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Effect of bleeding treatment and perfusion of yellowtail on lipid oxidation in post-mortem muscle

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Abstract

The present study investigated the effects of bleeding treatment and perfusion of antioxidant compounds on lipid oxidation in ordinary and dark muscles of yellowtail in the early stage of ice storage. The lipid hydroperoxide contents of dark muscles obtained from yellowtails with and without bleeding treatment were higher and increased more rapidly than those of ordinary muscles. There were no significant differences in the rates of change of the lipid hydroperoxide content (up to 48 h), fatty acid composition and metmyoglobin formation between dark muscles with and without bleeding treatment. Physiological saline containing ascorbic acid or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) was perfused into live yellowtail or added to minced dark muscle. Trolox® significantly ($P < 0.01$) delayed the accumulation of lipid hydroperoxide in dark muscle compared to ascorbic acid in perfusion experiment. These results indicate that simply removing a portion of the blood from live yellowtail by bleeding is not sufficient to prevent lipid oxidation in the early stage of ice storage. Contrary to this, addition of antioxidants into fish flesh is effective to delay lipid oxidation in post-mortem muscle.

Keywords: Bleeding treatment; Lipid hydroperoxide; Metmyoglobin; Ordinary muscle; Dark muscle; Antioxidant

1. Introduction

Lipid oxidation is one of the most important problems for quality control of flesh foods because it negatively affects food flavor, texture, consistency and appearance. The high content of polyunsaturated lipids in fish flesh is directly related to the development of rancidity and off-odors (Sohn, Taki, Ushio, Kohata, Shioya, & Ohshima, 2005). The color and odor of raw fish flesh are very important indices for consumers to evaluate the freshness and quality of fish. The color tone of fish flesh depends on the amount of the myoglobin and hemoglobin contents in the muscles (Matsuura & Hashimoto, 1959; Richards & Hultin, 2002). Moreover, methemoglobin and metmyoglobin, the oxidized analogues of hemoglobin and myoglobin, respectively, usually contribute to lipid oxidation in fish flesh (Yamamoto, 1980; Koizumi, Wada, & Ohshima, 1987; Chan, Faustman, Yin, & Decker, 1997). In contrast, heme iron has been reported to have no catalytic effect on lipid oxidation in cooked beef meat (Sato & Hegarty, 1971; Love & Pearson, 1974).

Generally, bleeding of fish is carried out to eliminate most of the hemoglobin from the tissues. It is well accepted that immediate bleeding of live fish delays rigor-mortis compared to the flesh of untreated fish during ice storage (Mochizuki, Norita, & Maeno, 1998). On the other hand, residual blood in fish tissues is one of the main factors that leads to the development of undesirable discoloration of the flesh (Flechtenmacher, 1975) and unpleasant flavor

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during ice storage. Therefore, fish without bleeding treatment are usually unsuitable for use as "sashimi", a Japanese dish of thinly sliced and garnished raw fish, because of their undesirable meat color and odor. There have been a number of reports on the effects of bleeding on post-mortem changes for several fish muscles in terms of rigor-mortis, softening and quality (Mochizuki et al., 1998; Ando, Nishiyabu, Tsukamasa, & Makinodan, 1999; Terayama & Yamanaka, 2000). However, little information has been available regarding the effects of bleeding treatment on lipid oxidation stability of fish muscles (Porter, Kennish, & Kramer, 1992; Richards & Hultin, 2002).

Recently, we reported that lipid hydroperoxide accumulation in the early stage of lipid oxidation differs not only between fish species but also between ordinary and dark muscles (Sohn, Taki, Ushio, Kohata, et al., 2005). In this stage, the rate of lipid oxidation of yellowtail dark muscle was significantly faster than that of ordinary muscle. Moreover, lipid oxidation of yellowtail dark muscle was closely related to darkening of the flesh color and development of a rancid off-odor during the early stage of ice storage.

The aim of the present study was to evaluate the effects of residual blood in fish muscles on the oxidative stability of lipid during the early stage of ice storage. Furthermore, the abilities of antioxidants to delay the lipid oxidation were investigated by adding antioxidant compounds to fish muscle.

2. Materials and methods

2.1. Fish

Live cultured yellowtail (*Seriola quinqueradiata*, 3.25 ± 0.21 cm in fork length and 52.5 ± 1.4 kg in body weight) were landed at the Misaki fishing port in Kanagawa prefecture, Japan. The obtained fish were immediately transported to a laboratory within 1 h for storage experiments.

2.2. Chemicals and Materials

Diphenyl-1-pyrenylphosphine (DPPP) was purchased from Dojindo Laboratories Co. Ltd. (Kumamoto, Japan). 2,6-Di-*tert*-butyl-*p*-cresol (BHT) was obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). 1-Myristoyl-2-(12-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl)-*sn*-glycero-3-phosphocholine (NBD-labeled PC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) and sodium L-ascorbate were obtained from Aldrich Chem. Co. (Milwaukee, WI, USA) and Sigma-Aldrich (St. Louis, MA, USA), respectively. HPLC grade methanol from Kokusan Chemical Co. Ltd. (Tokyo, Japan) and 1-butanol from Kishida Chemical Co. Ltd. (Osaka, Japan) were used after degassing. All other chemicals were of analytical grade.

2.3. Bleeding of fish and addition of antioxidants

To obtain bleeding-treated muscles, the medulla oblongata and tail vein were cut, and the fish were left in artificial seawater to bleed out for 1 h. Untreated fish were kept in artificial seawater until use for experiments.

