and 0.2 ml diluted fish muscle fluid. The reaction was initiated by adding 0.2 ml of ρ-nitrophenyl-*N*-acetyl-β-*D*-glucose amide and incubated at 37°C for 10 min. The reaction was stopped by adding 1 ml of 0.3 M KOH. The blank and negative

control were run as described above. The absorbance was measured at 405 nm. The amount of ρ-nitrophenol released was monitored at 405 nm and calculated using an extinction coefficient of 19,500 M^{-1} cm^{-1}. One unit of enzyme was defined as the activity which released 1 nmole of ρ-nitrophenol per min.

2.7. Thiobarbituric acid-reactive substance (TBARS) analysis

TBARS analysis was performed according to the method of Buege and Aust (1978). The chopped fillet sample (0.5 g) was dispersed in 2.5 ml of 0.0375% thiobarbituric acid 15% Trichloroacetic acid 0.25 N HCl solution. The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600×***g*** for 20 min in a Sigma 3K30 centrifuge (Laborzentrifugen GmbH, Germany) and the absorbance was measured at 532 nm using a U-3000 spectrophotometer (Hitachi, Tokyo, Japan). TBARS was calculated from the standard curve of malondialdehyde. TBARS was expressed as mg malondialdehyde/kg sample.

2.8. Determination of heme iron content

The heme iron content was determined according to the method of Gomez-Basauri and Regenstein (1992b) with a slight modification. A chopped fillet sample (2 g) was weighed into a 50-ml polypropylene centrifuge tube and 20 ml cold 40 mM phosphate buffer, pH 6.8, was added. The mixture was homogenised with an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 10 s. The homogenate was centrifuged at 3000×***g*** for 30 min at 4°C, using a Sorvall RC 26 Plus (Sorvall, Norwalk, CT, USA). The supernatant was filtered with Whatman #1 filter paper. Total heme pigment was determined by direct spectrophotometric measurement at 525 nm using a U-3000 spectrophotometer (Hatachi, Tokyo, Japan). Myoglobin content was calculated from the millimolar extinction coefficient of 7.6 and a molecular weight of 16,110. Heme iron was calculated based on myoglobin, which contains 0.35% iron. The heme iron content was expressed as mg/100 g sample.

2.9. Determination of non-heme iron content

Non-heme iron was determined as described by Schricker, Miller and Stouffer (1982) with a slight modification. The chopped fillet sample (1.0 g) was weighed into a screw cap test tube and 50 μl of 0.39% (w/v) sodium nitrite was added. A mixture (4 ml) of 40% trichloroacetic acid and 6 N HCl (ratio of 1:1 [v/v], prepared freshly) was added. The tightly capped tubes were placed in an incubator shaker at 65°C (GFL Model 1083, Burgwedel, Germany) for 22 h and then cooled down at ambient temperature for 2 h. The supernatant (400 μl) was mixed with 2 ml of the non-heme iron colour reagent (prepared freshly). After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The colour reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of: (1) bathophenanthroline (0.162 g, dissolved in 100 ml of double deionised water with 2 ml thioglycolic acid [96–99%]); (2) double-deionised water; and (3) saturated sodium acetate solution.

The non-heme iron content was calculated from iron standard curve. The iron standard solution, ranging from 0 to 2 ppm (400 μl) was mixed with 2 ml of the non-heme iron colour reagent. The concentration of non-heme iron was expressed as mg/100 mg sample.

3. Analysis of metmyoglobin

Metmyoglobin was determined according to the method of Krzywicki (1982) as described by Lee, Hendricks and Cornforth (1999). The sample solution was prepared by the method previously mentioned for heme iron determination. The supernatant was subjected to absorbance measurement at 700, 572, and 525 nm by scanning the visible spectrum using a U-3000 spectrophotometer (Hitachi, Tokyo, Japan). The metmyoglobin was calculated using the following equation:

$$\%\text{Metmyoglobin} = \left\{1.395 - [(A572 - A700)/(A525 - A700)]\right\} \times 100$$

3.1. Measurement of autolysis

The autolytic degradation products were measured by the method of Benjakul, Seymour, Morrissey and An (1997). Fish muscle (3 g) was homogenised in 27 ml of 5% (w/v) trichloroacetic acid with an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany). The homogenate was kept on ice for 1 h and centrifuged at 5000×***g*** for 10 min in a Sigma 3K30 centrifuge (Laborzentrifugen GmbH, Germany). Soluble peptides in supernatant were measured as an index of the autolytic degradation products, using tyrosine as a standard and expressed as μmol tyrosine/g muscle.

3.2. Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

3.3. Statistical analysis

Data were subjected to analysis of variance. Mean difference was determined by the least significant difference multiple range test (Steel & Torrie, 1980).

4. Results and discussion

4.1. Effect of freeze–thaw cycles and chilled storage on cell integrity and exudate

The activities of AG and NAG in catfish fillets are presented in Figs. 1 and 2. Both enzyme activities increased ($P < 0.05$) as the freeze–thaw cycles increased. After five cycles of freeze–thawing, the activities increased by 3-and 2.6-fold for AG and NAG, respectively. AG and NAG have been used to differentiate the fresh and freeze–thawed muscle (Rehbein, 1979; Shimomura, Takahashi, Morisita & Ueno, 1987). An increase in activities of both enzymes indicated the disintegration of membrane structure. Freeze–thaw processing potentially disrupts muscle cells, leading to the release of enzymes from mitochondria into sarcoplasm (Hamm, 1979). In this trial, both AG and NAG activities were found in fresh catfish fillet, even though it has been known that those enzymes are retained in the intracellular organelles in the fresh tissue. This result suggested that the fish experienced poor harvest or handling, resulting in the loss of membrane integrity.

