魚肉貯蔵・加工時における有毒アルデヒド，4－ヒドロキシヘキセナールの生成と抑制

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はしがき

脂質過酸化により生じるn-3高度不飽和脂肪酸（PUFA）より生じる4-ヒドロキシヘキセナール（HHE）は強い肝臓毒性および変異原性を持ち、食品衛生上問題となる。また、HHEは反応性に富み、タンパク質の変性や栄養の低下を引き起こす。そこで、市販の数種魚種筋肉および魚肉加工食品のHHEおよびマロンアルデヒド（MA：脂質過酸化の指標）含量を測定する。さらに、ブリ筋肉を0および-20Cに貯蔵した際、HHE、MAおよびn-3高度不飽和脂肪酸（PUFA）含量の変動を明らかにした。ついで、水産スモーク食品中のHHEおよびMA含量を測定後を、サクラおよびスギ木酢液添加による脂質過酸化の抑制についても検討した。さらに，すり身食品中のHHEおよびMA含量を測定するとともに，NaCl添加はブリ肉中のHHEの生成を抑制するがマダイ肉中ではHHE生成に影響を示さず，カンパチでは促進することを明らかにした。また，ブリ肉中ではHHEは脂質過酸化の進行が抑制された状況で促進し，逆に脂質過酸化が抑制されている状況では促進する可能性を示した。エピガロカテキンガレート添加は魚肉中の脂質過酸化およびHHE生成を抑制するが，αートコフェロール添加は脂質過酸化を促進しHHEの生成を抑制した。βーカロテンおよびアスタキサンチン添加はHHE生成を抑制できなかった。以上の結果を元に、HHEおを生じない安全で栄養価の低下しない魚肉および加工食品を供給するための貯蔵・加工条件を明らかにするした。以上の結果についてのとりまとめたものが、この報告書である。

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2.境　正・DMSムナシンハー・市丸憲一郎・植木暢彦・龍野巳代・河原　聡，すり身製品中の脂質過酸化由来アルデヒド4-ヒドロキシ-2-ヘキセナール生成に及ぼす食塩の影響，日本水産学会大会，2003年4月2日

カテキン添加実験

目的

魚肉保存におけるカテキンの脂質過酸化抑制作用と，脂質過酸化関連物質であるマロンジアルデヒド(以下MA)および4‐ヒドロキシヘキセナール(以下HHE)との関係を明らかにするため実験を行った。MAはPUFAの過酸化によってできる最終産物であり，このMA含量(TBA値)は食品および生体組織の酸化の指標として用いられている。HHEはn-6脂肪酸の過酸化によって生じるα，β不飽和アルデヒドである。このα，β不飽和アルデヒドには，多くの種類が存在することが確認されているが，中でもこのHHEは非常に強い細胞毒性を持つことで知られている。

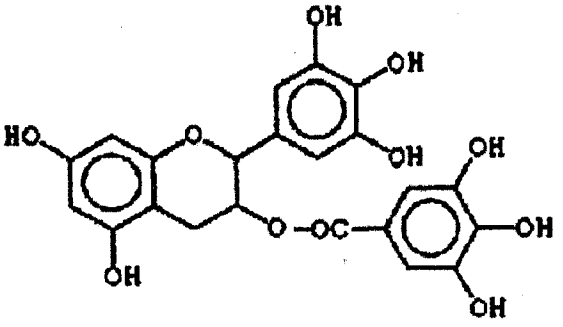
そこで，カテキンを試料に添加後4℃にて貯蔵し，脂質過酸化関連物質であるMA，HHEおよびCP含量の変動を測定した。

実験方法

1) 試薬

MAおよび HHE　測定用試薬

1.3-Diethyl-1-2-thiobarbituric acid (以下DETBA)は，Aldrich Chemicalsより購入した。(－)-EpigallocatechinGallateは和光純薬株式会社より購入した。構造式を以下に示す。（$C_{22}H_{18}O_{11}$＝458.38）



2) 試料

試料(カンパチ)は，一般の市場で購入した。皮および内臓を取り除き，脂身と血合筋を削除した後，筋肉組織のみをフードプロセッサーで細かく刻んだ。その試料を二等分した後，0.5%となるようにエピガロカテキンを添加した区とControl区の2試験区を調整した。それぞれをポリエチレンバックに入れて4℃で貯蔵後，試料のMAおよびHHE含量0，3，7日目に測定した。

3) MA含量の測定方法

試料をテフロンホモジナイザーに0.5gずつ正確に秤量し，生理的食塩水を20m加えホモジネートを作成した。その0.5mlを栓付試験管に採取し，0.125Mリン酸緩衝液（pH3，0.4%SDS，10mM DETBA，4mM BHTを含む）を3.5ml加えた。100℃のヒーターブロックで150分間反応させた。反応後，氷中冷却し，酢酸エチルを4ml加え，激しく振盪した。2500rpmで15分間遠心分離を行い，その上清0.5mlを共栓付試験管に採取し，エバポレーターで濃縮乾固した。これを200μlのメタノールで再溶解し，その20μlを以下の方法にてHPLC分析を行った。

分析用のポンプにはBIP I pump（日本分光工業）を，インジェクターには7725 Injecter（島津製作所），カラムにはInertsil ODS(5μm particle size 250×4.6mm i.d；GL Science)をそれぞれ用いた。検出波長はEx515nm-Em555nmを用い，検出器にはRF-10A分光蛍光検出器（島津製作所）を用いた。展開溶媒は，アセトニトリル：0.1M NaCl溶液＝3：1を用いた。なお溶出量は1ml/minとした。

4) HHE含量の測定方法

試料を三角フラスコに1.5gずつ正確に秤量し，試料に対して0.5%のBHTを添加した。この三角フラスコに2.5mM DNPHを含んだ1N-HClを20ml加え，低温(4℃)暗所で2時間放置し，抽出と誘導化を同時に行った。反応後，濾紙で濾過し，3倍量のジクロロメタンを加えて激しく撹拌し，分液漏斗を用いて，HHE-DNPH誘導体を含む下層をなす型フラスコに採取した。この操作を2回繰り返した。そのなす型フラスコをエバポレーターで濃縮乾固した。得られた残渣を2mlのクロロホルムで溶解し，あらかじめ3mlのヘキサン：クロロホルム＝2：1で洗浄したSilica Gel

Disposable Extraction Columnに注入した。注入後，ヘキサン：クロロホルム＝2：1混合液3mlを2回カラムに注入し試料を展開させ，HHE-DNPH誘導体を含むバンドを分離させた。分離したバンドをクロロホルム6mlで溶解させ，溶出液を10ml容共栓付褐色試験管に採取し，濃縮乾固した。これを500μlのメタノールで再溶解しHPLC用プレフィルターで濾過後，その20μlを以下の方法にてHPLC分析を行った。

結果と考察

結果をまとめて図に示した。カテキン添加区ではMAの増加はcontrol区に比べ有意に低く，脂質過酸化の進行は抑制された。しかしながらHHEに関してはカテキン添加により統計学的に有意ではないが増加の傾向を示した。従って，カテキン添加は脂質過酸化の進行を抑制するが，HHEの生成にかんしては抑制効果を示さなかった。

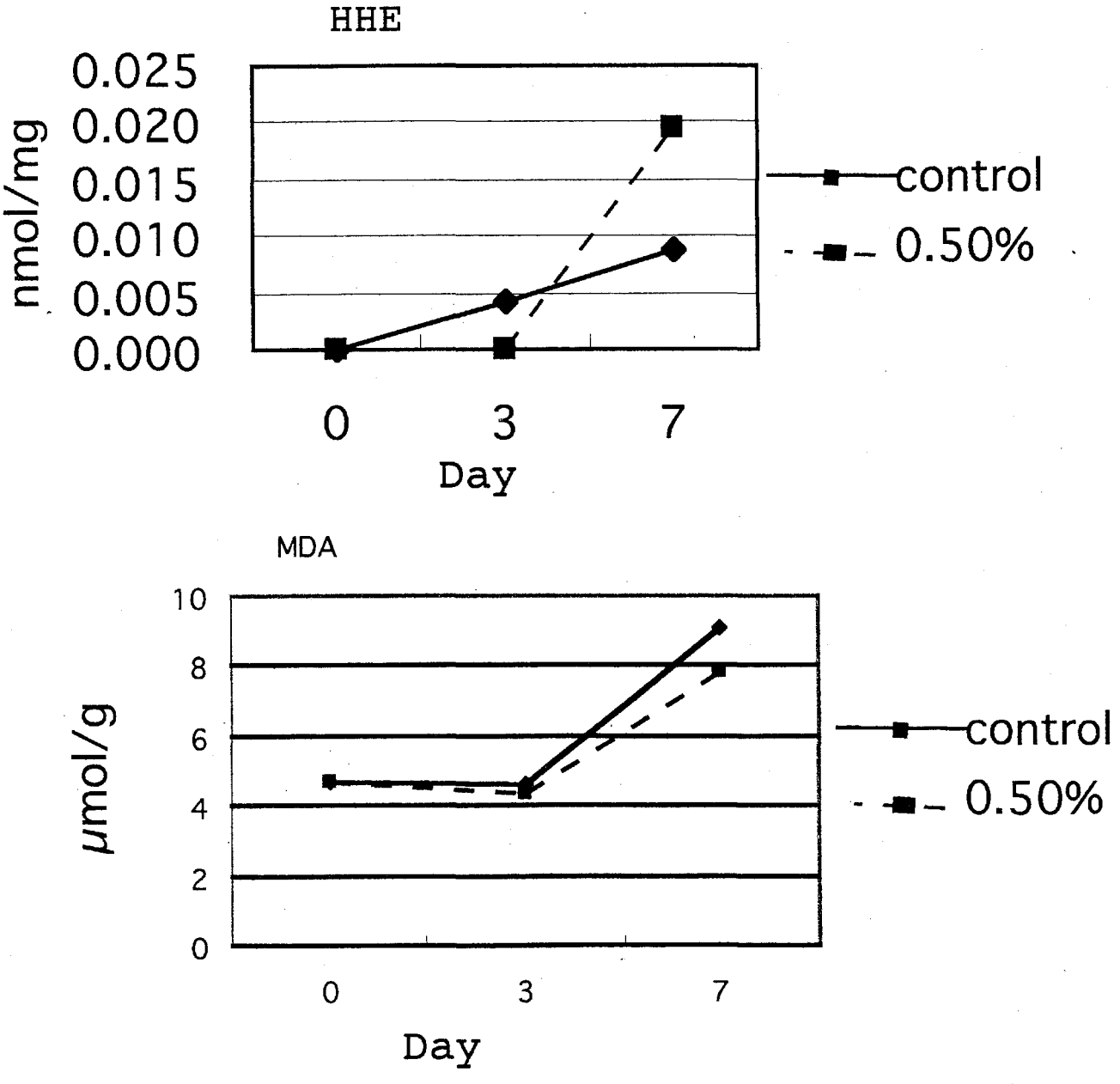


図1. カテキン添加によるHHEおよびMA含量の変動

The formation of 4-hydroxy-2-hexenal, a hepatotoxic aldehyde derived from n-3 fatty acids, may be suppressed in the yellowtail meats in which lipid oxidation progresses

Running title: HHE formation in yellowtail meats

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Abstract

Changes in 4-hydroxyhexenal (HHE) and malonaldehyde (MA) contents were investigated in yellowtail Seriola quinqueradiate meats containing 0, 0,1, 0.3, 0.6 and 0.9 M NaCl stored at 0 °C for seven days. The HHE contents in the meat containing NaCl were significantly lower than those of the control after seven days of storage. Judging from the MA contents, NaCl may act as a pro-oxidant in the meats. Changes in the HHE and MA contents were also analyzed in the meat of the control, those containing 0.3 M NaCl, 30 μM $FeSo_4$, and 0.3 M NaCl+30 μM $FeSo_4$. HHE contents in the meats containg 0.3 M NaCl+30 μM $FeSo_4$ were significantly lower than those of the ontrol after seven days of storage. Judging from MA contents, NaCl+$FeSo_4$ may act as pro-oxidant in the meats. The present results indicate that HHE formation may be supressed in the meats in which lipid oxidation progresses.