2.4. Perfusion of fish

For perfusion treatment, fish were anesthetized by immersion in tricaine methanesulfonate (MS222; Acros Organics, NJ, USA) and the heart was exposed by an incision from the abdomen to the gills. A cannula connected to a hypodermic syringe, which was filled with physiological saline (0.7489% NaCl, 0.1294% KCl, 0.199% $CaCl_2$) solution (control), 500 ppm of Trolox® (Trolox® group) or sodium L-ascorbate (ascorbic acid group) dissolved in physiological saline solution, was inserted into the bulbus arteriosus. A polyethylene tube (1.5 mm in outer diameter) was inserted into the ventricle to take venous blood out of the fish body, resulting in circulation of the venous blood and perfusion. Immediately after starting the perfusion, the color of the fish gills changed to pale gray and the color of the fluid from the outlet of the cannula became colorless and clear after approximately 10 min. Perfusion was carried out for 1 h at room temperature.

2.5. Ice storage of fish muscles

Ordinary and dark muscles were isolated and thoroughly minced individually using a food processor (model MK-K50; National, Osaka, Japan). The minced muscles were packed in polyethylene bags and kept on ice for up to 72 h.

2.6. Fatty acid analysis

Tricosanoic acid methyl ester was added to accurately weighed total lipids (ca. 50 mg) as an internal standard. Next the mixture was saponified by 1 M KOH in methanol, and methylated by 14% BF_3–CH_3OH to obtain fatty acid methyl esters (FAMEs). The FAMEs thus obtained were dissolved in *n*-hexane and analyzed using a GC-15A gas chromatograph (Shimdazu, Kyoto, Japan) equipped with an open-tubular capillary column (0.25 mm i.d. × 30 m, 0.25 μm in film thickness; Supelco, Bellefonte, PA). The oven temperature was initially kept at 140 °C for 1 min and then programmed to reach a final temperature of 240 °C at a rate of 1 °C/min. The temperatures of the injector and detector were controlled at 250 °C. Helium was used as a carrier gas at a column inlet pressure of 2 kg/cm^2.

2.7. Determination of lipid hydroperoxides

Lipid hydroperoxides were analyzed by a flow-injection analysis (FIA) system equipped with a fluorescence detection system using DPPP as a fluorescent reagent as previ-

ously described (Sohn, Taki, Ushio, & Ohshima, 2005). Briefly, a 1 ml aliquot of NBD-labeled PC (4.82 nmol/ml in methanol) was added to a 5 g portion of minced muscle as an internal standard, and total lipids were extracted and purified according to the Bligh and Dyer procedure (Bligh & Dyer, 1959). The homogenate was centrifuged at 1700*g* for 8 min at 4 °C, and the lower layer was dehydrated with anhydrous sodium sulfate and filtered through a membrane filter (PTFE, 0.20 μm; Advantec Toyo Roshi Kaisha Ltd., Tokyo, Japan). The filtrate thus obtained was made up to 10 ml with chloroform. A 20 μl portion of the sample solution was injected into the FIA system for determination of the lipid hydroperoxide contents.

2.8. Metmyoglobin analysis

The metmyoglobin percentages among the total myoglobin present in the muscles were measured. Briefly, a portion (3 g) of minced muscle was transferred into a polypropylene tube. Ice-cold distilled water (10 ml) was added to the tube and the contents were mixed with a Teflon®-coated magnetic stirring bar. The mixtures were stood at 4 °C for 10 min and centrifuged at 1700*g* for 5 min. The supernatant thus obtained was filtered through a Toyo No. 1 filter paper (Advantec Toyo Roshi Kaisha, Ltd.) and adjusted to pH 6.8–7.0 with 1 M NaOH. The filtrate was filtered again through a membrane filter (Cellulose acetate, 0.45 μm; Advantec Toyo Roshi Kaisha Ltd.) and a 100 μl portion of the filtrate was subjected to HPLC. A TSK-GEL HPLC column ($G3000SW_{XL}$, 300 × 7.8 mm i.d.; Tosoh, Tokyo, Japan) was used for separation of the myoglobin and a mobile phase of 0.01 M phosphate buffer (pH 6.8) containing 0.2 M of Na_2SO_4 was pumped into a model LC-10AS HPLC pump (Shimadzu) at a flow rate of 1.0 ml/min. The absorbances of the supernatant were measured at 540 nm (E_{540}) and 503 nm (E_{503}) with a model SPD-M10AVP diode array detector (Shimadzu) and the metmyoglobin percentage was calculated by using the absorption ratio (E_{540}/E_{503}) according to the formula reported for tuna myoglobin (Bito, 1965).

2.9. Analysis of α-tocopherol

Total lipids were extracted by a mixture of chloroform and methanol according to the Bligh and Dyer procedure (Bligh & Dyer, 1959). 2,2,5,7,8-Pentamethyl-6-hydroxychroman was added as an internal standard. Chloroform used as the solvent was replaced with 20 μl of *n*-hexane, and the sample was subjected to HPLC. A silica-gel HPLC column (LiChrosorb® Si-60, 5 μm, 250 × 4 mm i.d.; Merck, Darmstadt, Germany) was used for separation of the tocopherol analogues and the mobile phase consisting of a mixture of *n*-hexane:2-propanol (99.5:0.5, v/v) was pumped into a model LC-9A HPLC pump (Shimadzu) at a flow rate of 1.0 ml/min. The fluorescence intensity excited at a wavelength of 297 nm was monitored at an emission wavelength of 327 nm using a model RF-550 spectrofluorometric detector (Shimadzu). The fluorescence signals were integrated using Chromatopac C-R3A chromatographic integrators (Shimadzu). The detector signals were quantified by reference to a standard calibration curve that was previously prepared using an authentic standard.

2.10. Measurement of pH

A portion (1 g) of sample was homogenized with 10 ml of distilled water. The homogenate was centrifuged at 1700*g* for 5 min at 4 °C and the supernatant was filtered through a Toyo No. 1 filter paper. The pH of supernatant was then measured with a Horiba pH meter F-21 (Kyoto, Japan).