After 3 days of chilled storage, it was found that the activities of enzymes decreased to 90–95% and 73–89%, for AG and NAG, respectively (Figs. 1 and 2). This was probably due to the denaturation or instability of those enzymes during chilled storage. Ueno, Yuan and Horiguchi (1987) found that purified NAG had 85% relative activity when kept at 4°C for 1 week. However, Rehbein, Kress and Schrieberb (1978) reported that both AG and NAG activities in cod muscle increased during ice storage. The differences were possibly due to the differences in stability of enzymes from different sources. Therefore, use of those enzymes as the indicators of membrane integrity during the storage of catfish fillet is of limited value.

Volumes and protein contents of exudate of catfish fillets subjected to multiple freeze–thaw cycles are presented in Table 1. Higher amounts of exudate were observed ($P < 0.05$) when the freeze–thaw cycles increased. The increase in exudate indicates loss of water holding capacity of muscle. Therefore, more cycles of freeze–thaw cycles showed more detrimental effects on the fish muscle. This was due to repeated melting and reformation of ice crystals damaging the cell membranes and organelles. Repeated freeze–thawing was shown to increase the cook loss (Locker & Daines, 1973; Srinivasan, Xiong, Blanchard & Tidwell, 1997). Total amount of protein in exudate correlated with the amount of exudate (Table 1). Higher protein was found with the samples subjected to more cycles of freeze–thawing. The proteins are soluble proteins, which are localised in the cell and released when the cells are damaged. The drip loss of muscle can lead to less acceptability due to the loss of tasteful constituents, e.g. some amino acids or nucleotides.

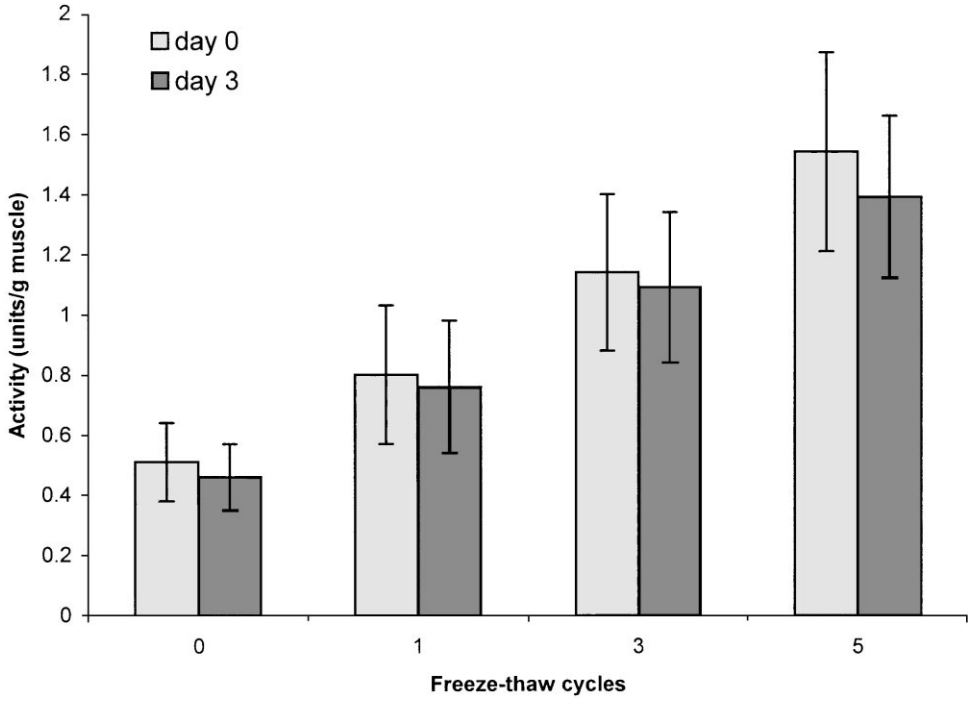

Fig. 1. α-Glucosidase activities in catfish fillets subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage. One unit of enzyme was defined as the activity, which released 1 nmole of ρ-nitrophenol per min. Bars indicate the standard deviation from four determinations.

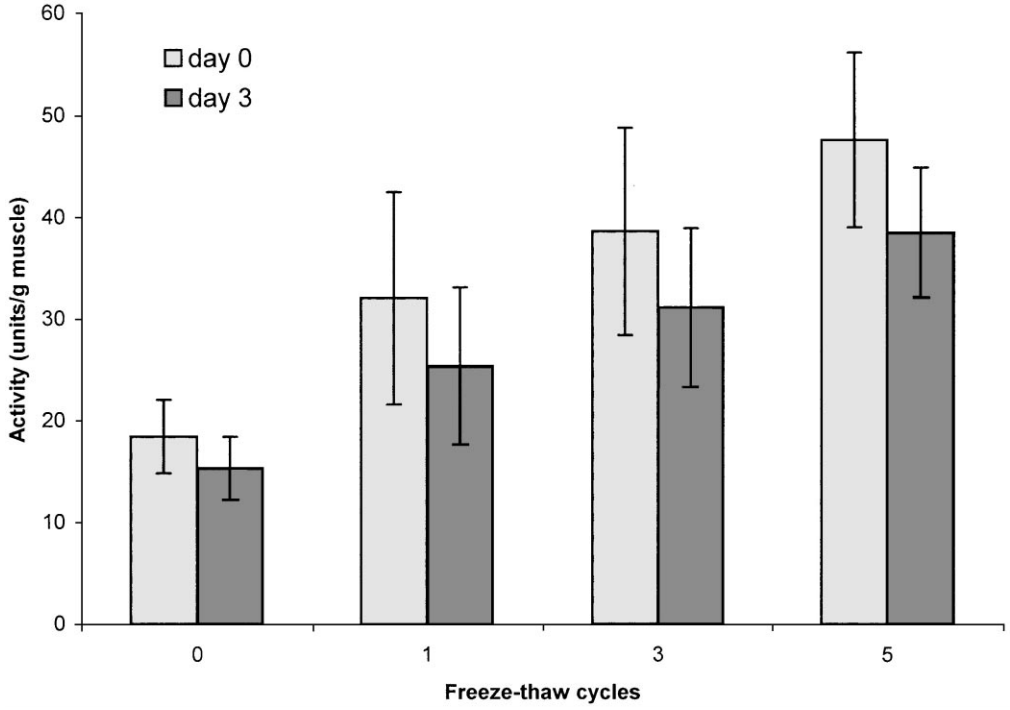

Fig. 2. β-*N*-acetyl-glucosaminidase activities in catfish fillets subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage. One unit of enzyme was defined as the activity, which released 1 nmole of ρ-nitrophenol per min. Bars indicate the standard deviation from four determinations.