Key words: Yellowtail; NaCl; 4-Hydroxy-2-hexenal; Malonaldehyde

1. Introduction

Lipid peroxidation is a major cause of quality deterioration in meat products (Ladikos & Lougovios, 1990). Fish is more prone to lipid peroxidation than meat, due to the high degree of unsaturation in fish lipids and also due to the high concentrations of metals in seafood (Khayat & Schwall, 1983). The n-3 series of polyunsaturated fatty acids are predominant in fish tissues. During lipid peroxidation, n-3 polyunsaturated fatty acids (PUFA) yield 4-hydroxy-2E-hexenal (HHE) (Van Kuijk, Holte & Dratz, 1990), which is α,β-unsaturated aldehyde. Crotonaldehyde administered in drinking water was shown to cause liver tumors in F344 rats (Chung, Tanaka & Hecht, 1986), suggesting that α,β-unsaturated aldehydes may be potential carcinogens (Witz, 1989). There are many papers reporting cytotoxicity of HHE (Brembilla, et al., 1986, Müller, et al., 1996, Sakai, Sugamoto & Eto, 2000). We have reported that HHE exists in the muscle of some fish (Sakai, Matsushita, Sugamoto & Uchida, 1997). Also, HHE increased in yellowtail meats stored at -20 °C (Sakai, et al., 2000) or containing cherry and sugi wood vinegar stored at 0 °C (Munasinghe, et al., 2003), although malonaldehyde (MA) contents scarcely increased in these meats. These results strongly suggest that HHE formation may accelerate in the yellowtail meats in which lipid oxidation is suppressed. To understand the formation mechanism of HHE in fish meats, it is necessary to clearify whether the HHE formation accelarates or suppresses in the meats in which lipid oxidation progresses.

Therefore, we investigated whether addition of NaCl and $FeSO_4$ which are known to accelerate the lipid oxidation in various kinds of meats (Ahn, Wolfe & Sim, 1993, Kanner, Harel & Jaffe, 1991, Ohshima, Wada & Koizumi, 1988, Osinchak, Hultin, Zajicek, Kelleher & Huang, 1992) accelerates or suppresses the formation of HHE in yellowtail meats.

2.Materials and Methods

2.1. Materials.

HHE was synthesized by the method of Sugamoto, Matsushita and Matsui (1997) and identified by ^{1}H-NMR and ^{13}C-NMR spectra. Yellowtail (Seriola quinqueradiata) samples were obtained from commercial markets. Butyl hydroxy toluene (BHT) was obtained from Tokyo Kasei (Tokyo, Japan), 2,4-dinitrophenylhydrazine (DPNH) was obtained from Wako Pure Chemicals (Tokyo, Japan), and 1,3-diethyl-2-thiobarbituric acid (DETBA) was obtained from Aldrich Chemicals (Milwaukee, WI. USA). Other reagents were of analytical grade.

2.2. Storage experiments.

The ordinary muscle was ground, mixed with relevant amount of NaCl and $FeSO_4$ and stored at 0°C. In all experiments, 3 samples from each group were analyzed.

2.3. HHE analysis.

2,4-Dinitrophenylhydrazine (DNPH) conversion of HHE essentially followed the procedure of Goldring, Casini, Maellaro, Del Bello and Comporti (1993). Five g of ground meat

was mixed with 25 mg of butyl hydroxy toluene (BHT). It was found in a preliminary experiment that the addition of 0.5% BHT to the ground meat prevented lipid peroxidation. Fifty ml of 1 N HCl containing 2.5 mmol DNPH was added to the BHT mixture, and the reaction was carried out in the dark for 2 h at room temperature. DNPH derivatives were extracted three times with 3 vol of dichloromethane. The dichloromethane extract thus obtained was evaporated *in vacuo* to dryness and redissolved in 0.2 ml of chloroform. The samples were applied to a disposable silica gel extraction column (Baker) which had been preequilibrated with n-hexane/chloroform (2:1 vol/vol). The same solvent mixture was used to wash off the highly lipophilic DNPH derivatives in a discrete band, the remaining material being eluted with chloroform. The resulting chloroform eluate was evaporated *in vacuo* to dryness and redissolved in 0.5 ml of methanol. The HHE-DNPH derivative was analyzed by the HPLC method reported by Sakai et al. (1993) under the following analytical conditions: column, Ultrasphere C18 (25 cm x 4.6 mm i.d., Beckman); mobile phase, 30 mM sodium citrate/27.7 mM acetate buffer (pH 4.75): methanol=35:65; flow rate, 1 ml/min; column temperature, 40°C; detection wavelength, 365 nm. The HHE-DNPH derivative was analyzed by the same method, except that a mobile phase of 30 mM sodium citrate/27.7 mM acetate buffer (pH 4.75): methanol=35:65 was used.

2.4. MA analysis.

The 1,3-diethyl-2-thiobarbituric acid (DETBA) assay is based on the method of Sakai, Habiro and Kawahara (1999). One g of the ordinary muscle were homogenized with 9 vol of ice-cooled 10 mM sodium phosphate buffer (pH 7.0) in a Polytron homogenizer

at 0 °C. An aliquot (less than 0.4ml) of the homogenate was transferred to a screw-capped tube containing 0.2 ml of 8% SDS and 0.2 ml of 20 mM butyl hydroxy toluene in ethanol, and the mixture was finally made up to 0.8 ml with distilled water. After adding 3.2 ml of 12.5 mM DETBA in sodium phosphate buffer (0.125 M, pH 3.0), the solution was mixed and heated in a water bath at 95°C for 15 min, and then cooled quickly with running tap water. To extract the DETBA-MA adduct, 4 ml of ethyl acetate was added, and the mixture was shaken vigorously. An ethyl acetate extract (2.4ml) containing the DETBA-MA adduct was transferred to another tube and evaporated *in vacuo*. The residue was dissolved in 150 μl of methanol, and 10 μl of the sample was applied to HPLC under the following conditions: column, Inertsil ODS (5 μm particle size, 250 x 4.6 mm i.d.; GL Sciences, Tokyo, Japan); mobile phase, acetonitrile-0.1 M sodium chloride (75:25, v/v); flow rate, 1.0 ml/min; detection, excitation 515 nm and emission 555 nm.

2.5. Fatty acid analysis.

Total lipids were extracted from meats by the method of Folch, Lefes and Sloane Stanley (1957). The lipids obtained were hydrolyzed with 0.5 M KOH in methanol at 100 ℃ for 5 min and esterifyed with aqueous HCl (35%)/methanol (1:1 v/v) at 100 ℃ for 5 min. The metyl esters obtained were separated by gas-liquid chromatograph equipped with a capillary column and determined using tricosanoic acid as an internal standard (Takenoyama, Kawahara, Murata & Yamauchi, 1999).

2.4.Statistical Analysis

All data was expressed as a mean ± SE. The results were

analyzed by the analysis of variance (ANOVA) and the significant differences among the means were determined by the Tukey-Kramer test.

3.Results and discussion

Table 1 shows the changes in the HHE and MA contents in yellowtail meat containing 0 and 0.1 M NaCl. After seven days of storage, the HHE contents in the samples containing NaCl were significantly lower than those of the control but the MA contents in the former were significantly higher than those in the latter.

Table 2 shows the changes in the HHE and MA contents in the yellowtail meat containing 0, 0.3, 0.6 and 0.9 M NaCl. All samples showed a significant increase in HHE content after three days of storage compared with that of after zero day of storage. After three days of storage, difference was not observed in the HHE contents in any of the samples. After seven days of storage, the HHE contents in the sample containing 0.9 M NaCl were significantly lower than those of the other samples. The contents in the control were significantly higher than those of samples containing NaCl. After three or seven days, the MA contents of the sample containing NaCl were significantly higher than those of the control. Judging from the changes of the MA contents, NaCl acts as a pro-oxidant in the meat.

Table 3 shows the changes in the contents of total fatty acid, eicosapentaenoic acid and docosahexaenoic acid in the meat containing 0, 0.3, 0.6 and 0.9 M NaCl. Total fatty acids (TFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

contents decreased during storage. This result might suggest that oxidation of these lipids induced the decrease.

Table 4 shows the changes in both aldhdes in the meats of the control and those containing 0.3 M NaCl, 30 μM FeSO4 and 0.3 M NaCl+30 μM $FeSO_4$. In the control, the HHE contents showed significant increase during the storage period. In other samples the contents fluctuated. The HHE contents in the meats containig NaCl+FeSO4 were significantly lower than those of the control. The contents in the meats containing NaCl or FeSO4 seemed to be higher than those of control although the difference was not significant. After three and seven days of storage, MA contents in the meats containing NaCl and/or $FeSO_4$ were significantly higher than those of the control. As shown in Table 5, TFA, EPA and DHA contents decreased during storage. This result might suggest that the xidation of these lipids induced the decrease.