2.11. Statistics

The chemical and instrumental analyses were carried out in triplicate and are represented as means ± standard deviation. Student's *t*-test (Box, Hunter, & Hunter, 1978) was applied to distinguish significant differences among the mean values. A statistically significant difference between two mean values was declared at $P < 0.01$.

3. Results

3.1. Total lipid contents and fatty acid compositions

The lipid contents of yellowtail ordinary and dark muscles were 4.1 ± 0.8 and 12.3 ± 1.4 g/100 g muscle, respectively. The changes in the fatty acid compositions of ordinary and dark muscles are shown in Table 1. The predominant fatty acids were 16:0, 16:1*n*−7, 18:1*n*−9, 20:5*n*−3 (EPA) and 22:6*n*−3 (DHA) in both ordinary and dark muscles. The percentages of EPA were higher in ordinary muscles than in dark muscles, whereas the percentages of DHA were higher in dark muscles than in ordinary muscles. There were no significant differences in the fatty acid compositions of ordinary and dark muscles with and without bleeding treatment during ice storage.

3.2. Effects of bleeding on lipid oxidation of fish flesh

The changes in the lipid hydroperoxide contents of muscles with and without bleeding treatment during ice storage are shown in Fig. 1. There was a significant ($P < 0.01$) difference in the lipid hydroperoxide contents between ordinary muscles with and without bleeding treatment during ice storage. The lipid hydroperoxide content of ordinary muscle with bleeding treatment was higher than that of ordinary muscle without bleeding treatment throughout ice storage for 68 h. The lipid hydroperoxide contents of both treated and untreated dark muscles were approximately twice those of treated and untreated ordinary muscles before ice storage, and increased to 40-fold the levels in ordinary muscles after 68 h of ice storage. Furthermore, the lipid hydroperoxide contents of dark muscles were

Table 1
Changes in the fatty acid compositions (wt%) of ordinary and dark muscles of yellowtail with and without bleeding treatment during ice storage for 3 days

Fatty acid	Ordinary muscle				Dark muscle			
	With bleeding treatment		Without bleeding treatment		With bleeding treatment		Without bleeding treatment	
	0 day	3 days	0 day	3 days	0 day	3 days	0 day	3 days
14:0	5.04	5.14	5.17	5.09	4.76	4.80	4.99	4.92
15:0	0.46	0.48	0.46	0.45	0.44	0.46	0.46	0.45
16:0	18.39	18.36	19.23	18.86	18.38	19.16	19.43	18.81
18:0	3.34	3.32	3.85	3.87	3.87	4.45	4.68	5.00
Saturated	27.23	27.30	28.71	28.27	27.45	28.87	29.56	29.18
16:1*n*−7	6.80	6.80	6.20	6.27	6.08	6.17	6.11	5.92
18:1*n*−9	18.12	17.65	16.81	16.77	17.66	18.24	17.49	17.51
18:1*n*−7	2.97	2.93	2.86	2.89	3.06	3.29	3.19	3.32
20:1*n*−11	2.29	2.20	2.42	2.39	2.29	2.19	2.39	2.38
20:1*n*−9	3.70	3.60	3.62	3.62	3.57	3.56	3.44	3.52
22:1*n*−11	4.59	4.36	4.89	4.80	4.60	4.00	4.19	4.26
22:1*n*−9	0.56	0.53	0.60	0.63	0.59	0.47	0.53	0.54
Monoenoic	39.02	38.07	37.40	37.37	37.84	37.93	37.34	37.45
16:2*n*−4	0.50	0.51	0.47	0.46	0.42	0.47	0.44	0.43
16:3*n*−4	0.38	0.41	0.39	0.40	0.40	0.48	0.45	0.40
16:4*n*−1	0.41	0.42	0.39	0.38	0.34	0.37	0.35	0.36
18:2*n*−6	4.67	4.53	4.11	4.09	4.34	4.43	4.17	4.28
18:3*n*−3	1.08	1.06	0.85	0.91	0.90	0.86	0.80	0.79
18:4*n*−3	1.90	1.92	1.61	1.63	1.49	1.50	1.40	1.46
20:4*n*−6	0.69	0.74	0.81	0.78	0.70	0.76	0.84	0.88
20:4*n*−3	0.79	0.79	0.70	0.69	0.70	0.64	0.60	0.60
20:5*n*−3	7.76	7.95	7.40	7.64	6.23	6.14	6.16	6.55
22:5*n*−6	0.45	0.45	0.40	0.40	0.39	0.37	0.35	0.36
22:5*n*−3	2.29	2.28	2.24	2.27	2.44	2.26	2.20	2.22
22:6*n*−3	12.83	13.57	14.57	14.64	16.36	14.91	15.35	15.05
Polyenoic	33.74	34.64	33.94	34.29	34.71	33.20	33.11	33.38
n−6	5.81	5.72	5.32	5.27	5.43	5.56	5.36	5.52
n−3	26.65	27.57	27.37	27.78	28.12	26.31	26.51	26.67
n−6/*n*−3	0.22	0.21	0.19	0.19	0.19	0.21	0.20	0.21
Total	100	100	100	100	100	100	100	100