Table 1
Volume and protein content of exudate from catfish fillets subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage

Freeze–thaw cycles	Exudate volume (ml/10 g)		Protein content (mg/g)	
	Day 0	Day 3	Day 0	Day 3
0	1.27±0.35[a]	1.35±0.30	12.31±1.81	13.6±2.05
1	1.55±0.30	1.67±0.33 12	8±2.02	14.6±2.33
3	2.05±0.29	2.22±0.25	14.6±2.08	15.6±1.99
5	2.30±0.36	2.52±0.41	15.3±2.34	16.5±1.85

[a] Mean±S.D. from four determinations.

4.2. Effect of freeze–thaw cycles and chilled storage on heme iron content

Heme iron content in catfish fillet was 0.72 mg/100 g. In general, the heme iron constituted 25–44% of the total iron in the fish (Fisher & Deng, 1977). Gordon and Roberts (1977) reported the total iron content in 14 Pacific species to be 0.2–1.0 mg/100 g. Leu, Jhaveri, Karakoltsidis and Constantinides (1981) found that mackerel contained 1.2 mg iron/100 g edible portion. Gomez-Basauri and Regenstein (1992a, b) reported that mackerel fillets had a total iron content of 0.7–0.9 mg/100 g and 46–65% of the iron in fillets was heme iron.

Heme iron content decreased ($P < 0.05$) as the number of freeze–thaw cycles increased (Fig. 3). The heme iron decreased to 93, 61 and 50% after freeze–thawing for one, three and five cycles, respectively. After keeping for 3 days at 2–4°C, the heme iron content decreased to 88–95%. The decrease in heme iron content in the muscle was coincident with the increase in non-heme iron content (Fig. 4). Therefore, it can be postulated that the heme iron, decreased due to heme breakdown, was affected by freeze–thaw cycles, resulting in increased non-heme iron content. More freeze–thaw cycles led to a greater decrease in heme iron. This result was in agreement with that of Gomez-Basauri and Regenstein (1992a) who reported that the decrease in heme iron was inversely related to non heme iron content.

4.3. Effect of freeze–thaw cycles and chilled storage on non-heme iron content

Non-heme iron contents in catfish fillets subjected to different freeze–thaw cycles are shown in Fig. 4. No significant changes in non-heme iron content were obtained ($P > 0.05$) when the freeze–thaw cycles increased. Nevertheless, a small non-significant increase in non-heme iron was observed with more freeze–thaw cycles. The content in the sample subjected to five freeze–thaw cycles was approximately 18.6% higher than that in the fresh sample. This suggested that the freeze–thaw process affected the distribution of iron in the catfish muscle. The non-heme iron found in the fresh sample (0.45 mg/100 g) suggested that the post harvest and handling were not

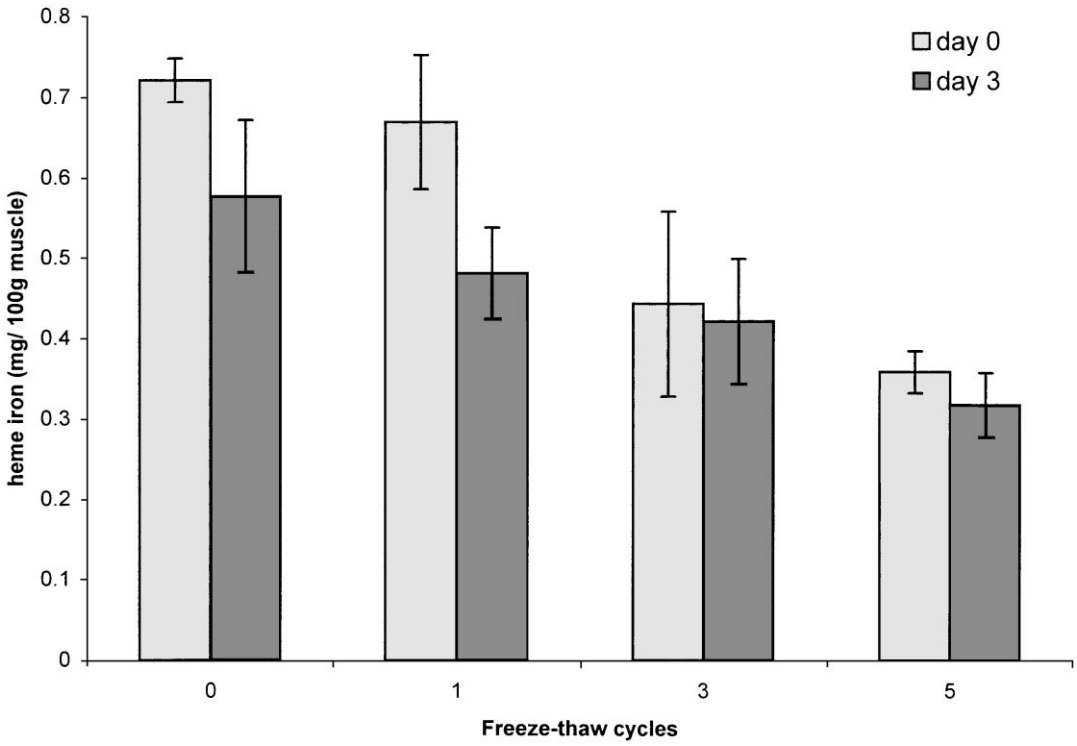

Fig. 3. Heme iron content in catfish fillet extract subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage. Bars indicate the standard deviation from four determinations.