Judging from the MA contents, addition of NaCl or NaCl+$FeSo_4$ accelerates the lipid oxidation in yellowtail meats (Table 1, 2 and 4). The decrease of TFA, EPA and DHA contents also indicates that lipid oxidation progresses in the meats cotaining NaCl and NaCl+$FeSO_4$ (Table 3 and 5). Changes in the HHE contents in the meats containing NaCl and NaCl+$FeSO_4$ indicate that the HHE formation may suppress in the meats in which lipid oxidation progresses. We have reported that the HHE formation may accelerate in yellowtail meats in which lipid oxidation suppresses (Munasinghe, et al., 2003, Sakai, et al., 2000). Esterbauer, Schaur and Zollner (1991)reported that HHE is not an intermediate of the MA formation in vivo. It is also

reported that the HHE reacts readily with sulfhydryl groups of proteins and low molecular weight substances, such as cysteine and glutathione, with ε-amino groups of lysine and with histidine residues in proteins (Esterbauer, et al., 1991) and that MA preferentially reacts with lysine, tyrosine, methionine and arginine in proteins (Buttkus, 1967). The formation and disappearance rate of HHE and MA is thought to be different from each other and the present and previous results in the yellowtail meats might be due to this difference. Considering the toxicity of HHE, it is necessary to examine the formation mechanism of this aldehyde in fish meat products. Studies along these lines are in progress in our laboratory.

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Table 1. Changes in HHE and MA contents in yellowtail meats containing 0 and 0.1 M NaCl stored at 0℃

Days	0	3	7
HHE(nmol/g tissue)			
Control	$0.21\pm0.02^{a,x}$	$0.44\pm0.04^{a,x}$	$2.59\pm0.22^{b,x}$
NaCl 0.1M	$0.21\pm0.02^{a,x}$	$2.68\pm0.29^{b,y}$	$1.79\pm0.17^{c,y}$
MA(μmol/g tissue)			
Control	$0.22\pm0.02^{a,x}$	$0.46\pm0.08^{b,x}$	$0.42\pm0.02^{b,x}$
NaCl 0.1M	$0.22\pm0.02^{a,x}$	$0.63\pm0.02^{b,x}$	$0.52\pm0.02^{c,y}$

a-b Means (n=4) ±SE in the same row with no common superscript differ significantly ($P<0.05$).

x-y Means (n=4) ±SE in same column with no common superscript differ significantly ($P<0.05$).

Table 2. Changes in HHE and MA contents in yellowtail meat containing 0, 0.3, 0.6 and 0.9 M NaCl stored at 0℃

Days	0	3	7
HHE(nmol/g tissue)			
Control	$2.22\pm0.30^{a,x}$	$5.07\pm0.32^{b,xy}$	$3.38\pm0.15^{c,x}$
NaCl 0.3M	$2.22\pm0.03^{a,x}$	$4.88\pm0.27^{b,xy}$	$2.39\pm0.10^{a,y}$
NaCl 0.6M	$2.22\pm0.03^{a,x}$	$5.44\pm0.43^{b,x}$	$2.74\pm0.12^{a,y}$
NaCl 0.9M	$2.22\pm0.03^{a,x}$	$4.21\pm0.11^{b,y}$	$0.16\pm0.12^{a,z}$
MA(μmol/g tissue)			
Control	$0.05\pm0.01^{a,x}$	$0.05\pm0.00^{a,x}$	$0.06\pm0.00^{a,x}$
NaCl 0.3M	$0.05\pm0.01^{a,x}$	$0.12\pm0.01^{b,y}$	$0.18\pm0.01^{c,y}$
NaCl 0.6M	$0.05\pm0.01^{a,x}$	$0.14\pm0.03^{b,y}$	$0.22\pm0.02^{c,z}$
NaCl 0.9M	$0.05\pm0.01^{a,x}$	$0.12\pm0.01^{b,y}$	$0.17\pm0.01^{c,y}$

a-c Means (n=3) ±SE in the same row with no common superscript differ significantly ($P<0.05$).

x-z Means (n=3) ±SE in the same column with no common superscript differ significantly ($P<0.05$).

Table. 3. Changes in contents of total fatty acid (TFA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in yellowtail meat containing 0, 0.3, 0.6 and 0.9 M NaCl after 0 and 7 days of storage stored at 0 °C.

	Control	0.3 M	0.6 M	0.9 M
TFA (mg/100 g tissue)				
0 days	470*	470	470	470
7 days	470	333	344	325
EPA (mg/100 g tissue)				
0 days	26.7	26.7	26.7	26.7
7 days	27.6	15.6	15.0	17.1
DHA (mg/100 g tissue)				
0 days	184	184	184	184
7 days	187	98.5	93.8	93.0

*mean (n=2)

Table 4. Changes in HHE and MA contents in yellowtail meats of control, those containing 0.3 M NaCl, 30 μM $FeSO_4$, and 0.3 M NaCl+30 μM $FeSO_4$ stored at 0℃

Days	0	3	7
HHE(nmol/g tissue)			
Control	$3.80\pm0.56^{a,x}$	$4.25\pm0.52^{b,x}$	$5.62\pm0.58^{c,x}$
NaCl	$3.80\pm0.56^{ab,x}$	$2.19\pm0.35^{a,yy}$	$4.56\pm00.70^{b,x}$
Fe	$3.80\pm0.56^{a,x}$	$5.47\pm0.08^{b,z}$	$3.75\pm0.61^{ab,xy}$
NaCl+Fe	$3.80\pm0.56^{a,x}$	$3.84\pm0.31^{a,x}$	$2.08\pm0.23^{b,y}$
MA(μmol/g tissue)			
Control	$0.23\pm0.04^{a,x}$	$0.72\pm0.05^{b,x}$	$0.66\pm0.01^{b,x}$
NaCl	$0.23\pm0.04^{a,x}$	$1.04\pm0.04^{b,y}$	$1.00\pm0.01^{b,y}$
Fe	$0.23\pm0.04^{a,x}$	$0.97\pm0.07^{b,y}$	$1.68\pm0.03^{c,y}$
NaCl+Fe	$0.23\pm0.04^{a,x}$	$1.89\pm0.06^{b,z}$	$2.14\pm0.16^{c,z}$

a-c Means (n=3) ±SE in the same row with no common superscript differ significantly ($P<0.05$).

x-z Means (n=3) ±SE in the same column with no common superscript differ significantly ($P<0.05$).

Table 5. Changes in contents of total fatty acid (TFA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in yellowtail meat of control, those containing 0.3 M NaCl, 30 μM $FeSO_4$, and 0.3 M NaCl+30 μM $FeSO_4$ stored at 0℃

	Control	NaCl	Fe	NaCl+Fe
TFA (mg/100 g tissue)				
0 days	768*	768	768	768
7 days	790	773	748	736
EPA (mg/100 g tissue)				
0 days	52.6	52.6	52.6	52.6
7 days	52.6	51.5	49.9	46.7
DHA (mg/100 g tissue)				
0 days	155	155	155	155
7 days	160	152	146	135

*mean (n=2)

Effects of NaCl on the formation of lipid peroxidation-derived cytotoxic aldehyde, 4-hydroxy-2E-hexenal in fish meats

Running title: Aldehydes in fish meat containing NaCl

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Effects of NaCl on the formation of lipid peroxidation-derived cytotoxic aldehyde, 4-hydroxy-2E-hexenal in fish meats

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Abstract

The contents of 4-hydroxy-2E-hexenal (HHE), hepatotoxic aldehydes, and malon aldehyde (MA) in some surimi based products were analyzed. Large differences in these contents between the different samples were observed, however low levels of HHE were detected in all surimi-based products. Changes of HHE and MA contents in yellowtail Seriola quinqueradiata meats containing 0, 0.3, 0.6 and 0.9 M NaCl and in red sea bream Pagrus major and great amberjack Seriola dumerili meats containing 0, 0.3, and 0.9 M NaCl stored at 0° were analyzed for 7 days. After 7 days of storage, HHE contents in the yellowtail meats containing NaCl were significantly lower than those of the control, but MA contents in the former were significantly higher than the latter. HHE was only detected in the red sea bream meats containing 0.3 M after 7 days of storage and MA contents were significantly higher in the meats containing NaCl than those of control after 7 days of storage. HHE contents in the great amberjack meats containing NaCl were significantly higher than those of control after 3 and 7 days of storage but difference in MA contents was not observed between each sample after 7 days of storage.

Key words:4-hydroxy-2E-hexenal; lipid peroxidation; surimi based products; NaCl

Introduction

Lipid peroxidation is a major cause of quality deterioration in meat products.[1] Fish is more prone to lipid peroxidation than meat, due to the high degree of unsaturation in fish lipids and to the high concentrations of metals in seafood.[2] The n-3 series of polyunsaturated fatty acids are predominant in fish tissues. During lipid peroxidation, n-3 polyunsaturated fatty acids (PUFA) yield 4-hydroxy-2E-hexenal (HHE),[3] which is α,β-unsaturated aldehyde. Crotonaldehyde administered in drinking water was shown to cause liver tumors in F344 rats,[4] suggesting that α,β-unsaturated aldehydes may be potential carcinogens.[5] There are many papers reporting cytotoxicity of HHE.[6-8] We have reported that HHE exists in the muscle of some fish,[9] and that it increased in yellowtail meats stored at -20 °C [8] or containing commercial,[10] cherry and sugi wood vinegar.[11] From the viewpoint of food hygiene, it is necessary to analyze HHE contents in many kinds of fish meat products.

Sodium chloride (NaCl) is added to muscle foods for a variety of purposes, including flavor and the inhibition of microorganisms. NaCl, nevertheless, has been shown to have an accelerating effect on lipid oxidation in a variety of meats, including beef, pork, chicken, and fish.[12-17] Other observers have seen inhibition in lipid oxidation rates by sodium chloride.[18-21] Kanner et al.[14] found that pro-oxidative activity of NaCl is due to its ability to release iron from heme pigments and other heme binding molecules. However, it is uncertain whether addition of NaCl to fish meat affects the HHE formation or not. Therefore, we analyzed HHE contents in some surimi-based products and investigated the effects of NaCl on HHE

formation in yellowtail, red sea bream and great amberjack meats. As an index of lipid peroxidation, we also analyzed malonaldehyde (MA) content in these samples.

MATERIALS AND METHODS

Materials.

HHE was synthesized by the method of Sugamoto et al.,[22] and identified by 1H-NMR and 13C-NMR spectra. Yellowtail Seriola quinqueradiata, red sea bream Pagrus major, and great amberjacks Seriola dumerili, samples were obtained from commercial markets, as well as surimi-based products (shown in Table 1). Butyl hydroxy toluene (BHT) was obtained from Tokyo Kasei (Tokyo, Japan), NaCl (analytical grade) and 2,4-dinitrophenylhydrazine (DNPH) from Wako Pure Chemicals (Tokyo, Japan), and 1,3-diethyl-2-thiobarbituric acid (DETBA) from Aldrich Chemicals (Milwaukee, WI. USA). Other reagents were of analytical grade.