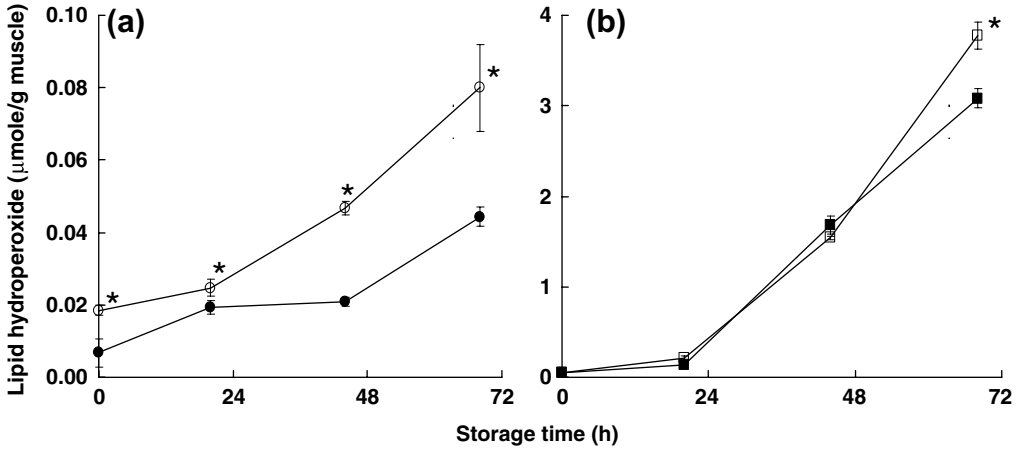

Fig. 1. Changes in lipid hydroperoxide in ordinary and dark muscles of yellowtail during ice storage. (a) Ordinary muscle and (b) Dark muscle. For muscle, significant differences ($P < 0.01$) between muscles with and without bleeding treatment are indicated by asterisks. ○, Ordinary muscle with bleeding treatment; ●, ordinary muscle without bleeding treatment; □, dark muscle with bleeding treatment; ■, dark muscle without bleeding treatment.

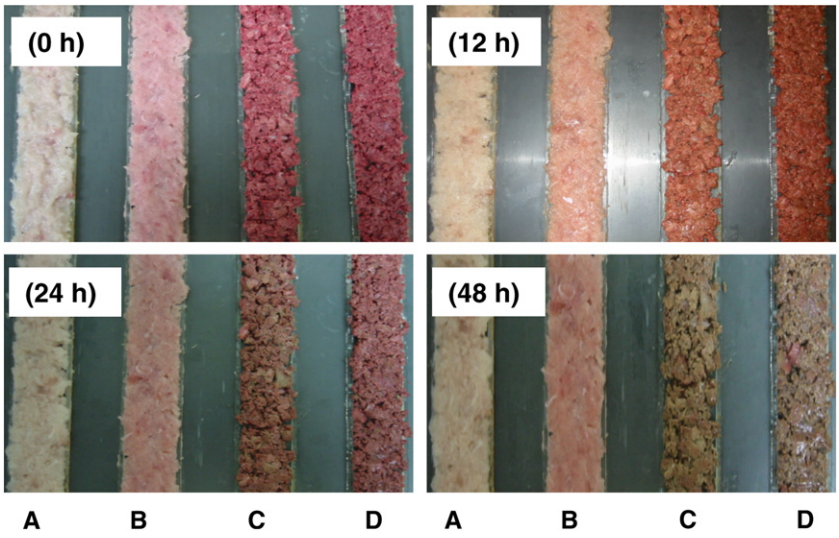

Fig. 2. Changes in the color tones of minced ordinary and dark muscles of yellowtail during ice storage for 48 h. A: Ordinary muscle with bleeding treatment; B: Ordinary muscle without bleeding treatment; C: Dark muscle with bleeding treatment; D: Dark muscle without bleeding treatment.

higher and increased more rapidly than those of ordinary muscles. The lipid hydroperoxide content of dark muscle without bleeding treatment was significantly ($P < 0.01$) less than that of dark muscle with bleeding treatment after 68 h of ice storage.

3.3. Changes in the color of fish muscles

The changes in the color tones of minced ordinary and dark muscles during 48 h of ice storage are shown in Fig. 2. The colors of ordinary muscles with and without bleeding treatment were white and pink, respectively. The pink color of ordinary muscle without bleeding was mainly due to higher amounts of residual blood in the muscle than the residual blood remaining in ordinary muscle with bleeding. No dramatic color changes were observed for ordinary muscles with and without bleeding treatment throughout the ice storage. On the other hand, the colors of dark muscles with and without bleeding treatment changed from reddish-brown to dark brown after 12 h of ice storage and a rancid off-odor developed after 24 h of ice storage (data not shown).

3.4. Formation of metmyoglobin

The percentages of metmyoglobin in ordinary and dark muscles are shown in Fig. 3. The metmyoglobin percentages of ordinary muscles with and without bleeding treatment were 4.6% and 0.2%, respectively, at 0 h of ice storage, and increased to 30.9% and 33.9%, respectively, after 48 h of ice storage. The metmyoglobin percentages of ordinary muscles were low and increased slowly during ice storage. There was no significant difference ($P < 0.01$) in the metmyoglobin percentage between ordinary muscles with and without bleeding treatment. On the other hand, the metmyoglobin percentages of dark muscles were high and increased rapidly with increasing storage time. The percentage of metmyoglobin increased rapidly from 20% to 70% after 48 h of ice storage, but there was no significant difference ($P < 0.01$) between dark muscles with and without bleeding treatment.