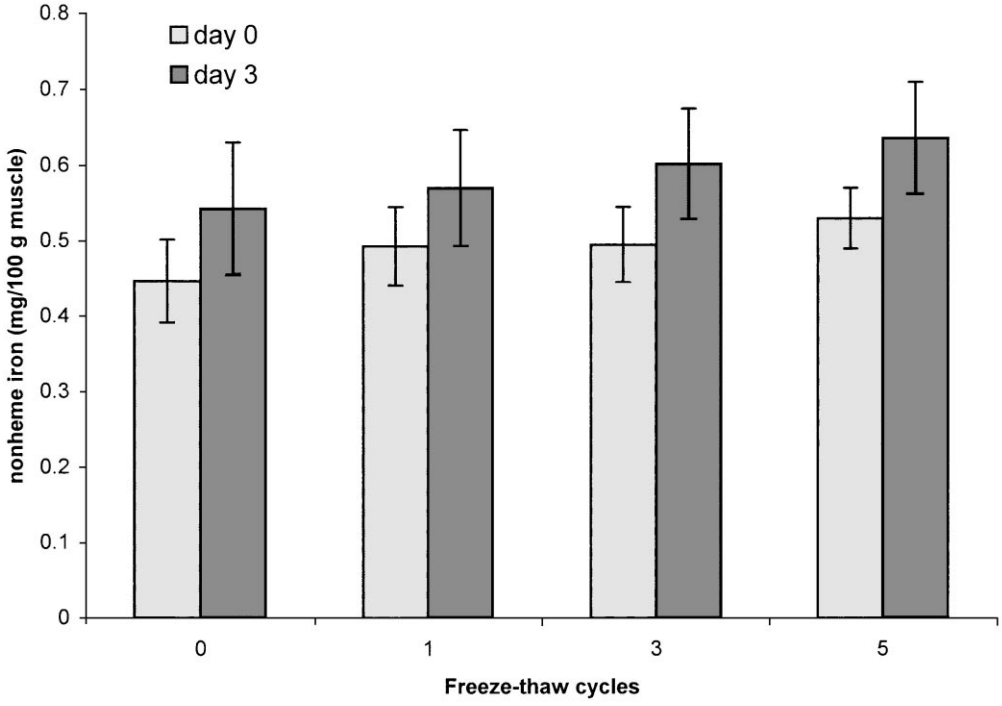

Fig. 4. Non-heme iron content in catfish fillet extract subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage. Bars indicate the standard deviation from four determinations.

properly conducted, resulting in release of iron from the heme pigment or else from iron-containing protein. In general, iron was distributed between five main components, including insoluble fraction, ferritin, haemoglobin, myoglobin and a low molecular weight fraction (Hazell, 1982). The denaturation of those components, especially after repeated freeze–thaw process, possibly contributed to the increase in non-heme iron. Deterioration of subcellular organelles, e.g. the mitochondria, and the release of cytochrome c, could be responsible for the increase in soluble hemin (Decker & Hultin, 1990a, b). Decker and Welch (1990) suggest that heating could cause the conformational changes in ferritin, which may increase the accessibility of ascorbate, to interact with and stimulate the release of iron bound to ferritin. Freeze–thawing, which has been known to induce protein denaturation, was presumed to affect the conformation of those components and allow ascorbate, cysteine or superoxide anion to release more iron. The ferritin releases iron in the presence of reducing agents such as superoxide anion, ascorbate and thiols (Boyer & McCleary, 1987; Thomas, Morehouse & Aust, 1985). Reduction of the oxygen generates a superoxide anion. In addition, autoxidation of oxymyoglobin and oxyhaemoglobin may result in the formation of superoxide anion and metmyoglobin and methaemoglobin, respectively (Harris & Tall, 1994).

After 3 days of chilled storage, the non-heme iron increased about 16–22% more than that observed at day 0 (Fig. 4). This result indicated that the non-heme iron was released more when stored at a low temperature for a longer time. This result was in accordance with those of Decker and Hultin (1990a) who found that the soluble hemin concentration increased within 13 days of iced storage. Furthermore, the increase in non-heme iron content of muscle was probably due to the degradation of insoluble hemoprotein or other iron-containing proteins, resulting in the release of non-heme iron. This assumption was in agreement with the increase in autolysis of muscle at the longer storage time (Table 4). In addition, the oxidation intermediates or products, particularly superoxide anion, generated during 3 days of storage can induce the release of iron from some compounds. This assumption coincided with the increase in TBARS values observed in all samples at day 3 (Fig. 6). Gomez-Basauri and Regenstein (1992a,b) found that non-heme iron increased during frozen storage of cod and mackerel due to heme breakdown. Storage at $-14^{\circ}C$ appeared more deleterious to the heme molecule than storage at lower temperatures (-20 and $-40^{\circ}C$).