Storage experiments.

Ordinary muscle was ground, mixed with different amounts of NaCl and stored at 0°C. In all the experiments, 3 or 4 samples from each group were analyzed. HHE and MA contents were analyzed after 0, 3 and 7 days of storage.

HHE analysis.

The 2.4-Dinitrophenylhydrazine (DNPH) conversion of HHE essentially followed the procedure of Goldring et al.[23] Five g of ground meat was mixed with 25 mg of butyl hydroxy toluene (BHT). It was found in a preliminary experiment that the addition of 0.5% BHT to the ground meat prevented lipid peroxidation. Fifty mL of 1 N HCl containing 2.5 mmol DNPH was added to the BHT mixture, and the reaction was carried out in

the dark for 2 h at room temperature. DNPH derivatives were extracted three times with 3 vol of dichloromethane. The dichloromethane extract thus obtained was evaporated *in vacuo* to dryness and redissolved in 0.2 mL of chloroform. The samples were applied to a disposable silica gel extraction column, (Baker) which had been preequilibrated with n-hexane/chloroform (2:1 vol/vol). The same solvent mixture was used to wash off the highly lipophilic DNPH derivatives in a discrete band, the remaining material being eluted with chloroform. The resulting chloroform eluate was evaporated *in vacuo* to dryness and redissolved in 0.5 mL of methanol. The HHE-DNPH derivative was analyzed by the HPLC method reported by Sakai *et al.*[9] under the following analytical conditions: column, Ultrasphere C18 (25 cm x 4.6 mm i.d., Beckman); mobile phase, 30 mM sodium citrate/27.7 mM acetate buffer (pH 4.75): methanol=35:65; flow rate, 1 mL/min; column temperature, 40°C; detection wavelength, 365 nm.

MA analysis.

The 1,3-diethyl-2-thiobarbituric acid (DETBA) assay is based on the method of Sakai *et al.*[24] One g of ordinary muscle or 0.5 g of red muscle were homogenized with 9 vol of ice-cooled 10 mM sodium phosphate buffer (pH 7.0) in a Polytron homogenizer at 0 °C. An aliquot (less than 0.4mL) of the homogenate was transferred to a screw-capped tube containing 0.2 ml of 8% SDS and 0.2 mL of 20 mM butyl hydroxy toluene in ethanol, and the mixture was finally made up to 0.8 mL with distilled water. After adding 3.2 mL of 12.5 mM DETBA in sodium phosphate buffer (0.125 M, pH 3.0), the solution was mixed and heated in a water bath at 95°C for 15 min, and then cooled quickly with running tap water. To extract the DETBA-MA adduct, 4 mL of ethyl acetate was added, and the mixture was shaken vigorously. An ethyl acetate extract (2.4mL) containing the DETBA-MA adduct was

transferred to another tube and evaporated in vacuo. The residue was dissolved in 150 μL of methanol, and 10 μL of the sample was applied to HPLC under the following conditions: column, Inertsil ODS (5 μm particle size, 250 x 4.6 mm i.d.; GL Sciences, Tokyo, Japan); mobile phase, acetonitrile-0.1 M sodium chloride (75:25, v/v); flow rate, 1.0 mL/min; detection, excitation 515 nm and emission 555 nm.

Fatty acid analysis.

Total lipids were extracted from meats by the method of Folch et al.[25] Lipids obtained were hydrolyzed with 0.5 M KOH in methanol at 100 ℃ for 5 min and esterifyed with aqueous HCl (35%)/methanol (1:1 v/v) at 100 ℃ for 5 min. The metyl esters obtained were separated by gas-liquid chromatograph equipped with a capillary column and determined using tricosanoic acid as an internal standard.[26]

Statistical Analysis

All data was expressed as the mean ± SE. The results were analyzed by the analysis of variance (ANOVA) and the significant differences among the means were determined by the Tukey-Kramer test.

Results and Discussion

Table 1 shows the HHE and MA contents of commercial surimi-based products. Large differences of these contents among the different samples were observed in all food products. Similar results were observed in the pork and beef obtained from commercial markets.[27] The diversity of these contents in the surimi-based products is probably due to their history in respect to source, storage time, additives, and storage

conditions. In contrast to our previous results that the linear correlation was observed between the MA and HHE contents in raw meats stored at 0 or -20 °C,[28] this correlation was not observed in surimi-based products. Similar results were obtained from smoked foods as well.[11] HHE contents of all surimi-based products analyzed were lower than 1 μmol/kg, which may be much lower than that of the toxic level.

Table 2 shows the changes in HHE and MA contents in yellowtail meat containing 0, 0.3, 0.6 and 0.9 M NaCl. All samples showed a significant increase in HHE content after 3 days of the storage compared with that of after 0 days of storage. Ensuingly, a significant decrease was observed after 7 days of storage. After 3 days of storage, the HHE contents in the sample containing 0.9 M NaCl were lower than those of other samples, although the difference was not significant. After 7 days of storage, the HHE contents in the sample containing 0.9 M NaCl were significantly lower than those of other samples, and the contents in the control were significantly higher than those of other samples. After 3 or 7 days, MA contents of the sample containing NaCl were significantly higher than those of the control. Following 3 days of storage, the highest level of MA were observed in the sample containing 0.6 M NaCl. Judging from the changes of MA contents, NaCl acts as a pro-oxidant in the meat. Table 3 shows the changes in total fatty acid, eicosapentaenoic acid and docosahexaenoic acid in the meat containing 0, 0.3, 0.6 and 0.9 M NaCl. Total fatty acids, eicosapentaenoic acid and docosahexaenoic acid contents decreased during storage. This result might suggest that oxidation of these lipids induced the decrease. As shown in Table 4, in the meats of red sea bream HHE was only detected in the sample containing 0.9 M NaCl after 7 days of storage. After

7 days of storage, MA contents in the sample containing NaCl were significantly higher than those of control. As shown in Table 5, in the great amberjack meats HHE contents increased during the storage periods. HHE contents in the samples containing NaCl were significantly higher than those of control after 3 and 7 days of storage. MA contents in the sample containing NaCl were significantly higher than those of control after 3 days of storage but the difference was not observed between each samples after 7 days of storage.

Judging from MA contents, NaCl seemed to act as a pro-oxidant in the meats of all fish species analyzed. On the other hand, addition of NaCl may suppress the HHE formation in yellowtail meats, progress the formation in great amberjack meats and not relate to the formation in red sea bream meats. Esterbauer et al.[29] reported that HHE is not an intermediate of the MA formation in vivo and that HHE reacts readily with sulfhydryl groups of proteins and low molecular weight substances, such as cysteine and glutathione, with ε-amino groups of lysine and with histidine residues in proteins. In addition, Buttkus[30] reported that MA preferentially reacts with lysine, tyrosine, methionine and arginine in proteins. Considering the results reported previously,[8, 11] the formation and disappearance rate of HHE and MA are thought to be different from each other, and also the accumulation mechanism of HHE might be different from that of MA in fish meats. Contents of antioxidants (α-tocopherol, β-carotene etc.), pro-oxidants (transition metals) and oxidation substrates (fatty acids composition) in the meats may be different from species to species, which consequently change the formation of HHE. Seasonal variation of these contents is known to be observed in

the fish meats, which also change the formation of HHE. Considering the toxicity of HHE, it is necessary to examine the formation mechanism of this aldehyde in the meat of many fish species. Studies along these lines are in progress in our laboratory.

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Table 1. HHE and MA contents in surimi-based products obtained from commercial market

Sample	n	HHE(μmol/kg)	MA(μmol/g)
Kamaboko-I	3	0.97 ± 0.19	2.90 ± 1.50
Kamaboko-II	3	0.48 ± 0.11	0.34 ± 0.12
Maru-ten	3	nd.	3.88 ± 0.49
Obi-ten	3	nd.	1.83 ± 0.14
Iwashi-ten	3	nd.	1.55 ± 0.15
Chikuwa	3	0.42 ± 0.02	1.37 ± 0.71
Satuma-age	3	0.37 ± 0.08	4.82 ± 1.77
Kani-kama	3	0.32 ± 0.01	2.39 ± 1.04

(Mean ± Standard deviation). n: Sample number.

nd.: not detected (< 0.1 nmol/kg)

Table 2. The variation of HHE and MA contents in yellowtail meats that contained 0, 0.3, 0.6 and 0.9 M NaCl during day 0, 3, and 7 at 0°C storage

Days	0	3	7
HHE (nmol/g)			
Control	2.22 ± 0.30 ^{x,a}	5.07 ± 0.32 ^{xy,b}	3.38 ± 0.15 ^{x,c}
0.3 M	2.22 ± 0.30 ^{x,a}	4.88 ± 0.27 ^{xy,b}	2.39 ± 0.10 ^{y,a}
0.6 M	2.22 ± 0.30 ^{x,a}	5.44 ± 0.43 ^{x,b}	2.74 ± 0.12 ^{y,a}
0.9 M	2.22 ± 0.30 ^{x,a}	4.24 ± 0.11 ^{y,b}	1.86 ± 0.12 ^{z,a}
MA (μmol/g)			
Control	50.94 ± 5.87 ^{x,a}	50.26 ± 0.90 ^{x,a}	61.86 ± 0.93 ^{x,a}
0.3 M	50.94 ± 5.87 ^{x,a}	123.1 ± 10.8 ^{y,b}	177.6 ± 12.3 ^{y,c}
0.6 M	50.94 ± 5.87 ^{x,a}	138.2 ± 31.8 ^{y,b}	222.5 ± 18.1 ^{z,c}
0.9 M	50.94 ± 5.87 ^{x,a}	116.5 ± 12.1 ^{y,b}	116.1 ± 11.4 ^{y,c}

a-c Means (n=3) ± standard error within the same row with no common superscript differs significantly ($P < 0.05$)

x-z Means (n=3) ±standard error within the same column with no common superscript differs significantly ($P < 0.05$).

Table. 3. Changes in contents of total fatty acid (TFA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in yellowtail meat containing 0, 0.3, 0.6 and 0.9 M NaCl after 0 and 7 days of storage stored at 0 °C.