3.5. Changes in the contents of α-tocopherol

The changes in the α-tocopherol contents of yellowtail ordinary and dark muscles during ice storage are illustrated in Fig. 4. The α-tocopherol content of ordinary muscle without bleeding treatment was significantly higher ($P < 0.01$) than that of ordinary muscle with bleeding treatment throughout the ice storage. It is well known that α-tocopherol in muscles plays a role in preventing lipid peroxidation (Scaife, Onibi, Murray, Fletcher, & Houlihan, 2000). The α-tocopherol remaining in ordinary muscle

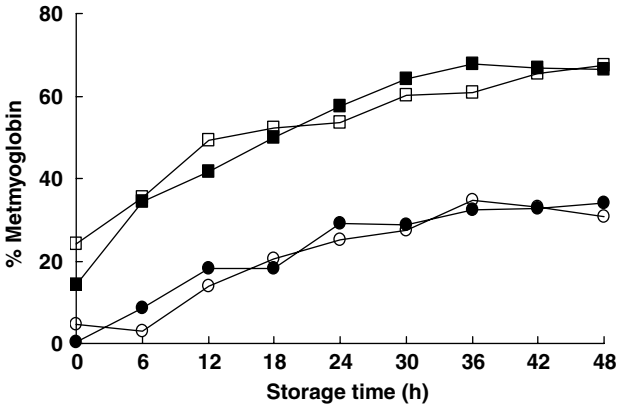

Fig. 3. Changes in the percentage of metmyoglobin in ordinary and dark muscles of yellowtail during ice storage for 48 h. ○, Ordinary muscle with bleeding treatment; ●, ordinary muscle without bleeding treatment; □, dark muscle with bleeding treatment; ■, dark muscle without bleeding treatment.

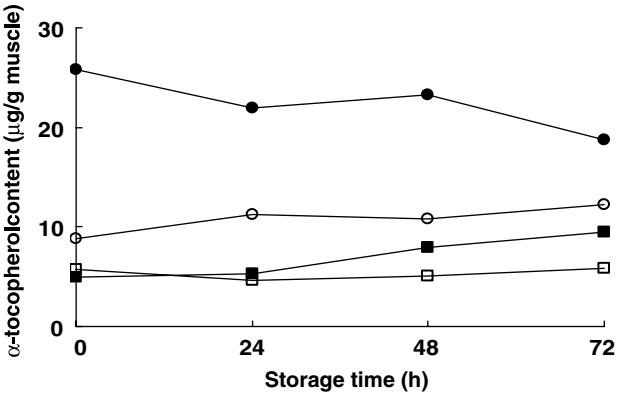

Fig. 4. Changes in α-tocopherol in ordinary and dark muscles of yellowtail during ice storage for 72 h. ○, Ordinary muscle with bleeding treatment; ●, ordinary muscle without bleeding treatment; □, dark muscle with bleeding treatment; ■, dark muscle without bleeding treatment.

without bleeding treatment may scavenge free radicals involved in the initiation and propagation steps of lipid peroxidation. Contrary to this, the α-tocopherol contents in dark muscles with and without bleeding treatment were below 10 μg/g muscle and remained unchanged during ice storage period for 72 h.

3.6. Changes in pH

The changes in pH of ordinary and dark muscles of yellowtail during ice storage are shown in Fig. 5. All the pH except for that of ordinary muscle with bleeding treatment fluctuated between 6.4 and 6.1 throughout the ice storage. Between 0 to 6 h of ice storage, the pH of ordinary muscle with bleeding treatment decreased rapidly and became significantly lower ($P < 0.01$) than these of ordinary muscle without bleeding treatment. On the other hand, the pH of dark muscles showed no significant different ($P < 0.01$) between with and without bleeding treatment during ice storage.

3.7. Effects of perfusion and antioxidants addition on hydroperoxide accumulation

The changes in the lipid hydroperoxide contents of dark muscles in the physiological saline (control), Trolox® and ascorbic acid perfusion groups are shown in Fig. 6. The total hydroperoxide contents of all three groups were low and remained unchanged ($P < 0.01$) from 0 to 21 h of ice storage. The lipid hydroperoxide contents of the control and ascorbic acid groups increased rapidly after 21 h of ice storage, and increased up to 304 and 337 nmol/g muscle, respectively, after 93 h of ice storage. In contrast, the total hydroperoxide content of the Trolox® group was low increased slightly with prolongation of the storage time to 93 h, and was significantly lower ($P < 0.01$) than those of the control and ascorbic acid groups between 45 and 93 h of ice storage. Thus, perfusion of Trolox® remarkably suppressed accumulation of lipid hydroperoxide in dark muscle, whereas perfusion of ascorbic acid was not sufficient to control the accumulation in dark muscle under the same conditions.

3.8. Changes in metmyoglobin formation

The changes in metmyoglobin formation in yellowtail dark muscles during ice storage are shown in Fig. 7. The metmyoglobin percentages in the control, Trolox® and ascorbic acid groups were 25%, 27% and 26%, respectively, at the beginning of ice storage, and increased to 47%, 45% and 42%, respectively, after 68 h of ice storage. The formation of metmyoglobin slightly increased with prolongation of the storage period in all groups. The formation rates of metmyoglobin were lower in the Trolox® and ascorbic acid

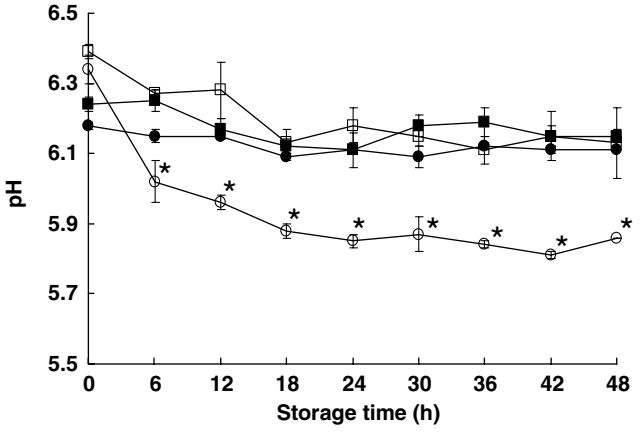

Fig. 5. Changes in pH in ordinary and dark muscles of yellowtail during ice storage for 48 h. For ordinary muscle, significant differences ($P < 0.01$) between muscles with and without bleeding treatment are indicated by asterisks. ○, Ordinary muscle with bleeding treatment; ●, ordinary muscle without bleeding treatment; □, dark muscle with bleeding treatment; ■, dark muscle without bleeding treatment.