Non-heme iron content was also determined in the exudate from freeze–thawed samples at day 0 and 3 (Fig. 5). The results were similar to those found in muscle extract. The samples, subjected to more cycles of the freeze–thaw process rendered non-significantly higher contents of non-heme iron. The exudate obtained from five freeze–thawed cycles had 27% higher non-heme iron content than the fresh sample. An increase in non-heme iron

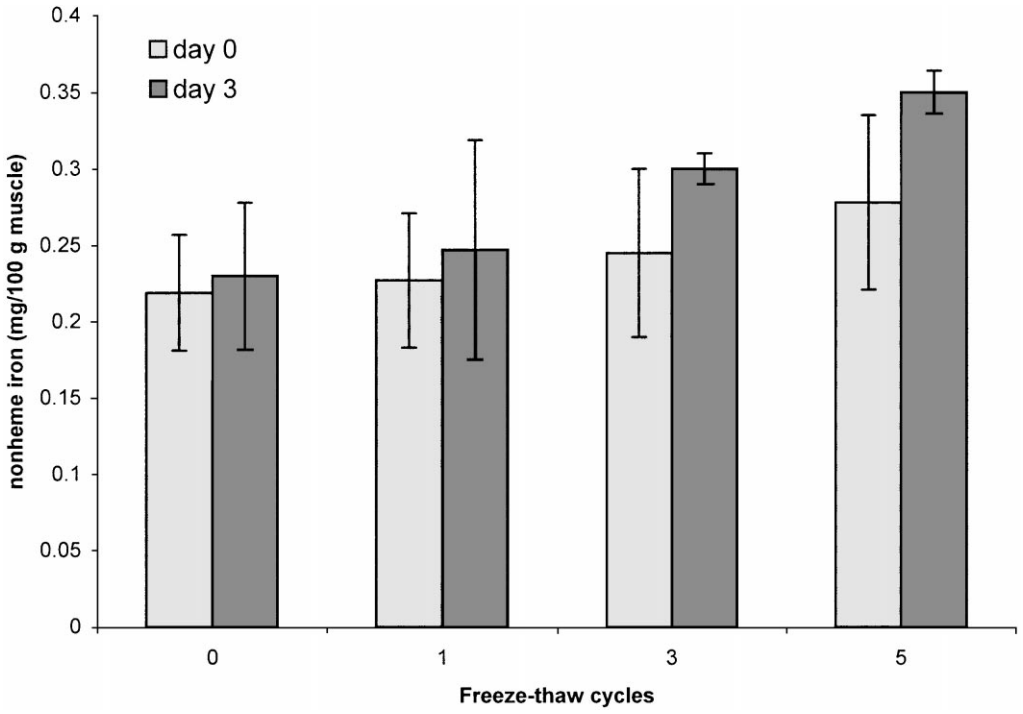

Fig. 5. Non-heme iron content in catfish fillet exudate subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage. Bars indicate the standard deviation from four determinations.

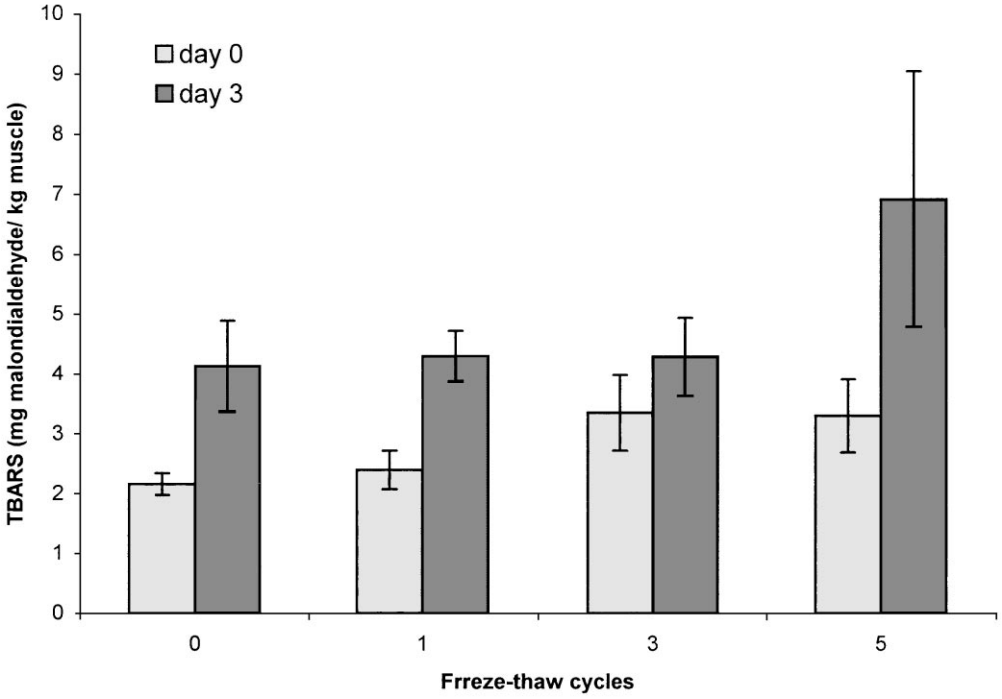

Fig. 6. TBARS formation in catfish fillets subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage. Bars indicate the standard deviation from four determinations.

content of all samples was found when stored at 2–4°C, indicating more release of non-heme iron during chilled storage. The highest non-heme iron content was obtained in the five-cycle freeze–thawed sample. Haemoglobin and other soluble iron-containing components mainly contributed to the pigment as well as the iron content in the exudate. Haemoglobin is basically a tetramer of myoglobin and is a component of red blood cells (Lehninger, 1982). Moreover, Decker and Hultin (1990a, b) have reported the existence of two non-enzymatic catalysts of lipid oxidation in the soluble fraction from mackerel ordinary muscle. The below-5 kDa fraction contained iron complexes while the above-5 kDa fraction contained H_2O_2-activated heme proteins. From these results, it can be concluded that non-heme iron, as well as H_2O_2-activated heme proteins were released as soluble substances into the exudate as of consequence of muscle cell injury caused by ice-crystals or changes in ionic strength of the cell system.