	Control	0.3 M	0.6 M	0.9 M
TFA (mg/100 g tissue)				
0 days	470*	470	470	470
7 days	470	333	344	325
EPA (mg/100 g tissue)				
0 days	26.7	26.7	26.7	26.7
7 days	27.6	15.6	15.0	17.1
DHA (mg/100 g tissue)				
0 days	184	184	184	184
7 days	187	98.5	93.8	93.0

*mean (n=2)

Table 4. The variation of HHE and MA contents in the red sea bream meats that contained 0, 0.3, 0.9 M NaCl during day 0, 3, 7 at 0°C storage

Days	0	3	7
HHE (nmol/g)			
Control	nd. ^{x,a}	nd. ^{x,a}	nd. ^{x,a}
0.3 M	nd. ^{x,a}	nd. ^{x,a}	5.03 ± 3.90 ^{x,a}
0.9 M	nd. ^{x,a}	nd. ^{x,a}	nd. ^{x,a}
MA (μmol/g)			
Control	0.48 ± 0.06 ^{x,a}	1.36 ± 0.07 ^{x,b}	1.42 ± 0.36 ^{x,b}
0.3 M	0.48 ± 0.06 ^{x,a}	1.67 ± 0.07 ^{x,b}	3.41 ± 0.18 ^{y,c}
0.9 M	0.48 ± 0.06 ^{x,a}	3.21 ± 0.39 ^{y,b}	3.67 ± 0.09 ^{y,b}

a-c Means (n=3) ± standard error within the same row with no common superscript differs significantly ($P < 0.05$)

x-z Means (n=3) ±standard error within the same column with no common superscript differs significantly ($P < 0.05$).

nd.: not detected (< 0.1 nmol/kg)

Table 5. The variation of HHE and MA contents in the great amberjack meats that contained 0, 0.3, 0.9 M NaCl during day 0, 3, 7 at 0°C storage

Days	0	3	7
HHE (nmol/g)			
Control	0.01±0.00 ^{x,a}	nd. ^{x,a}	0.13±0.07 ^{x,b}
0.3 M	0.01±0.00 ^{x,a}	0.09±0.01 ^{y,a}	1.24±0.41 ^{y,b}
0.9 M	0.01±0.00 ^{x,a}	0.19±0.04 ^{z,a}	1.45±0.30 ^{y,b}
MA (μmol/g)			
Control	0.61±0.07 ^{x,a}	1.93±0.29 ^{x,a}	4.78±0.79 ^{x,b}
0.3 M	0.61±0.07 ^{x,a}	4.05±0.23 ^{y,b}	4.72±0.91 ^{x,c}
0.9 M	0.61±0.07 ^{x,a}	2.83±0.71 ^{xy,a}	6.30±1.08 ^{x,b}

a-c Means (n=3) ± standard error within the same row with no common superscript differs significantly ($P < 0.05$)

x-z Means (n=3) ±standard error within the same column with no common superscript differs significantly ($P < 0.05$).

nd.: not detected (< 0.1 nmol/kg)

アスタキサンチン添加によるカンパチ肉の脂質過酸化抑制の試み

目的

1970年にFoote et alは、β－カロテンが一重項酸素消去活性を有することを報告した。また1982年には幹らは、アスタキサンチンが一重項酸素消去活性、およびこれに伴う脂質の過酸化に対する抗酸化作用を有することを報告した。またカロテノイドによるフリーラジカル補足能も明らかにされている。しかしこれらの実験は、メカニズム解明のために全て厳しい条件下で行われており、実際に食品添加物として応用するための実験はほとんど行われていない。そこで，本実験では前述に研究を基に、魚肉に食品添加物としてアスタキサンチンを添加することにより脂質過酸化を抑制することが可能かどうかを明らかにするために、カンパチ肉にアスタキサンチンを添加後，脂質過酸化の指標であるマロンアルデヒド（MA）および4-ヒドロキシヘキセナール（HHE）含量の変動を経時的に調べた。

実験方法

1）MDAおよびHHE測定用試薬

1,3-diethyl-2-thiobarbituric acid（以下DETBA）は、Aldrich Chemicalsより購入した。抗酸化剤Butyl Hydroxy Toluene(以下BHT)は、東京化成工業より購入した。Ethylenediaminetetraacetic acid, disodium salt（以下EDTA），Sodium dodecyl sulfate（以下SDS），1,1,3,3-tetraethoxypropane, 2,4-Dinitrophenylhydrazine(以下DNPH)，尿素は、和光純薬より購入した。Bio-Rad protein Assay Kid（以下PAK）は、Bio-Rad Laboratoriesより購入した。なお、このPAKは標準として牛血清アルブミンを使用した。高速液体クロマトグラフィー(以下HPLC)用の溶媒は、すべて、HPLC用の試薬を用いた。他の試薬については、できうる限り特級を用いた。

2）試料

試料のカンパチは、一般の市場で購入した。皮および内臓を取り除き、脂身と血合筋を削除した後、筋肉組織のみをフードプロセッサーで細かくした。その試料を4等分し、Control，0.05%アスタキサンチン、0.1%アスタキサンチン、0.2%アスタキサンチンとなるように調整した。それぞれをポリエチレンバックに入れ、4℃で貯蔵した。以上の条件で貯蔵した試料のMDA，CP，HHE含量を0、3、7、10日目に測定し、それぞれの値を測定した。

3）MAの測定法

試料をテフロンホモジナイザーに1gずつ正確に秤量し、生理的食塩水を20ml加えホモジネートを作成した。それを0.5ml栓付試験管に採取し、0.125M リン酸緩衝液（pH3，0.4%SDS，10mM DETBA，4mM BHTを含む）を3.5ml加えた。100℃のヒーターブロックで150分間反応させた。反応後、氷中で冷却しながら酢酸エチルを4ml加え、激しく振盪した。2500rpmで15分間遠心分離を行い、その上清0.5mlを共栓付試験管に採取し、エバポレーターで濃縮乾固した。これを200μlのメタノールで再溶解し、その20μlをHPLCに注入し測定した。

分析用のポンプにはBIP-I pump（日本分光工業）を、インジェクターには7725 Injector（島津製作所）、カラムにはInertsil ODS（5μm particle size250×4.6mm i.d.;GL Science）をそれぞれ用いた。検出波長は、Ex515nm-Em555nmの条件で行い、検出器にはRF-10A分光蛍光検出器（島津製作所）を用いた。展開溶媒は、アセトニトリル：0.1M NaCl溶液=3：1を用いた。なお溶出量は、1ml/minとした。

4）HHEの測定法

試料を三角フラスコに1gずつ正確に秤量し、試料に対して0.5%のBHTを添加した。この三角フラスコに、DNPH 2.5mMを含んだ1N-HClを20ml加え、低温(4℃)暗所で2時間反応させ、抽出と誘導化を同時に行った。反応後、試料を濾過し、3倍量のジクロロメタンを加えて、分液漏斗を用いて激しく撹拌し、HHE-DNPH誘導体を含む下層を500ml容なす型フラスコに採取した。この操作を2回繰り返した。その後、エバポレーターを用いて、濃縮乾固した。得られた残渣を2mlのクロロホルムで溶解し、あらかじめヘキサン：クロロホルム=2：1混合液3mlで洗浄したSilica Gel Disposable Extraction Columnに注入した。注入後、ヘキサン：クロロホルム=2：1混合液3mlを2回カラムに注入し、試料を展開させ、HHE-DNPH誘導体を含む

バンドを分離させた。分離したバンドをクロロホルム6mlで溶解させ、溶出液を10ml容共栓付試験管に採取し、濃縮乾固した。これをメタノール500μlで再溶解し、HPLC用プレフィルターで濾過後、20μlをHPLCに注入し測定した。

分析用のポンプには880-PU pump（日本分光工業）を、インジェクターには7725i Injector（島津製作所）、カラムにはUltrasphere（25cm×4.6mm i.d. Beckman）をそれぞれ用いた。検出波長は365nmで、検出器にはSPD-M10AVP紫外可視検出器（島津製作所）を用いた。展開溶媒は、30mM sodium citrate/27.7mM acetate buffer(pH4.75)：メタノール=35：65を用いた。なお溶出量は、1ml/minとした。この測定方法による検出限界は、0.01nmol/g以下である。HHE-DNPH誘導体のピークの同定は、その保持時間およびスパークテストにより行った。

結果

MDAおよびHHE含量の測定結果をTable 1 に示し。

MA含量は、Control区において、3日目まで有意的に増加し、その後、10日目まで有意的に減少し続けた。0.05%区では3日目までは変化せず、その後7日目まで有意的に増加した。さらにその後10日目までは有意的に減少した。0.1%区では3日目まで有意的に増加し、その後有意的に減少し続けた。0.2%区では3日目まで有意的に増加し、その後有意的に減少し続けた。

HHE含量は、Control区において、3日目までは変化せず、その後3日目から10日目にかけて有意的に減少した。0.05%区では変化しなかった。0.1%区では変化しなかった。0.2%区では3日目までは変化せず、その後7日目までは有意的に増加した。その後10日目までは有意的に減少した。

考察

MA含量は、7日目に0.05%区において生成が促進された。またHHE含量は、3日目に0.2%区で生成が抑制された。また7日目に0.2%区で生成が促進された。また10日目には0.1%区で生成が促進された。以上の結果から、今回実験を行った条件下では、アスタキサンチンを添加しても脂質過酸化は抑制されず、むしろ促進されることが明らかになった。これはアスタキサンチンが一重項酸素の消去、及びフリーラジカルの捕捉には有効であるものの、その他の、スーパーオキシドや過酸化水素などによる酸化を抑制する機能が弱いため、全体としての脂質過酸化を抑制できなかったためだと考えられる。また，幹らの研究によれば、アスタキサンチンと同じカロテノイド類であるβ－カロテンは、低酸素分圧下ではフリーラジカル捕捉剤として働くものの、高酸素分圧下ではむしろフリーラジカル連鎖反応を促進することが報告されている。そのため，アスタキサンチンが一重項酸素の消去やフリーラジカルの捕捉自体を行なわなかったことも考えられる。

Table1,アスタキサンチン添加カンパチ魚肉中のMAおよびHHEの変動

	0日目	3日目	7日目	10日目
MA				
コントロール	0.27	0.46	0.3	0.23
0.05%アスタキサンチン	0.27	0.35	0.49	0.29
0.1%アスタキサンチン	0.27	0.56	0.32	0.25
0.2%アスタキサンチン	0.27	0.55	0.31	0.26
HHE				
コントロール	0.23	0.33	0.19	0.01
0.05%アスタキサンチン	0.23	0.21	0.28	0.06
0.1%アスタキサンチン	0.23	0.23	0.25	0.18
0.2%アスタキサンチン	0.23	0.07	0.55	0.07