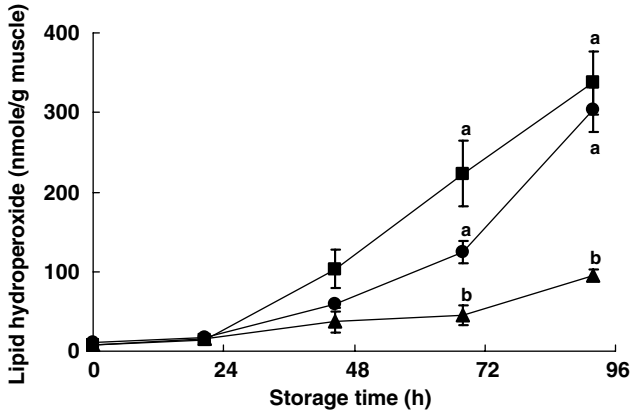

Fig. 6. Changes in lipid hydroperoxide in dark muscles of yellowtail previously treated by perfusion during ice storage for 92 h. Within each muscle type, different letters for a fixed storage period indicate a significant difference ($P < 0.01$). ●, Control; ■, 500 ppm of ascorbic acid; ▲, 500 ppm Trolox®.

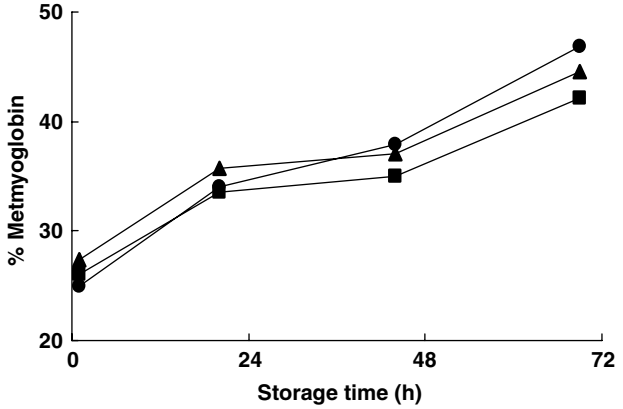

Fig. 7. Changes in the percentage of metmyoglobin in dark muscles of yellowtail during storage for 68 h. ●, Control; ■, 500 ppm of ascorbic acid; ▲, 500 ppm Trolox®.

groups than in the control group, although significant differences were not obtained.

4. Discussion

In the present study, the ordinary muscle with bleeding treatment was more pro-oxidative than the ordinary muscle without bleeding and the bled dark muscle was prooxidative compared to the unbled dark muscle after 68 h of ice storage. Moreover, the lipid hydroperoxide content in ordinary muscle with bleeding treatment was higher and subsequently increased compared with ordinary muscle without bleeding treatment. Porter et al. (1992) reported that there was no significant difference in lipid oxidation between salmon muscles with and without bleeding treatment during frozen storage. Itazawa, Takeda, and Yamamoto (1983) reported that the whole volume of blood in yellowtail was 48.9 ± 10.7 ml/kg. Bleeding treatment of yellowtail was reported to remove a blood volume of 12.8 ± 1.2 ml/kg, being equivalent to $49.6 \pm 6.0\%$ of the blood cells (Ando et al., 1999). These results suggest that bleeding treatment is not sufficient to reduce hemoglobin to a low concentration enough to reduce lipid oxidation, although removal of blood from the muscles delays muscle softening. On the other hand, a difference in the amounts of lipid hydroperoxide was confirmed between ordinary and dark muscles. The lipid hydroperoxide content in dark muscle was much higher and increased rapidly compared to that in ordinary muscle. The higher lipid oxidation in dark muscle may be due to the higher lipid and heme protein contents. The hemoglobin and myoglobin contents in ordinary and dark muscles of yellowtail were reported to be 30 and 560–800 mg/100 g muscle, respectively (Matsuura & Hashimoto, 1959). The high contents of hemoglobin and myoglobin in dark muscle may explain the susceptibility to lipid oxidation of dark muscle compared to ordinary muscle, suggesting that lipid oxidation of yellowtail whole muscle is predominantly due to dark muscle.

A drastic color changes in dark muscles compared to that in ordinary muscles are mainly due to higher heme protein contents in dark muscles. The formation of metmyoglobin by the oxidation of myoglobin predominantly in dark muscle promotes discoloration and leads to the rancid off-odor in dark muscle during ice storage.

Ascorbic acid is used to prevent undesirable oxidative changes in fresh meat and various foodstuffs. Indeed, addition of ascorbic acid was reported to prevent lipid oxidation and color changes in ground beef (Ahn & Nam, 2004). On the other hand, ascorbic acid sometimes acts as a prooxidant in foods. Ohshima, Wada, and Koizumi (1988) reported that addition of ascorbic acid accelerated lipid oxidation in cooked mackerel meat during storage at 4 °C, and a prooxidant effect of ascorbic acid was also observed in homogenates of Japanese oyster (Hatate & Kochi, 1992). Yin, Faustman, Riesen, and Williams (1993) reported that the prooxidant effect was further enhanced by increasing the concentration of ascorbic acid. Moreover, ascorbic acid acted as a prooxidant at lower concentrations (Apte & Morrissey, 1987), but as an antioxidant at higher concentrations when added to cooked beef meat (Sato & Hegarty, 1971). Therefore, the functions of ascorbic acid added to flesh are influenced by many factors, including unsaturated fatty acids, enzymes and metal ions, as well as the storage conditions. In the present study, addition of ascorbic acid to the dark muscle of yellowtail by perfusion treatment did not delay the accumulation of lipid hydroperoxide. The radical species generated from oxidized ascorbic acid may act as a prooxidants, and accelerate the accumulation of lipid hydroperoxide in dark muscle.