4.4. Effect of freeze–thaw cycles and chilled storage on lipid oxidation

TBARS in catfish fillet increased ($P < 0.05$) when the number of freeze–thaw cycles increased (Fig. 6). Increase in TBARS was observed with three and five freeze–thaw cycles ($P < 0.05$). A significant increase in TBARS was found when the samples were stored for 3 days ($P < 0.05$). Therefore, the freeze–thaw cycles played an essential role in the oxidation acceleration. This was due to the fact that the freeze–thawing caused some loss of integrity in the muscle system. The ice crystals formed could injure the cell and cause the release of pro-oxidants for lipid oxidation, especially free iron (Figs. 4 and 5). The increase in TBARS value was correlated with the increase in non-heme iron. The sample with a high content of non-heme iron showed the higher TBARS value. Iron is able to react with hydrogen peroxide via the Fenton-Haber-Wiess reaction (Kubow, 1992). Also, 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 & Tall, 1994). Generally, it has been accepted that intact iron containing proteins do not react with hydrogen peroxide or superoxide anion to form hydroxy radical unless the iron is released from the protein (Harris & Tall, 1994). However, the prosthetic ferric haem group can be activated by H_2O_2 to a higher redox state ($P^{+}-Fe^{4+}=O$), which can attack a lipid as a true initiator (Kanner, German & Kinsella, 1987). This result was in agreement with that reported by Srinivasan, Xiong, Blanchard and Tidwell (1997) who found that TBARS in freshwater prawn increased gradually during the first three freeze–thaw cycles and then rapidly after four cycles. Therefore, the result reflected a redistribution of pro-oxidants resulting from denaturation of lipoprotein and destabilisation of the muscle structure caused by the freeze–thaw process.

In addition, the freeze–thaw process was presumed to denature some antioxidant enzymes, which play a role in biological prevention of lipid oxidation. Antioxidant

defences, which exist in different compartments of muscle cells, are either enzymatic or chain-breaking lipophilic and hydrophilic components. Among the enzymic defences, the superoxide anion is scavenged by superoxide dismutase, while catalase and gluthathione peroxidase decompose hydrogen peroxide and fatty acid hydroperoxide (Renerre, Dumont & Gatellier, 1996). Superoxide dismutase in beef decreased significantly after 8 days of storage at 2°C and there was a small non-significant decrease in catalase and glutathione peroxidase after storage (Renerre, Dumont & Gatellier, 1996). The higher the number of freeze–thaw cycles, the more the loss in enzymic activity was. From this investigation, it was presumed that some antioxidant enzymes underwent denaturation caused by freeze–thawing and activity loss subsequently occurred during prolonged storage.

4.5. *Effect of freeze–thaw cycles and chilled storage on the absorption maxima in soret region and metmyoglobin*

The soret absorption peaks, of muscle extract and exudates, from catfish fillets subjected to different freeze–thaw cycles and kept for 3 days under chilled temperature, are shown in Table 2. A blue shift was observed in both extract and exudate after freeze–thawing. A marked shift occurred with the samples subjected to five freeze–thaw cycles. A blue shift from 416 to 408 nm was observed in frozen-thawed mackerel ordinary muscle within 1 day of iced storage, while it took 10 days for unfrozen samples to shift from 414 to 409 nm (Decker & Hultin, 1990b). A blue shift from 418 to 409 nm was obtained when sperm whale oxymyoglobin was changed to metmyoglobin (Antonini & Brunori, 1971). The blue shift observed in this investigation suggested that the hemoproteins were converted to oxidised states. The blue shift coincided with the slight increase in metmyoglobin in samples subjected to multiple freeze–thaw cycles.

After keeping the fresh and one-cycle freeze–thawed samples for 3 days, there were no differences in absorption maxima compared to those observed at day 0. However, a red shift was obtained in three and five-cycle freeze–thawed samples (Table 2). A red shift from 408–409 to 411–412 nm in extract, and a red shift from 409–410 to 413–414 nm, in exudate of five-cycle freeze–thawed sample suggested that oxidation products, including hydrogen peroxide, contributed to the activation of hemoproteins. Antonini and Brunori (1971) found that a red shift of sperm whale metmyoglobin from 409 to 421 nm resulted from the activation of hemoproteins by hydrogen peroxide. This result was in agreement with the increase in TBARS, which was used as an index of oxidation. A red shift was coincidental with the decrease in metmyoglobin (Table 3). The freeze–thaw process increased the oxidation of hemoprotein and formed the compounds with spectral characteristics similar to hydrogen peroxide during storage (Decker & Hultin, 1990b). The inactivation of antioxidant enzymes, especially catalase, due to the freeze–thaw process possibly resulted in the accumulation of hydrogen peroxide.

No differences in metmyoglobin were observed in the samples subjected to different freeze–thaw cycles ($P > 0.05$). However, metmyoglobin formation tended to increase with the freeze–thawed cycles (Table 3). The myoglobin formation in fresh sample indicated that some myoglobin underwent oxidation to form metmyoglobin. There are many factors known to increase myoglobin oxidation, including pH, salt concentration and species (Trout, 1990). The inactivation of enzymes which maintain the reduced state of hemoproteins was also presumed to increase the formation of metmyoglobin. Tajima and Shikama (1987) showed that oxymyoglobin oxidation can produce hydrogen peroxide. The combination of hydrogen peroxide and metmyoglobin results in a pro-oxidative complex (Bodaness, Leclair & Zigler, 1984; Kanner & Harel, 1985). A red shift means that the

Table 2
Absorption maxima in the soret region (350–450 nm) of extract and exudate from catfish fillets subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage

Freeze-thaw cycles	Extract (nm)		Exudate (nm)	
	Day 0	Day 3	Day 0	Day 3
0	412–413	412–413	412–413	413–414
1	410–411	410–411	410–411	410–411
3	409–410	410–411	408–409	410–411
5	408–409	411–412	409–410	413–414

Table 3
Metmyoglobin formation in catfish fillets subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage

Freeze-thaw cycles	Day 0	Day 3
0	50.66±8.10[a]	50.17±7.42
1	53.25±6.40	48.09±5.55
3	54.53±5.80	42.68±2.28
5	55.23±5.30	43.77±2.97

[a] Mean±S.D. from four determinations.

