Note

Cytotoxicity of 4-Hydroxy-2E-hexenal, a Lipid Peroxidation-derived Aldehyde, and Changes of Its Content in Frozen Yellowtail Meat

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Cytotoxicity of 4-hydroxy-2E-hexenal (HHE), a hepatotoxic aldehyde, to Chinese hamster lung fibroblast V79 and Balb/c mouse semi-normal cell line BALB/3T3 clone A31 cells was examined. The 50%-inhibitory concentrations to V79 and A31 were 3 μ mol/L and 16 μ mol/L, respectively. Judging from the sensitivity, HHE might show cytotoxicity below these concentrations. Changes in HHE and malonaldehyde (MA) contents were also examined in yellowtail meat stored at -20°C . HHE content increased up to $60.82 \pm 4.39 \mu\text{mol/kg}$ meat during 28 weeks of storage. In contrast to HHE, MA content increased only slightly in the meat during 28 weeks of storage. From the viewpoint of food hygiene, it is noteworthy that the HHE content increased even when meat was stored at -20°C .

Key words: 4-hydroxy-2E-hexenal; cytotoxicity; malonaldehyde; lipid peroxidation; stored meat; yellowtail

Introduction

Polyunsaturated fatty acids of the n-3 series are predominant in fish tissues. During lipid peroxidation, n-3 polyunsaturated fatty acids (PUFA) yield 4-hydroxy-2E-hexenal (HHE)¹⁾, a α, β -unsaturated aldehyde. Crotonaldehyde administered in drinking water was shown to cause liver tumors in F344 rats²⁾, suggesting that α, β -unsaturated aldehydes may be potential carcinogens³⁾. There are many papers reporting cytotoxicity of several α, β -unsaturated aldehydes, such as 4-hydroxy-2E-nonenal (HNE)^{4), 5)}. However to our knowledge, only a few papers deal with the cytotoxicity of HHE^{6), 7)}. In addition, Segall *et al.* reported that the *in vivo* pathology caused by HHE appears to be identical to that previously attributed to the toxicity of macrocyclic pyrrolizidine alkaloids in the livers of man and animals⁸⁾. From the viewpoint of food hygiene, it is necessary to clarify the cytotoxicity of HHE and to analyze HHE content in fish foodstuffs. Therefore, we examined the cytotoxicity of HHE and changes in its content in yellowtail meat stored at -20°C . 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.*⁹⁾, and identified on the basis of the ^1H -NMR and ^{13}C -NMR spectra. Chinese hamster lung fibroblast V79 and Balb/c mouse semi-normal cell line BALB/3T3 clone

A31 were obtained from RIKEN Cell Bank (Tsukuba, Japan). They were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Biosciences Pty Ltd., Australia), 100,000 units/L of penicillin and 0.1 g/L of streptomycin. Yellowtail *Seriola quinqueradiata* samples were obtained from commercial markets. The ordinary muscle was ground and stored at -20°C . Butyl hydroxy toluene (BHT) was obtained from Tokyo Kasei (Tokyo, Japan), 2,4-dinitrophenylhydrazine (DPNH) 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.

Growth inhibition test

Methanol concentration in MEM was 0.01%, a level which had no observable cytotoxic effect on the cells. Cells were inoculated in 60 mm dishes at 5×10^4 cells/mL and cultured at 37°C in a humidified atmosphere of 5% CO_2 /95% air for 48 hr in HHE-containing MEM supplemented with 5% fetal bovine serum. Cultured cells were stained with 5% trypan blue after having been harvested with 40 USP trypsin units/mL of trypsin in Ca- and Mg-free phosphate-buffered saline (PBS) containing 1 mmol/L EDTA. Non-stained cells were counted as viable cells. Percent inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = \left\{ 1 - \frac{\text{viable cell number in experiment}}{\text{viable cell number in control}} \right\} \times 100$$

Fig. 1. Growth-inhibitory effect of HHE on V79 and BALB/3T3 A31 cells
Percent inhibition of V79 (—□—) and A31 (—○—) cells by HHE was determined by growth inhibition assay. Error bars indicate the standard deviations of the mean of triplicate determinations.

Storage experiments

In all experiments 3 samples from each group were analyzed. HHE and MA contents were analyzed after 0, 4, 8, 12, 16, 20, 24 and 28 weeks of storage.