α-Tocopherol is an active antioxidant in a number of food systems. High residual contents of α-tocopherol in ordinary muscle without bleeding treatment compared to that in ordinary muscle with bleeding treatment was more effective in terms of controlling lipid hydroperoxide formation. Indeed, the pH of ordinary muscle with bleeding treatment became significantly lower than that of ordinary muscle without bleeding treatment in the present study as shown in Fig. 5. The decline in muscle pH is one of the factor to accelerate hemoglobin-mediated lipid oxidation (Chiba et al., 1991; Yin & Faustman, 1993).

Trolox® is a hydrophilic analogue of tocopherol in which a carboxylic-acid group is replaced by a phytol side chain of tocopherol (Cort et al., 1975). The effects of Trolox® against oxidative damage, particularly against lipid and myoglobin oxidation, have been reported in bovine muscle (O'Grady, Monahan, & Brunton, 2001; Mielnik, Aaby, & Skrede, 2003; Nam & Ahn, 2003). In the present study, Trolox® was observed to show more active antioxidant effects compared to ascorbic acid treatment. Thin layer tests in soybean oil revealed that the comparative antioxidant activity decreased in the order tertiary butylhydroquinone (TBHQ) = Trolox® > ascorbyl palmitate (AP) > butylated hydroxytoluene (BHT) > butylated hydroxyanisole (BHA) > α-tocopherols. For chicken fat, the

antioxidant activity decreased as follows: TBHQ = Trolox® > BHA > BHT > α-tocopherols > AP (Cort et al., 1975). Trolox® delayed the accumulation of lipid hydroperoxide compared to the control groups in the present study, suggesting that Trolox® acts as a scavenger of free radicals under the hydrophilic conditions of fish muscles in the early stage of metmyoglobin formation.

5. Conclusions

A simple bleeding treatment did not suppress lipid and myoglobin oxidation in yellowtail muscles during ice storage. In dark muscle lipid oxidation proceeded more rapidly compared to ordinary muscle. These results suggest that myoglobin in dark muscle which is usually 47-times higher than that in ordinary muscle contributed to the onset of lipid oxidation in dark muscle. Hemoglobin that remained in the dark muscle after bleeding may also have promoted lipid oxidation. The relative percentage of hemoglobin and myoglobin on a weight basis in dark muscle from bled mackerel was 56% hemoglobin and 44% myoglobin (Richards & Hultin, 2002). Hemoglobin was more effective than myoglobin in oxidizing lipids in lipoproteins (Grinshtein, Bamm, Tsemakhovich, & Shaklai, 2003). Decline in ordinary muscle pH due to bleeding (by some removal of buffering components in blood) may explain why ordinary muscle from bled fish underwent lipid oxidation more rapidly than ordinary muscle from unbled fish. Residual hemoglobin may be the primary promoter of lipid oxidation in ordinary muscle since myoglobin content in ordinary muscle is very low (Matsuura & Hashimoto, 1959). Only hemoglobin was present in extracts from ordinary muscle of unbled and bled mackerel while myoglobin was not detected (Richards & Hultin, 2002). Addition of ascorbic acid to yellowtail dark muscle delayed the formation of metmyoglobin but could not inhibit the accumulation of lipid hydroperoxides, while the stronger antioxidant effects of Trolox® significantly suppressed the accumulation of lipid hydroperoxides in yellowtail dark muscle during ice storage.

In conclusion, oxidation of lipid and heme pigments is the main cause of the development of undesirable odor and unpleasant color during ice storage of yellowtail muscle. Removing a portion of the total hemoglobin from yellowtail muscle by bleeding did not prevent the oxidation of lipid and myoglobin. Residual hemoglobin content in fish muscle after bleeding is substantial; on average the percentages of hemoglobin removed from mackerel ordinary and dark muscles by bleeding treatment were 44% and 23%, respectively (Richards & Hultin, 2002). These results suggested that the bleeding treatment of yellowtail may be insufficient to remove enough blood to prevent lipid oxidation. However, bleeding treatment is commonly carried out for fishing-up and live tuna to delay significantly the development of fishy smell and muscle color darkening. An improvement in the oxidative stability of yellowtail muscle during ice storage may be achieved by the addition of an antioxidant such as Trolox®. However, Trolox® has been not accepted as food additive. Further studies will be required to establish the appropriate level of Trolox® relative to the levels of myoglobin and total lipid that provides the best protection against oxidation.

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References

Ahn, D. U., & Nam, K. C. (2004). Effects of ascorbic acid and antioxidants on color, lipid oxidation and volatiles of irradiated ground beef. *Radiation Physics and Chemistry, 71*, 149–154.

Ando, M., Nishiyabu, A., Tsukamasa, Y., & Makinodan, Y. (1999). Postmortem softening of fish muscle during chilled storage as affected by bleeding. *Journal of Food Science, 64*, 423–428.

Apte, S., & Morrissey, P. A. (1987). Effect of haemoglobin and ferritin on lipid oxidation in raw and cooked muscle systems. *Food Chemistry, 25*, 127–134.

Bito, M. (1965). Studies on the retention of meat color of frozen tuna-II. Effect of storage temperature on preventing discoloration of tuna meat during freezing storage. *Bulletin of the Japanese Society of Scientific Fisheries, 31*, 534–539.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*, 911–917.

Box, G. E. P., Hunter, W. G., & Hunter, J. S. (1978). *Statistics for experimenters*. New York: Wiley, pp. 21–56.