α-トコフェロールおよびβ-カロテン添加カンパチ魚肉における 4-ヒドロキシヘキセナールとマロンアルデヒドの生成-1。0.1%添加試験

目的

食品添加物として既に一般的に広く用いられているα-トコフェロール（α-Toc）およびβ-カロテン（β-Car）のそれぞれの坑酸化作用と、α-Toc＋β-Car混合物の抗酸化作用相乗効果の有無を明らかにするために、α-Toc、β-Car およびα-Toc＋β-Car混合物をそれぞれ0.10%添加したカンパチを貯蔵し、0、3、7、10日目における MA および HHE 含量の変動を測定した。

実験方法

1）試薬

α-Toc およびβ-Car は和光純薬より購入した。

MA、CP および HHE 測定用試薬

1、3-Diethyl-2-thiobarbituric acid（以下 DETBA）は、Aldrich Chemicals より購入した。SODiumdodecyl sulfate（以下 SDS）と 1、1、3、3-tetraethoxypropane、2.4- ジニトロフェニルヒドラジン（以下 DNPH)、尿素は和光純薬より購入した。Bio-Rad Proteinn Assay Kid (以下 PAK) は Bio-Rad Laboratories より購入した。なおこの PAK は標準として牛血清アルブミンを使用した。抗酸化剤 Butyl Hydroxy Toluene（以下 BHT）は、東京化成工業より購入した。高速液体クロマトグラフィー（以下 HPLC）用の溶媒は、すべて HPLC 用の試薬を用いた。他の試薬については、できうる限り特級を用いた。

2）試料

試料（カンパチ）は、一般の市場で購入した。内臓、 皮および脂身は取

り除き、筋肉組織のみをフードプロセッサーで細かく刻んだ。その試料を四等分し、何も加えないものを Control とし、あとの３つの試料に濃度がそれぞれ 0.1％α-Toc、 0.1％β-Car、および 0.1％α-Toc＋0.1％β-Car となるように添加した後、ラップとアルミホイルで包み、それぞれをポリエチレンバックに入れて 4℃で貯蔵した。以上の条件で貯蔵した試料の MA、CP、HHE 含量を 0、3、7、10 日目に測定した。

3）MA の測定方法

試料をテフロンホモジナイザーに 1ｇずつ正確に秤量し、生理的食塩水を 20ml 加えホモジネートを作成した。その 0.5ml を、栓付試験管に採取し、0.125Mリン酸緩衝 pH3、0.4%SDS、10mM DETBA、4mM BHT を含む）を 3.5ml 加えた。100℃のヒーターブロックで 150 分間反応させた。反応後、氷中冷却し、酢酸エチルを 4 mℓ加え、激しく振盪した。2500rpm で 15 分間遠心分離を行い、その上清 0.5 mℓを共栓付試験管に採取し、エバポレーターで濃縮乾固した。これを 200μℓのメタノールで再溶解し、その 20μℓを HPLC に注入し分析した。分析用のポンプには、BIP I pump （日本分光工業）を、インジェクターには 7725Injector（島津製作所）、カラムには Inertsil ODS(5μm particle size 250×4.6mm i.d.;GL Science)をそれぞれ用いた。検出波長は Ex515nm-Em555nm の条件で 行い、検出器にはＲＦ-１０A 分光蛍光検出器（島津製作所）を用いた。展開溶媒は、アセトニトリル：0.1M NaCl 溶液=3：1 を用いた。なお溶出量は 1ml/min とした。

4）HHE の測定方法

試料を三角フラスコに 1ｇずつ正確に秤量し、試料に対して 0.5%の BHT を添加した。この三角フラスコに 2.5mM DNPH を含んだ 1N-HCl を 20 mℓ加え、低温（4℃）暗所で 2 時間放置し、抽出と誘導化を同時に行った。反応後、

濾紙で濾過し3倍量のジクロロメタンを加えて激しく撹拌、分液漏斗を用いて、HHE-DNPH 誘導体を含む下層をなす型フラスコに採取した。この操作を 2 回繰り返した。そのなす型フラスコをエバポレーターで濃縮乾固した。得られた残渣を 2 mℓのクロロホルムで溶解し、あらかじめ 3 mℓのヘキサン：クロロホルム＝2：1 で洗浄した Silica Gel Disposable Extraction Column に注入した。注入後、ヘキサン：クロロホルム＝2：1 混合液 3 mℓを 2 回カラムに注入し試料を展開させHHE-DNPH誘導体を含むバンドを分離させた。分離したバンドをクロロホルム 6 mℓで溶解させ、溶出液を 10 mℓ容共栓付褐色試験管に採取し濃縮乾固した。これを 500μℓのメタノールで再溶解しHPLC 用プレフィルターで濾過後その 20μℓを HPLC に注入し測定した。分析用のポンプには、880-PU pump（日本分光工業）を、インジェクターには 7725i Injector（島津製作所)、カラムには Ultrasphere C18（25cm×4.6mm i.d.Beckman）をそれぞれ用いた。検出波長は 365nm で、検出器には、SPD-M10AVP 紫外可視検出器（島津製作所）を用いた。展開溶媒は、30mM SODium citrate/27.7mM asetate buffer (pH4.75)：methanol=35：65 を用いた。なお溶出量は 1ml/min とした。この測定法による検出限界は、0.1nmol/g 以下である。HHE－DNPH 誘導体のピーク同定は、その保持時間およびスパークテストにより行った。

結果

MA および HHE 含量の変動を Table 1 に示した。MA 含量は control、α-Toc、β-Car、α-Toc＋β-Car の全てにおいて、0～3 日目の間に有意に減少し、control、α-Toc においては、3～10 日目の間では変動がほとんど見られなかった。β-Car において、3～7 日目の間では変動がほとんど見られず、7～10 日目の間に有

意に増加した。α-Toc＋β-Car において、３～７日目の間に有意に増加し、７～10 日目の間では変動がほとんどは見られなかった。また、control とその他のサンプルを比較して、3 日目においては、β-Car、α-Toc、α-Toc＋β-Car 全てに有意差は認められなかった。７日目においては、α-Toc＋β-Car のみが有意に多かった。10 日目においては、β-Car、α-Toc＋β-Car が有意に多かった。HHE 含量は control、α-Toc において、０～３日目の間に有意に増加し、３～７日目に有意に減少し、７～10 日目の間では変動がほとんど見られなかった。β-Car において、０～７日目の間では変動がほとんど見られず、７～10 日目の間に有意に増加した。α-Toc＋β-Car において、０～３日目の間に有意に増加し、３～７日目の間に有意に減少し、７～10 日目の間に再び有意に増加した。また、control とその他のサンプルを比較して、3 日目においては、β-Car のみ有意に少なかった。7 日目においては、α-Toc、β-Car、α-Toc＋β-Car が有意に少なく、α-Toc＋β-Car については、α-Toc、β-Car よりさらに有意に少なかった。10 日目においては、α-Toc、β-Car、α-Toc＋β-Car が有意に多く、β-Car については、α-Toc、α-Toc＋β-Car よりさらに有意に多かった。

考察

MA HHE 含量の測定結果を考えると、α-Toc の添加は HHE 生成を抑制するという点を除いて、期待されたほどの脂質過酸化抑制効果が得られなかった。一般的に坑酸化剤として使われているα-Toc が脂質過酸化に対して効果がないということは、食品化学上重要な問題である。β-Car の添加は、脂質過酸化に対して多少の抑制を示すものの、最終的には促進する傾向があり，α-Toc＋β-Car 混合物の添加はむしろ全体的に脂質過酸化を促進していると考えられる。

α-トコフェロールおよびβ-カロテン添加カンパチ魚肉における4-ヒドロキシヘキセナールとマロンアルデヒドの生成-2。0.05%添加試験

目的

前章の結果はα-Tocおよびβ-Car添加による期待した脂質化酸化抑制効果は、MA および CP 含量の変動結果からみると認められず、むしろ促進させる結果となった。α-Toc の坑酸化作用が添加濃度によって変化するという報告に着目し、添加濃度を0.05%α-Toc、0.05%β-Car、および0.05%α-Toc+0.05%β-Carに変更した場合のそれぞれの脂質過酸化に及ぼす影響を見るために、MA およびHHE含量の変動の測定を行い、0.10%添加時の結果と比較した。

実験方法

1）試薬

α-Toc、β-Car および測定用の試薬は、前章で述べたもの同様のものを使用した。

2）試料

試料にはカンパチを用いて、α-Toc、β-Car およびα-Toc+β-Car の添加量を 0.05%に変更し、他の条件は第1章と同様に調整し、4_で貯蔵した。以上の条件で貯蔵した試料の MDA、CP、HHE 含量を 0、3、7、10 日目に測定した。

3）MDA およびHHE の測定法

MDA およびHHE の測定は、前章で述べた方法に従った。

結果

MA および HHE 含量の変動を Table 2.に示した。MA 含量は control において、0～3 日目の間に増加し、3～10 日目の間では変動がほとんど見られなかった。β-Car において、0～7 日目の間に有意に増加し、7～10 日目の間では変動がほとんど見られなかった。α-Toc、α-Toc＋β-Car において、0～10 日目の間では変動がほとんど見られなかった。また、control とその他のサンプルを比較して、3 日目においては、β-Car、α-Toc、α-Toc＋β-Car の全てに有意差は認められなかった。7 日目においては、β-Car のみ有意に多かった。10 日目においては、α-Toc、α-Toc＋β-Car が有意に少なかった。HHE 含量は control において、0～7 日目の間では変動がほとんど見られず、7～10 日目の間に有意に減少した。β-Car において、0～10 日目の間では変動がほとんど見られなかった。α-Toc において、0～3 日目の間では変動がほとんど見られず、3～7 日目の間に有意に減少し、7～10 日目の間では変動がほとんど見られなかった。α-Toc＋β-Car において、0～3 日目の間に有意に増加し、3～10 日目の間に有意に減少した。また、control とその他のサンプルを比較して、3 日目、7 日目においては、β-Car、α-Toc、α-Toc＋β-Car の全てに有意差は認められなかった。10 日目においては、α-Toc、α-Toc＋β-Car が有意に少なく、α-Toc についてはα-Toc＋β-Car よりさらに有意に少なかった。

考察

α-Toc およびα-Toc＋β-Car 混合物の添加により、MA および HHE の生成は抑制され。一方，β-Car の添加は MA 生成を促進したが、HHE 生成に対しての影響は認められなかった。

以上の結果と前章の結果より、α-Toc 添加においては、濃度を 0.10%から

0.05%に下げることで、MA 生成を抑制することが明らかとなり、「過剰量のα-Toc の添加は抗酸化作用を低下させる。」という報告を裏付ける結果となった。また、HHE 含量の測定では、0.10%添加で見られた HHE 生成の抑制効果が 0.50%添加においても認められた。このため、0.10%および 0.05%のα-Toc 添加は HHE の生成の抑制に有効であることが明らかとなった。

