Formation of Free Polyunsaturated Fatty Acids and Their Metabolites in Oyster, *Crassostrea gigas*, by Treatment with Acetate

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High toxicity in mice was observed in crude fractions obtained from oysters (*Crassostrea gigas*) pickled in 4% acetic acid solutions for 3 h at 37 °C. The toxic fraction, obtained from thin-layer chromatograms, consisted of a mixture of free fatty acids [including polyunsaturated fatty acids (PUFA)], oxidative metabolites, monoglycerides, and sterols. The marinade process at 37 °C appeared to have a strong influence in the formation of PUFA and the appearance of toxicity in the treated oysters. Free PUFA and oxidative metabolites of eicosapentaenoic acid were determined in fresh and live oysters, before and after treatment with 4% acetic acid, using high-performance liquid chromatography.

Keywords: *Polyunsaturated fatty acids, oyster, Crassostrea gigas, acetate, mouse toxicity*

INTRODUCTION

It is well-known that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) prevent atherosclerosis or thrombosis and improve ischemic heart disease (Dyerberg et al., 1978; Hornstra et al., 1979). On the other hand, it has been feared that oxidized metabolites of these acids have toxic influences on animals (Oarada et al., 1989; Jurgens et al., 1986; Kanazawa and Ashida, 1991). Free-type polyunsaturated fatty acids (PUFA) are autoxidized more reasily than the isolated ester type (Miyashita and Takagi, 1986). Moreover, PUFA having 20 carbons are also enzymatically oxidized to eicosanoids such as prostaglandins and leukotrienes in animals.

We have reported that the viscera of Japanese anchovy contained more PUFA, mainly EPA and DHA, than the flesh (Sajiki and Takashashi, 1991, 1992). High concentrations of EPA, besides autoxidized EPA, can cause diarrhea in rabbits (Sajiki et al., 1993). There are many outbreaks of gastroenteritis from consumption of vinegar-pickled oyster in Japan. It appears that this method of preparing shellfish influences the change of PUFA and the oxidative metabolites.

The cause of gastroenteritis by raw oysters is primarily attributed to bacteria, virus, or lipid-soluble toxins such as okadaic acid and dinophysistoxins (DTXs) (Ahmed, 1991) but has yet to be clarified. The question whether PUFA formation in oysters, due to vinegar treatment, causes the poisoning prompted us to analyze the oxysters for PUFA.

This study aimed to (1) examine the effect of addition of acetate and heating on the concentration of PUFA and (2) investigate the formation of PUFA and their oxidative metabolites at ambient temperature and their relation to toxicity in oysters using mice.

MATERIALS AND INSTRUMENTS

Oysters, *Crassostrea gigas*, from the Setonaikai coast in Japan obtained in January 1992, which poisoned one of three persons, were used. Live oysters were collected in the Tohoku district, Japan, in July (summer) and December (winter) in 1992.

Male mice, 4 weeks old and weighing 16-20 g, were purchased at Japan SLC. Inc. (Shizuoka, Japan).

Standard reagents such as linolenic acid (LNA, 18:3), stearidonic acid (SDA, 18:4), arachidonic acid (AA, 20:4), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6) were purchased from Sigma Chemical Co. (St. Louis, MO), and (5)-hydroxyeicosapentaenoic acid (5-HEPE), 8-HEPE, 12-HEPE, 15-HEPE, and 12-hydroperoxyeicosapentaenoic acid (12-HPEPE) were from Cayman Chemical Co. (Ann Arbor, MI). Oxidative DHA were prepared by incubating authentic DHA at 37 °C for 2 h. Oxidized EPA, whose peroxide value (POV) and oxidative decomposition ratio were 1720 and 61.6%, respectively, was prepared by incubation at 37 °C for 1 week in air (Sajiki et al., 1992).

ODS-silica Sep-Pak (Waters Associates, Milford, MA), used for extraction of PUFA and its oxidative metabolites, was washed with 20 mL of ethanol (EtOH) and 20 mL of water before use.