Table 4
Degradation products in catfish fillets subjected to different freeze–thaw cycles at days 0 and 3 of chilled storage (μmol tyrosine/g muscle)

Freeze-thaw cycles	Day 0	Day 3
0	0.46±0.23[a]	0.93±0.18
1	0.51±0.29	0.93±0.19
3	0.46±0.21	0.87±0.26
5	0.55±0.21	1.08±0.28

[a] Mean±S.D. from four determinations.

hydrogen peroxide metmyoglobin complex could be generated and work as a pro-oxidant in the oxidation. As a result, TBARS value was markedly high for the sample subjected to the five cycles freeze–thaw process.

Metmyoglobin content surprisingly decreased after keeping at a chilled temperature for 3 days (Table 3). A comparable decrease was observed in the three and five-cycle freeze–thawed samples with a concomitant red shift. This probably resulted from the production of substances with spectral characteristics similar to hydrogen peroxide-activated hemoproteins, leading to a red shift (Table 2). A small change in metmyoglobin of catfish fillets after the freeze–thaw process was probably due to the activity of metmyoglobin reductase, which could be released and activated. It has been known that metmyoglobin reductase is a component of red blood cells and can be found in fish muscle (Al-Shaibani, Price & Brown, 1977). Since some bloods retained in the fillets, residual activity of this enzyme could be present and resulted in the retardation of colour deterioration.

4.6. Effect of freeze–thaw cycles and chilled storage on autolysis of muscle

The increase in the autolytic degradation products was observed at 3 days of chilled storage ($P < 0.05$; Table 4). No differences in degradation products were obtained among the samples subjected to different freeze–thaw cycles ($P > 0.05$). However, a slight non-significant increase in degradation products was found in five freeze–thawed cycles, especially after 3 days of storage, suggesting that a higher release of proteolytic enzyme possibly occurred, due to the freeze–thaw process. Catheptic enzymes have been implicated in autolytic spoilage of some species of fish (Haard, 1990). Degradation of myosin of Pacific whiting was found during iced storage for 8 days (Benjakul et al., 1997). Serine proteinase can cause the degradation of myofibrils at low temperature (Busconi, Folco, Martone, Trucco & Sanchez, 1989). In addition, calpain was reported to be involved in postmortem tenderization of tilapia muscle (Jiang, Wang & Chen, 1991). Therefore, freeze–thawing may affect the distribution of proteinase in the muscle, leading to the hydrolysis of muscle proteins. In addition, the loss of native conformation of muscle proteins, due to the freeze–thaw process, could make them more susceptible to enzymic hydrolysis.

5. Conclusion

Freeze–thaw cycles directly affected the biochemical and physicochemical properties of catfish fillets. The freeze–thaw process caused the destabilisation of the muscle structure, leading to the redistribution of pro-oxidants and acceleration of lipid oxidation.

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References

Al-Shaibani, K. A., Price, R. J., & Brown, W. D. (1977). Purification of metmyoglobin reductase from bluefin tuna. *Journal of Food Science, 42*, 1013–1015.

Andersen, H. J., Bertelsen, G., & Skibsted, L. H. (1990). Colour and colour stability of hot processed frozen mince beef. Results from chemical model experiments tested under storage conditions. *Meat Science, 28*, 87–97.

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

Antonini, E., & Brunori, M. (1971). *Hemoglobin and myoglobin in their reactions with ligands*. New York: American Elsevier Publishing Co., Inc.

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

Bodaness, R. S., Leclair, M., & Zigler, J. S. (1984). Analysis of the H_2O_2-mediated crosslinking of lens crystallins catalyzed by the heme-undecapeptide from cytochrome c. *Archives of Biochemistry and Biophysics, 231*, 461–469.

Boyer, R. F., & McCleary, C. J. (1987). Superoxide ions as a primary reductant in ascorbate-mediated ferritin iron release. *Free Radical Biology and Medicine, 3*, 389–395.

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology, 52*, 302–304.

Busconi, L., Folco, E. J., Martone, C. B., Trucco, R. E., & Sanchez, J. J. (1989). Fish muscle cytoskeletal network: its spatial organisation and its degradation by an endogenous serine proteinase. *Archives of Biochemistry and Biophysics, 268*, 203–208.

Decker, E. A., & Hultin, H. O. (1990a). Factors influencing catalysis of lipid oxidation by the soluble fraction of mackerel muscle. *Journal of Food Science, 55*, 947–950.

Decker, E. A., & Hultin, H. O. (1990b). Nonenzymic catalysts of lipid oxidation in mackerel ordinary muscle. *Journal of Food Science, 55*, 951–953.

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry, 38*, 674–677.

Faustman, C., Specht, S. M., Malkus, L. A., & Kinsman, D. M. (1992). Pigment oxidation in ground veal: influence of lipid oxidation, iron and zinc. *Meat Science, 31*, 351–362.

Fisher, J., & Deng, J. C. (1977). Catalysis of lipid oxidation: a study of mullet (*Mugil cephalus*) dark flesh and emulsion model system. *Journal of Food Science, 42*, 610–614.

Gomez-Basauri, J. V., & Regenstein, J. M. (1992a). Processing and frozen storage effects on the iron content of cod and mackerel. *Journal of Food Science, 57*, 1332–1336.

Gomez-Basauri, J. V., & Regenstein, J. M. (1992b). Vacuum packaging, ascorbic and frozen storage effects on heme and nonheme iron content of mackerel. *Journal of Food Science, 57*, 1337–1339.

Gordon, D. T., & Roberts, G. L. (1977). Mineral and proximate composition of Pacific coast fish. *J. Agric. Food Chem., 25*, 1262–1268.

Haard, N. F. (1990). Enzymes from food myosystems. *Journal Muscle Foods, 1*, 293–338.

Hamm, R. (1979). Delocalization of mitochondria enzymes during freezing and thawing of skeletal muscle. In Fennema, O. R.,

Proteins at low temperatures (Advances in Chemistry Series, No. 180; p. 191). Washington, DC: American Chemical Society.