β-Car 添加においては、0.10%添加で見られた CP 生成の促進が 0.05%への濃度変更で見られなくなったが、CP 生成の抑制には至らなかった。また、MA、HHE 含量の測定結果を見ても、0.10%添加とほとんど変わらず、添加濃度による抗酸化作用の増強が認められなかった。

α-Toc＋β-Car 混合物添加においては、前章での結果と同様に、MA、CP および HHE 測定結果の全てにおいて 0.05%α-Toc 添加の結果とほぼ一致しているため、α-Toc 添加の脂質過酸化に対する作用が寄与するところが大きいと考えられる。このため、β-Car との脂質過酸化の相乗効果が低い、もしくは無いのではないかと考えられる。

Table.1　α-Toc 0.10%、β-Car 0.10%、およびα-Toc 0.10%＋β-Car 0.10%添加カンパチ肉中の、MA、CP、およびHHE含量の測定結果。

Days		0日目	3日目	7日目	10日目
MA（μmol/g tissue）	Control	2.86±0.41^{a,w}	0.46±0.02^{a,x}	1.80±0.10^{a,x}	1.64±0.14^{a,x}
	α-Tocopherol	2.86±0.41^{a,w}	0.35±0.03^{a,x}	1.47±0.18^{a,x}	2.35±0.07^{bc,w}
	β-Carotene	2.86±0.41^{a,w}	0.56±0.17^{a,x}	1.46±0.21^{a,x}	2.02±0.02^{ac,x}
	α-Toc+β-Car	2.86±0.41^{a,w}	0.55±0.13^{a,x}	2.67±0.19^{b,w}	2.64±0.44^{b,w}
HHE (nmol/g tissue)	Control	0.35±0.16^{a,w}	0.88±0.17^{a,x}	0.57±0.16^{a,w}	0.30±0.02^{a,w}
	α-Tocopherol	0.35±0.16^{a,w}	0.38±0.25^{b,w}	0.22±0.04^{bc,w}	0.76±0.06^{b,x}
	β-Carotene	0.35±0.16^{a,w}	1.06±0.17^{a,x}	0.36±0.10^{b,w}	0.60±0.14^{c,w}
	α-Toc+β-Car	0.35±0.16^{a,w}	0.77±0.07^{a,x}	0.16±0.03^{c,y}	0.48±0.04^{c,w}

平均±標準偏差（n＝3）　有意差：a～d（日）　W～Z（試験区）

Table.2　α-Toc 0.05%、β-Car 0.05%、およびα-Toc 0.05%＋β-Car 0.05%添加カンパチ肉中の、MA、CP、およびHHE含量の測定結果。

	Days	0日目	3日目	7日目	10日目
MA (μmol/g tissue)	Control	4.88±0.24^{a,w}	2.53±0.14^{a,x}	3.20±0.61^{a,y}	3.69±0.06^{a,wx}
	α-Tocopherol	4.88±0.24^{a,w}	3.23±0.20^{a,x}	3.02±0.40^{ab,y}	2.66±0.11^{b,z}
	β-Carotene	4.88±0.24^{a,w}	3.30±0.10^{a,w}	3.16±0.14^{b,x}	2.65±0.68^{b,w}
	α-Toc+β-Car	4.88±0.24^{a,w}	2.79±0.08^{a,w}	2.79±0.61^{ab,x}	1.34±0.27^{c,y}
HHE (nmol/g tissue)	Control	0.37±0.06^{a,w}	0.50±0.13^{a,wx}	0.57±0.16^{a,wx}	0.81±0.38^{a,x}
	α-Tocopherol	0.37±0.06^{a,w}	0.36±0.14^{ab,w}	0.39±0.04^{ab,w}	0.39±0.13^{a,w}
	β-Carotene	0.37±0.06^{a,wx}	0.21±0.03^{b,w}	0.43±0.10^{ab,wx}	0.87±0.63^{a,x}
	α-Toc+β-Car	0.37±0.06^{a,w}	0.20±0.05^{b,w}	0.31±0.11^{b,w}	0.47±0.34^{a,w}

平均±標準偏差（n＝3）　有意差：a～d（日）　W～Z（試験区）

Influence of sodium chloride on superoxide dismutase activity and lipid peroxidation in refrigerated and frozen yellowtail meat

Running title: SOD activity in fish meat

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Influence of sodium chloride on superoxide dismutase activity and lipid peroxidation in refrigerated and frozen yellowtail meat

Abstract

Changes of superoxide dismutase (SOD) activities and malon aldehyde (MA) contents in yellowtail Seriola quinqueradiata meat stored at 0 °C were analyzed for 8 days. During storage periods changes of MA contents and SOD activities were not so large. SOD activities and MA contents in the meats containing 0, 0.3, 0.6 and 0.9 M NaCl stored at 0 and -20 °C were analyzed for 7 days and 12 weeks, respectively. In the meats stored at 0 °C, SOD activities in the meats containing 0.9 M NaCl were lower than those of the control, however MA contents in the former meats were significantly higher than those of the latter during storage. In the meats stored at -20 °C, SOD activities in the meats containing NaCl were lower than those of the control, but again MA contents in the former meats were higher than those of the latter during storage. A weak reverse correlation was observed between SOD acitivities and MA contents in the meats.

Key words: superoxide dismutase; lipid oxidation; malon aldehyde; yellowytail; NaCl

INTRODUCTION

Fish meat is considered a valuable protein source, especially in respect to health, due to presence of high levels of unsaturated fatty acids, particularly n-3 fatty acids. Moreover, it also has a higher mineral content. However, both these favorable factors make fish meat highly susceptible for lipid peroxidation; the major course of quality deterioration. Sodium chloride (NaCl) is added to muscle foods for a variety of purposes, including flavor and the inhibition of microorganisms. NaCl, nevertheless, has been shown to have an accelerating effect on lipid oxidation in a variety of meats, including beef, pork, chicken, and fish.[1-6] Lee et al.[7] have reported that the ability of NaCl to reduce the activity of antioxidant enzymes could be partially responsible for the lower oxidative stability of salted muscle foods. Glutathione peroxidase activity was not detected in horse mackerel Trachurus japonicus, Japanese sea-base Lateolabrax japonicus and yellowtail Seriola quinqueradiata meats,[8] although Nakano et al.[9] and Watanabe et al.[10] have reported that the enzyme existed in some fish muscle and might show potential in preventing oxidative detrioration in muscle during storage and processing. In addition, we found that catalase activities were very low in some fish muscle (Sakai unpablished result). Superoxide dismutase (E.C.1.15.1.1), which is found in all living cells utilizing molecular oxygen, converts superoxide anion to hydrogen peroxide and oxygen.[11] There are two varieties of superoxide dismutase in eucaryotic organisms. One contains Cu and Zn and the other contains Mg. Both are very stable against denaturation and proteolysis. Superoxide dismutases might be important enzymes protecting against oxidative detrioration during storage. However, to our

knowledge, there are no papers that have reported the changes in the enzyme activities in stored fish meats. Therefore, we analyzed the changes of the acitivities in yellowtail meat containing NaCl stored at 0 and -20 °C.

MATERIALS AND METHODS

Materials

Yellowtail Seriola quinqueradiata were obtained from commercial markets. Butyl hydroxy toluene (BHT) was obtained from Tokyo Kasei (Tokyo, Japan), NaCl (analytical grage) from Wako Pure Chemicals (Tokyo, Japan), and 1,3-diethyl-2-thiobarbituric acid (DETBA) from Aldrich Chemicals (Milwaukee, WI. USA). Other reagents were of analytical grade.

Storage experiments

In Experiment I, ordinary muscle of yellowtail was ground and stored at 0°C. In Experiment II, the muscle was ground ,mixed with 0, 0.3, 0.6 and 0.9 M NaCl and stored at 0°C. In Experiment III, the muscle was ground ,mixed with 0, 0.3, 0.6 and 0.9 M NaCl and stored at -20°C. In Experiment I and II, 4 samples from each group were analyzed and in Experiment III, 3 samples from each group were analyed. SOD activities and MA contents were analyzed after 0, 2, 4, 6 and 8 days of storage in Experiment I, after 0, 3 and 7 days of storage in Experiment II and after, 4, 8 and 12 weeks of storage in Experiment III.

MA analysis

The 1,3-diethyl-2-thiobarbituric acid (DETBA) assay is based on the method of Sakai et al.[12] One g of ordinary muscle or 0.5 g of red muscle were homogenized with 9 vol of ice-cooled 10 mM

sodium phosphate buffer (pH 7.0) in a Polytron homogenizer at 0 °C. An aliquot (less than 0.4mL) of the homogenate was transferred to a screw-capped tube containing 0.2 mL of 8% SDS and 0.2 mL of 20 mM butyl hydroxy toluene in ethanol, and the mixture was finally made up to 0.8 mL with distilled water. After adding 3.2 mL of 12.5 mM DETBA in sodium phosphate buffer (0.125 M, pH 3.0), the solution was mixed and heated in a water bath at 95°C for 15 min, and then cooled quickly with running tap water. To extract the DETBA-MA adduct, 4 mL of ethyl acetate was added, and the mixture was shaken vigorously. An ethyl acetate extract (2.4 mL) containing the DETBA-MA adduct was transferred to another tube and evaporated *in vacuo*. The residue was dissolved in 150 μL of methanol, and 10 μL of the sample was applied to HPLC under the following conditions: column, Inertsil ODS (5 μm particle size, 250 x 4.6 mm i.d.; GL Sciences, Tokyo, Japan); mobile phase, acetonitrile-0.1 M sodium chloride (75:25, v/v); flow rate, 1.0 ml/min; detection, excitation 515 nm and emission 555 nm.

Superoxide Dismutase (SOD) assay

SOD activities of the meats were assayed by the nitrite method of Ôyanagi[13] and expressed as NU/mg protein. Protein was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories). Total SOD activities were shown in the results.

Statistical Analysis

All data was expressed as the mean ± SE. The results were analyzed by the analysis of variance (ANOVA) and the significant differences among the means were determined by the Tukey-Kramer test.

Results and Discussion

Table 1 shows the changes in SOD activities and MA contents in the yellowtail meats stored at 0 °C. During storage periods changes of MA contents and SOD activities were not so large although the former was slightly increased and the latter fluctuated. From these results, it is uncertain whether or notchanges in SOD activities may affect the lipid oxidation in the fish meat. Therefore, we undertook Experiment II and III.