Silica gel plates (20 cm × 20 cm, 0.5-mm thickness, E. Merck, Darmstadt, Germany) were activated by heating for 2 h at 120 °C and kept in a desiccator until used.

High-performance liquid chromatography was performed using a Model 880-PU with Finepak SIL C_{18} column and a UV-970 detector (UV-vis) equipped with an on-flow spectrum system (Nihonbunko Industries Ltd., Tokyo, Japan).

EXPERIMENTAL METHODS

Preparation of Samples. *Experiment 1.* (A) Six fresh whole oysters (collected at Setonaikai) were removed from their shells and cut into pieces. Immediately thereafter, the meats were divided into five portions which received the following treatments: (a) heated at 100 °C for 1 h; (b) addition of 1.0 mL of 4% acetate (pH 2.45) to 0.5 g of oyster and standing at 5 °C for 3 h; (c) treated with acetic acid and allowed to stand at 5 °C for 20 h; (d) treated with acetic acid and allowed to stand at 37 °C for 3 h; (e) a control that was analyzed immediately for PUFA. (B) Another six oysters were collected at the same time as those in (A) but placed in frozen storage at −70 °C for 1 month. The oysters were cut and divided into three portions: (a) a control; (b) treated with 4% acetate and allowed to stand at 37 °C for 3 h; (c) treated with phosphate-buffered saline (PBS, pH 7.4) and allowed to stand at 37 °C for 3 h.

Experiment 2. In this experiment, the digestive glands of winter and summer live oyster (collected at Tohoku district) were used to obtain uniform data. They were cut into two or

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0021-8561/94/1442-1519$04.50/0

three pieces and were analyzed in the same manner as experiment 1B.

Extraction of PUFA from Oysters. Each sample (0.5 g) was added to 8 mL of EtOH containing 0.002% butylated hydroxytoluene (BHT) and homogenized for 30 s. The homogenates were placed in a cold room (5 °C) and shaken for 1 h. EtOH-soluble fat was separated by centrifugation at 3000 rpm for 20 min. After addition of 3 mL of water to the same volume of EtOH solution, the pH was adjusted to 3.0 with 1 N HCl. Extractions of PUFA and its metabolites were carried out according to the method of Powell (1980). Two milliliters of the solution was applied to the Sep-Pak cartridge, and polar and nonpolar lipids were removed from the column with 20 mL of 15% EtOH, 10 mL of water, and 10 mL of petroleum ether. PUFA and other related substances were eluted with 6 mL of ethyl acetate. The solvent was evaporated under N_2. The residue was dissolved in 0.1 mL of methanol (MeOH).

Analysis of PUFA and Identification of Conjugated Diene Compounds. PUFA were analyzed by HPLC at 195 nm according to the method previously reported (Sajiki and Takahashi, 1992). For PUFA conjugated diene compounds, 20 μL of MeOH sample was injected into the HPLC and monitored at 235 nm (Sajiki et al., 1992). Identification was made by comparing HPLC peak retention times with those of authentic standards. The concentration of conjugated diene compounds of EPA is reported as a relative value to 15-HEPE. Conditions of HPLC analysis were as follows: solvent, acetonitrile/MeOH/water/phosphate (200:100:125:0.4, by volume); flow rate, 1.0 mL/min; column temperature, 40 °C. Identification of peaks was made by comparison with the absorbance spectrum pattern of authentic samples from 200 to 370 nm.

Mouse Bioassay. Forty grams of digestive glands was homogenized in 300 mL of chloroform and methanol (2:1 v/v) containing 0.001% BHT. After filtration, the residue was reextracted with 150 mL of the solvent. Solvent was removed, and finally lipid was emulsified with PBS containing 1% Tween 60 [2 g (wet weight) of digestive gland/mL of PBS]. The samples were peritoneally injected to mice. Toxicity was expressed as mouse units (MU) per gram of digestive glands according to the definition that 1 MU was the amount of toxin to kill a mouse weighing 20 g within 24 h after injection (Yasumoto et al., 1978).