Harris, P., & Tall, J. (1994). Rancidity in fish. In J. C. Allen, & R. J. Hamilton, *Rancidity in Foods* (pp. 256–272). London, UK: Chapman & Hall.

Hazell, T. (1982). Iron and zinc compounds in the muscle meats of beef, lamb, pork and chicken. *Journal of the Science of Food and Agriculture, 33*, 1049–1056.

Igene, J. O., King, J. A., Pearson, A. M., & Gray, J. I. (1979). Influence of heme pigments, nitrite and non-heme iron on development of warmed-over flavor in cooked meats. *Journal of Agirucltural and Food Chemistry, 27*, 838–842.

Jiang, S. T., Wang, J. W., & Chen, C. S. (1991). Purification and some properties of calpain II from tilapia muscle (*Tilapia nilotica X Tilapia aurea*) muscle myofibrils. *Journal of Agiruclturaland Food Chemistry, 39*, 237–241.

Kanner, J., German, J. B., & Kinsella, J. E. (1987). Initiation of lipid oxidation in biological systems. *CRC Critical Reviews in Food Science and Nutrition, 25*, 317–364.

Kanner, J., & Harel, S. (1985). Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin. *Archives of Biochemistry and Biophysics, 237*, 314–321.

Krzywicki, K. (1982). The determination of haem pigment in meat. *Meat Science, 7*, 29–35.

Kubow, S. (1992). Routes of formation and toxic consequences of lipid oxidation products in foods. *Free Radical Biology and Medicine, 12*, 63–81.

Lee, B. J., Hendricks, D. G., & Cornforth, D. P. (1999). A comparison of carnosine and ascorbic acid on color and lipid stability in a ground beef pattie model system. *Meat Science, 51*, 245–253.

Lehninger, A. L. (1982). *Principles of biochemistry*. New York: Worth Publishers.

Leu, S. S., Jhaveri, S. N., Karakoltsidis, P. A., & Constantinides, S. M. (1981). Atlantic mackerel (*Scomber scombrus*, L): seasonal variation in proximate composition and distribution of chemical nutrients. *Journal of Food Science, 46*, 1635–1638.

Locker, R. H., & Daines, G. J. (1973). The effected of repeated freeze–thaw cycles on tenderness and cooking loss in beef. *Journal of the Science of Food and Agriculture, 24*, 1273–1275.

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

Nilsson, K., & Ekstrand, B. (1993). The effect of storage on ice and various freezing treatments on enzyme leakage in muscle tissue of rainbow trout (*Oncorhynchus mykiss*). *Zeitschrift für Lebensmittel-Untersuchung und -Forchung, 197*, 3–7.

Nilsson, K., & Ekstrand, B. (1994). Enzyme leakage in muscle tissue of rainbow trout (*Oncorhynchus mykiss*). *Zeitschrift für Lebensmittel-Untersuchung und –Forchung, 198*, 253–257.

Nilsson, K., & Ekstrand, B. (1995). Frozen storage and thawing methods affect biochemical and sensory attributes of rainbow trout. *Journal of Food Science, 60*, 627–630.

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

Rehbein, H., Kress, G., & Schreiber, W. (1978). An enzymic method for differentiating thawed and fresh fish fillets. *Journal of the Science of Food and Agriculture, 29*, 1076–1082.

Rehbein, H. (1979). Development of an enzymatic method to differentiate fresh and sea-frozen and thawed fish fillets. *Zeitschrift fur Lebensmittel-Untersuchung und-Forchung, 196*, 263–268.

Renerre, M., Dumont, F., & Gatellier, P. H. (1996). Antioxidant enzyme activities in beef in relation to oxidation of lipid and myoglobin. *Meat Science, 43*, 111–121.

Schricker, B. R., Miller, D. D., & Stouffer, J. R. (1982). Measurement and content of nonheme and total iron in muscle. *Journal of Food Science, 47*, 740–743.

Shimomura, H., Takahashi, T., Morishita, T., & Ueno, R. (1987). Investigation of the differentiation of frozen-thawed fish from unfrozen fish by comparison of lysosomal enzyme activity. *Nippon Suisan Gakkaishi, 53*, 1841–1845.

Srinivasan, S., Xiong, Y. L., & Blanchard, S. P. (1997). Effects of freezing and thawing methods and storage on thermal properties of freshwater prawns (*Machrobrachium rosenbergii*). *Journal of the Science of Food and Agriculture, 75*, 37–44.

Srinivasan, S., Xiong, Y. L., Blanchard, S. P., & Tidwell, J. H. (1997). Physicochemical changes in prawns (*Machrobrachium rosenbergii*) subjected to multiple freeze–thaw cycles. *Journal of Food Science, 62*, 123–127.

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

Tajima, G., & Shikama, K. (1987). Autoxidation of oxymyoglobin: an overall stoichiometry including subsequent side reaction. *Journal of Biological Chemistry, 262*, 12603–12606.

Thomas, C. E., Morehouse, L. A., & Aust, S. D. (1985). Ferritin and superoxide-dependent lipid peroxidation. *Journal of Biological Chemistry, 260*, 3275–3280.

Thyholt, K., & Isaksson, T. (1997). Differentiation of frozen and unfrozen beef using near-infrared spectroscopy. *Journal of the Science of Food and Agriculture, 73*, 525–532.

Tichivangana, J. Z., & Morrissey, P. A. (1985). Metmyoglobin and inorganic metals as pro-oxidants in raw and cooked muscle systems. *Meat Science, 15*, 107–116.

Trout, G. R. (1990). The rate of metmyoglobin formation in beef, pork, and turkey meat as influenced by pH, sodium chloride and sodium tripolyphosphate. *Meat Science, 28*, 203–210.

Ueno, R., Yuan, C., & Horiguchi, Y. (1987). Characterization of neutral β-N-acetylglucosaminidase in carp blood. *Nippon Suisan Gakkaishi, 53*, 1017–1024.