Chan, W. K. M., Faustman, C., Yin, M., & Decker, E. A. (1997). Lipid oxidation induced by oxymyoglobin and metmyoglobin with involvement of H_2O_2 and superoxide anion. *Meat Science, 46*, 181–190.

Chiba, A., Hamaguchi, M., Kosaka, M., Tokuno, T., Asai, T., & Chichibu, S. (1991). Quality evaluation of fish meat by ^{31}phosphorus-nuclear magnetic resonance. *Journal of Food Science, 56*, 660–664.

Cort, W. M., Scott, J. W., Araujo, M., Mergens, W. J., Cannalonga, M. A., Osadca, M., et al. (1975). Antioxidant activity and stability of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. *Journal of the American Oil Chemists Society, 52*, 174–178.

Flechtenmacher, W. (1975). Bleeding of cod on board factory trawlers. *Archiv Fur Fischereiwissenschaft, 26*, 53–56.

Grinshtein, N., Bamm, V. V., Tsemakhovich, V. A., & Shaklai, N. (2003). Mechanism of low-density lipoprotein oxidation by hemoglobin-derived iron. *Biochemistry, 42*, 6977–6985.

Hatate, H., & Kochi, M. (1992). Effect of antioxidants on lipid oxidation in homogenate of Japanese oyster during storage at 4 and −25 °C. *Nippon Suisan Gakkaishi, 58*, 2397.

Itazawa, Y., Takeda, T., & Yamamoto, K. (1983). Determination of circulating blood bolume in three teleosts, carp, yellowtail and porgy. *Japanese Journal of Ichthyology, 30*, 94–101.

Koizumi, C., Wada, S., & Ohshima, T. (1987). Factors affecting development of rancid odor in cooked fish meats during storage at 5 °C. *Nippon Suisan Gakkaishi, 53*, 2003–2009.

Love, J. D., & Pearson, A. M. (1974). Metmyoglobin and non-haem iron as prooxidants in cooked meat. *Journal of Agricultural Food Chemistry, 22*, 1032–1034.

Matsuura, F., & Hashimoto, K. (1959). Chemical studies on the red muscle (“Chiai”) of fishes-X.A new method for determination of myoglobin. *Bulletin of the Japanese Society of Scientific Fisheries, 24*, 809–815.

Mielnik, M. B., Aaby, K., & Skrede, G. (2003). Commercial antioxidants control lipid oxidation in mechanically deboned turkey meat. *Meat Science, 65*, 1147–1155.

Mochizuki, S., Norita, Y., & Maeno, K. (1998). Effects of bleeding on post-mortem changes in the muscle of horse mackerel. *Nippon Suisan Gakkaishi, 68*, 276–279.

Nam, K. C., & Ahn, D. U. (2003). Use of antioxidants to reduce lipid oxidation and off-odor volatiles of irradiated pork homogenates and patties. *Meat Science, 63*, 1–8.

O'Grady, M. N., Monahan, F. J., & Brunton, N. P. (2001). Oxymyoglobin oxidation and lipid oxidation in bovine muscle-Mechanistic studies. *Journal of Food Science, 66*, 386–391.

Ohshima, T., Wada, S., & Koizumi, C. (1988). Influences of heme pigment, non-heme iron, and nitrite on lipid oxidation in cooked mackerel meat. *Nippon Suisan Gakkaishi, 54*, 2165–2171.

Porter, P. J., Kennish, J. M., & Kramer, D. E. (1992). The effects of exsanguinations of sockeye salmon on the changes in lipid composition during frozen storage. In E. G. Bligh (Ed.), *Seafood science and technology* (pp. 76–83). Oxford: Fishing News Books.

Richards, M. P., & Hultin, H. O. (2002). Contributions of blood and blood components to lipid oxidation in fish muscle. *Journal of Agricultural and Food Chemistry, 50*, 555–564.

Sato, K., & Hegarty, G. R. (1971). Warmed-over flavor in cooked meats. *Journal of Food Science, 36*, 1098–1102.

Scaife, J. R., Onibi, G. E., Murray, I., Fletcher, T. C., & Houlihan, D. F. (2000). Influence of a-tocopherol acetate on the short- and long-term storage properties of fillets from Atlantic salmon Salmo salar fed a high lipid diet. *Aquaculture Nutrition, 6*, 65–71.

Sohn, J. H., Taki, Y., Ushio, H., Kohata, T., Shioya, W., & Ohshima, T. (2005). Lipid oxidations in ordinary and dark muscles of fish. Influence on rancid off-odor development and color darkening of yellowtail flesh during ice storage. *Journal of Food Science, 70*, S490–496.

Sohn, J. H., Taki, Y., Ushio, H., & Ohshima, T. (2005b). Quantitative determination of total lipid hydroperoxides by a flow injection analysis system. *Lipids, 40*, 203–209.

Terayama, M., & Yamanaka, H. (2000). Effects of bleeding on the quality of skipjack. *Nippon Suisan Gakkaishi, 66*, 852–858.

Yamamoto, Y. (1980). In S. Ikeda (Ed.), *Minor constituents in marine foods – These biochemical and food chemical characteristics* (pp. 232–236). Tokyo: Koseisha-Koseikaku.

Yin, M. C., & Faustman, C. (1993). Influence of temperature, pH, and phospholipid composition upon the stability of myoglobin and phospholipid: A liposome model. *Journal of Agricultural Food Chemistry, 41*, 853–857.

Yin, M. C., Faustman, C., Riesen, J. W., & Williams, S. N. (1993). α-Tocopherol and ascorbate delay oxymyoglobin and phospholipid oxidation in vitro. *Journal of Food Science, 58*, 1273–1276.