Fractionation of Lipids from the Extract of Acetate-Treated Oyster by Thin-Layer Chromatography (TLC). The original lipid extracts from the acetate-treated summer oysters were applied to silica gel plates (20 cm × 20 cm) and developed with petroleum ether/diethyl ether/acetic acid (74:15:1 v/v/v) according to our previously reported method (Sajiki and Takahashi, 1992). Lipids were then fractionated into four zones as shown in Figure 1. Fraction 1 consisted mainly of phospholipids (PL); fraction 2 contained monoglyceride (MG), free fatty acids (FFA) including PUFA, their oxidative metabolites, and cholesterol (Chol); fraction 3 contained triglycerides (TG); and fraction 4 consisted of cholesterol esters (Chol-Es). Fractions 1 and 2 were removed from the TLC plates and extracted from the silica gel with methanol. Fractions 3 and 4 were extracted with diethyl ether. After the solvents were evaporated under N_2, test samples for mouse bioassay were prepared by using the same concentration with the original lipid extracts mentioned above.

Statistical Analysis. Data were analyzed by two-way analysis of variance. Significant difference was obtained by multiple tests using least significant difference (Snedecor and Cochran, 1969).

RESULTS

When 0.5 and 3.0 mg of authentic EPA were added to oyster homogenate, the recoveries by HPLC method with ethanol were 115 and 74.8%, respectively.

Figure 2 shows the average concentrations of PUFA (18:3, 18:4, 20:4, 20:5, 22:6) in six fresh oysters held under various conditions. The values of the 4% acetate treated oysters left at 5 °C for 3 and 20 h increased about 8- and 30-fold, respectively. The values for treated oysters incubated at 37 °C for 3 h increased about 43-fold. On the contrary, fresh oysters heated at 100 °C for 1 h had values similar to those of untreated oysters.

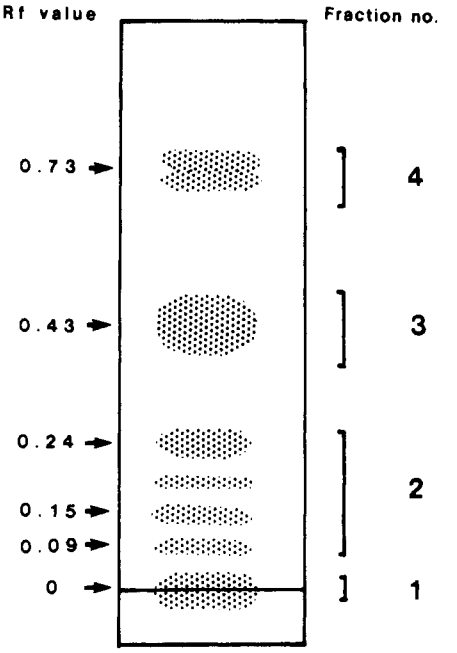

Figure 1. Thin-layer chromatogram of the lipids extracted from oyster digestive glands treated with 4% acetate at 37 °C for 3 h. Solvent: petroleum ether/diethyl ether/acetic acid (74:15:1 v/v/v). R_f values: 0, phospholipids; 0.09, monoglycerides and 15-HEPE; 0.15, cholesterol; 0.24, free fatty acids; 0.43, triglycerides; 0.73, cholesterol esters.

HPLC and absorbance spectra of main peaks monitored at 195 and 235 nm of the MeOH samples of fresh oysters and oysters frozen under three conditions are shown in Figure 3. The increase in PUFA concentrations of oysters treated with PBS at 37 °C was the same as those of oysters treated with acid. Peak 4 in Figure 3B (not found in nontreated, Figure 3A) was not identified. The absorbance pattern of this peak showed the same characteristic as that of 18:4 (Figure 3D5). This unidentified substance might be considered a PUFA that is easily increased by acetate.