Table 2 shows the changes in SOD activities and MA contents in the meats containing 0, 0.3, 0.6 and 0.9 M NaCl stored at 0 °C. MA contents in all the meats increased during storage periods. After 7 days of storage, the contents in the meats containing 0.9 M NaCl were significantly higher than those of other samples, while those containing 0.6 M NaCl were significantly higher than those of the control and 0.3 M NaCl. During storage periods, a difference was not observed between the control and 0.3 M NaCl containing the samples. SOD activities in the meats containing 0 and 0.3 M NaCl increased; those in the meats containig 0.6 M NaCl did not change; and those containing 0.9 M NaCl decreased during the storage periods. After 7 days of storage, SOD activites in the meats containing 0.6 and 0.9 M NaCl were significantly lower than those of other samples. A large derease of SOD activities seemed to accelerate the lipid peroxidation in the meats as seen in the meats containing 0.9 M NaCl. However, it is not always clear that changes of SOD activities may influence the lipid peroxidation in fish meats, therefore we conducted Experiment III.

Table 3 shows the changes in SOD activities and MA contents in the meats containing 0, 0.3, 0.6 and 0.9 M NaCl stored at -20 °C. In contrast to refrigerated meats, MA contents increased and SOD actitities decreased in all samples

during storage periods. After 12 weeks of storage, SOD activites in the samples containing NaCl were significantly lower than those of the control, and MA contents in the meats containing 0.6 and 0.9 M NaCl were significantly higher than those of the control. Figure 1 demonstrates the correlation between SOD activities and MA contents in the meats. A weak reverse correlation was observed between SOD acitivities and MA contents ($r=0.55$, $n=27$) in yellowtail meats.

Lee et al. have reported that NaCl could alter the activity of glutathione peroxidase, catalase and superoxide dismutase in salted pork and that the ability of NaCl to reduce the activity of these enzymes could be partially responsible for the lower oxidative stability of salted muscle foods.[7] Nakano et al.[9] and Watanabe et al.[10] have reported that glutathione peroxidase existed in some fish muscle and might show potential for preventing oxidative detrioration in muscle during storage and processing. However, Nagai et al. have reported that the enzyme does not exist in yellowtail muscle. We detected only a very low activity of glutathione peroxidase in the ordinary muscle of yellowtail (Sakai et al. unpublishd result). We also detected only a very low activity of catalase in the ordinary muscle of yellowtail (Sakai et al. unpublished result). Both enzyme activities might not be responsible for preventing oxidative detrioration in the meat of yellowtail during storage and processing. In contrast to the above enzymes, present results indicate that a decrease of SOD activities may accelerate the lipid oxidation in the fish meats and that the enzyme may play some roles in protecting yellowtail meats from oxidative deterioration during storage.

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Table 1. Changes in SOD activity and MA content in yellowtail meat during 8 day storage period at 0℃.

Days	0	2	4	6	8
SOD (NU/mg protein)	17.7±2.3[a]	28.8±1.5[b]	18.4±1.0[ac]	24.7±0.7[bc]	18.9±3.5[ac]
MA (μmol/g tissue)	0.062±0.017[a]	0.132±0.039[b]	0.064±0.002[ab]	0.098±0.013[ab]	0.115±0.014[ab]

a-c Means (n=4) ±SE within the same row with no common superscript differing significantly ($P<0.05$).

Table 2. Changes in SOD activities and MA content in the yellowtail meats containing 0, 0.3, 0.6 and 0.9 M NaCl during day 0, 3, and 7 at 0°C storage

Days	0	3	7
Total SOD (NU/mg protein)			
control	3.83 ± 0.03 [a,x]	8.63 ± 0.87 [b,xy]	8.23 ± 0.67 [b,x]
0.3 M	3.83 ± 0.03 [a,x]	9.83 ± 0.33 [b,x]	8.47 ± 1.07 [b,x]
0.6 M	3.83 ± 0.03 [a,x]	5.37 ± 1.73 [a,y]	3.77 ± 0.78 [a,y]
0.9 M	3.83 ± 0.03 [a,x]	0.80 ± 0.46 [b,z]	1.80 ± 0.55 [b,y]
MA (μmol/g)			
control	1.33 ± 0.32 [a,x]	2.18 ± 0.07 [b,x]	3.49 ± 0.16 [c,x]
0.3 M	1.33 ± 0.32 [a,x]	2.14 ± 0.24 [b,x]	2.77 ± 0.16 [b,x]
0.6 M	1.33 ± 0.32 [a,x]	2.55 ± 0.40 [a,xy]	5.44 ± 0.98 [b,y]
0.9 M	1.33 ± 0.32 [a,x]	3.22 ± 0.24 [b,y]	8.17 ± 0.34 [c,z]

a-c Means (n=4) ± standard error within the same row with no common superscript differing significantly ($P < 0.05$)

x-z Means (n=4) ±standard error within the same column with no common superscript differing significantly ($P < 0.05$).

Table 3. Chagnges in MA content and SOD activity in yellowtail meat containing NaCl during a 12 week storage period at -20℃.

Weeks	0	4	8	12
Total SOD activity (NU/mg protein)				
Control	$28.13\pm3.05^{a,w}$	$17.17\pm2.00^{b,w}$	$20.67\pm3.81^{ab,w}$	$10.90\pm3.48^{b,w}$
NaCl 0.3M	$28.13\pm3.05^{a,w}$	$13.8\pm3.55^{b,w}$	$9.57\pm4.03^{bc,wx}$	$2.53\pm1.16^{c,x}$
NaCl 0.6M	$28.13\pm3.05^{a,w}$	$3.40\pm1.52^{b,x}$	$3.73\pm3.38^{b,x}$	$4.30\pm1.25^{b,x}$
NaCl 0.9M	$28.13\pm3.05^{a,w}$	$3.90\pm1.47^{b,x}$	$5.33\pm3.99^{b,x}$	$1.13\pm1.13^{b,x}$
MA(μmol/g tissue)				
Control	$0.07\pm0.01^{a,w}$	$0.05\pm0.00^{a,w}$	$0.11\pm0.01^{a,w}$	$1.60\pm0.28^{b,w}$
NaCl 0.3M	$0.07\pm0.01^{a,w}$	$0.13\pm0.01^{a,y}$	$0.24\pm0.04^{a,x}$	$2.01\pm0.18^{b,wx}$
NaCl 0.6M	$0.07\pm0.01^{a,w}$	$0.26\pm0.01^{a,y}$	$0.37\pm0.02^{a,y}$	$2.45\pm0.21^{b,x}$
NaCl 0.9M	$0.07\pm0.01^{a,w}$	$0.39\pm0.01^{b,z}$	$0.66\pm0.06^{c,z}$	$2.61\pm0.14^{d,x}$

a-d Means (n=3) ±SE within the same row with no common superscript differing significantly ($P<0.05$).
w-z Means (n=3) ±SE within the same column with no common superscript differing significantly ($P<0.05$).

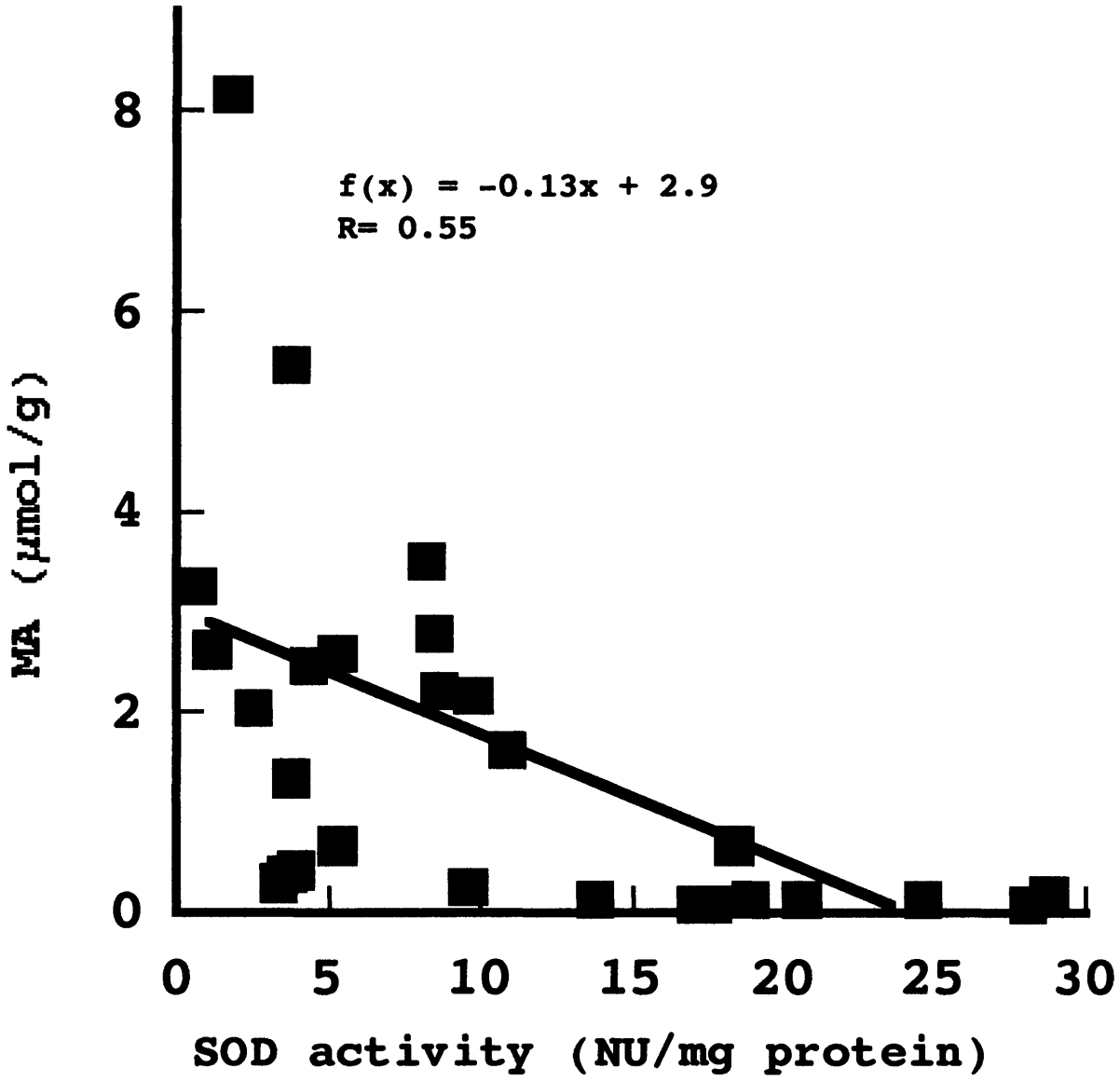

Figure 1. Relationship between SOD activities and MA contents in the yellowtail meats.