HHE analysis

2,4-Dinitrophenylhydrazine (DNPH) conversion of HHE essentially followed the procedure of Goldring *et al.*¹⁰ 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 mol/L HCl containing 2.5 mmol DNPH was added to the BHT mixture, and the reaction was carried out in the dark for 2 hr at room temperature. DNPH derivatives were extracted three times with 150 mL of dichloromethane. The dichloromethane extract thus obtained was evaporated to dryness *in vacuo* and the residue was redissolved in 0.2 mL of chloroform. The solution was applied to a disposable silica gel extraction column (Baker) which had been preequilibrated with *n*-hexane-chloroform (2:1 v/v). 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 to dryness *in vacuo* and the residue was redissolved in 0.5 mL of methanol. The HHE-DNPH derivative was analyzed by a slight modification of the HPLC method reported by Sakai *et al.*¹¹ under the following analytical conditions: column, Ultrasphere C18 (250×4.6 mm i.d., Beckman); mobile phase, 30 mmol/L sodium citrate/27.7 mmol/L acetate buffer (pH 4.75)-methanol=35:65; flow rate, 1 mL/min; column temperature, 40°C; detection wavelength,

Fig. 2. Changes in HHE and MA contents in yellowtail meat stored at -20°C

Error bars indicate the standard deviations of the mean of triplicate determinations.

■: HHE; ●: MA

365 nm.

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 was homogenized with 9 mL of ice-cooled 10 mmol/L sodium phosphate buffer (pH 7.0) in a Polytron homogenizer at 0°C . An aliquot (less than 0.4 mL) of the homogenate was transferred to a screw-capped tube containing 0.2 mL of 8% SDS and 0.2 mL of 20 mmol/L butyl hydroxy toluene in ethanol, and the mixture was finally made up to 0.8 mL with distilled water. After addition of 3.2 mL of 12.5 mmol/L DETBA in 0.125 mol/L sodium phosphate buffer (pH 3.0), the solution was mixed, 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 subjected to HPLC under the following conditions: column, Inertsil ODS (5 μm particle size, 250×4.6 mm i.d.; GL Sciences, Tokyo, Japan); mobile phase, acetonitrile-0.1 mol/L sodium chloride (75:25, v/v); flow rate, 1.0 mL/min; detection, excitation 515 nm and emission 555 nm.

Results and Discussion

Figure 1 shows growth-inhibitory effect of HHE on V79 and A31 cells. The 50%-inhibitory concentrations to V79 and A31 were calculated to be 3 $\mu\text{mol/L}$ and 16 $\mu\text{mol/L}$, respectively. Because Tsuchiya *et al.*^{13, 14} reported that the sensitivity of the growth inhibition test is much lower (about 1/10~1/30) than that of modified colony assay, which showed the highest sensitivity

among cytotoxicity tests, HHE may show cytotoxicity to V 79 and A 32 cells at concentrations below 0.3 $\mu\text{mol/L}$ and 1.6 $\mu\text{mol/L}$, respectively.

Figure 2 shows the changes in HHE and MA contents in yellowtail meat stored at -20°C . MA content increased slightly in the meats during 28 weeks of storage at -20°C , indicating that lipid peroxidation was suppressed in the meat during storage. However, in contrast to MA content, HHE content ($3.20 \pm 1.92 \mu\text{mol/kg}$ meat) hardly changed through 12 weeks of storage and then increased rapidly to $60.82 \pm 4.39 \mu\text{mol/kg}$ meat after 28 weeks of storage. HHE content was higher in the meat of red sea bream *Pagrus major* containing 34.7 $\mu\text{g/g}$ of α -tocopherol than in that containing 23.7 $\mu\text{g/g}$ of α -tocopherol (Murata *et al.*: unpublished results). In addition, the HHE content was higher in the meat of yellowtail containing 1.0% wood vinegar than in that not containing wood vinegar (Sakai *et al.**³). These results suggest that HHE may accumulate in meat in which the progress of lipid peroxidation is suppressed. Because the formation and accumulation mechanisms of 4-hydroxyalkenals, such as 4-hydroxy-2E-nonenal (HNE) derived from n-6 PUFA and HHE, are different from those of MA *in vivo*^{15), 16)}, the formation and accumulation of MA might be inhibited more strongly than those of HHE in meat in which the progress of lipid peroxidation is suppressed.

Overall assessment of the cancer risk of aldehydes in diet revealed that crotonaldehyde containing HHE is a dietary risk factor⁴⁾. Brembilla *et al.*⁶⁾ and Müller *et al.*⁷⁾ have reported that the toxicity and carcinogenicity of HHE are lower than those of HNE. However, HNE contents in pork increased to only $15.0 \pm 6.90 \mu\text{mol/kg}$ meat during 8 months of storage at -20°C ¹⁷⁾. Intake of frozen fish meat stored for long time might be more risky than that of frozen pork stored for long time. Therefore it is necessary to examine more precisely the cytotoxicity of HHE and the accumulation mechanisms of HHE in fish meat. Studies along this line are in progress in our laboratory.

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