The spectrum obtained by monitoring the PBS-treated sample at 235 nm (Figure 3C, 235 nm) was similar to that of the untreated one (Figure 3A, 235 nm). Although no peak was detected after 15 min in the HPLC chromatogram of both samples, several peaks appeared after 15 min in the spectra of the acetate-treated one (Figure 3B, 235 nm). On the basis of retention time and spectrum pattern of authentic substances (Figure 3D,E), peak 1 (Figure 3B, 235 nm) was considered to be a mixture of conjugated diene compounds such as 8-HEPE and 15-HEPE. Peak 2 agreed with the peak which appeared in the autoxidized authentic DHA and in EtOH blank, and it is suspected that peak 2 is a combination of two or more compounds having different characteristics. It was not possible to identify either of the peaks that appeared after 15 min and which possessed maximum absorbance at 235 nm (Figure 3D3).

Concentrations of EPA, DHA, and conjugated diene compounds of EPA in six oysters treated under three conditions are shown in Table 1. The EPA and DHA of samples treated with acetate and PBS showed significantly higher values than those of the untreated one. On the other hand, no significant difference in concentrations of conjugated diene compounds in three samples was observed. PUFA concentrations in nontreated and acetate-treated oysters before and after freezing for a month are listed in Table 2. Freezing increased the PUFA formation in nontreated oysters ($p < 0.05$) but not in the acetate-treated one.

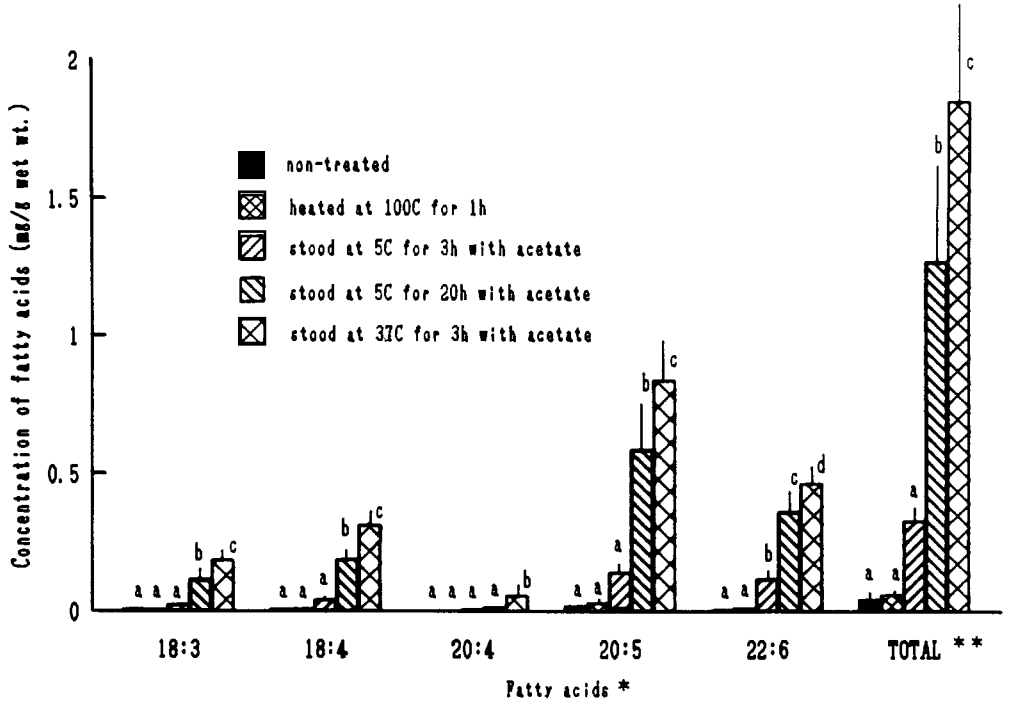

Figure 2. Changes in various PUFA concentrations of fresh oysters under different treatments. Values are mean ± SD of six oysters. Superscripts in different letters in the same fatty acid are significantly different at $p < 0.01$. The same supercripts in the same fatty acid are not significantly different. *Fatty acids correspond to the number of carbons and double bonds. **Total of five fatty acids.

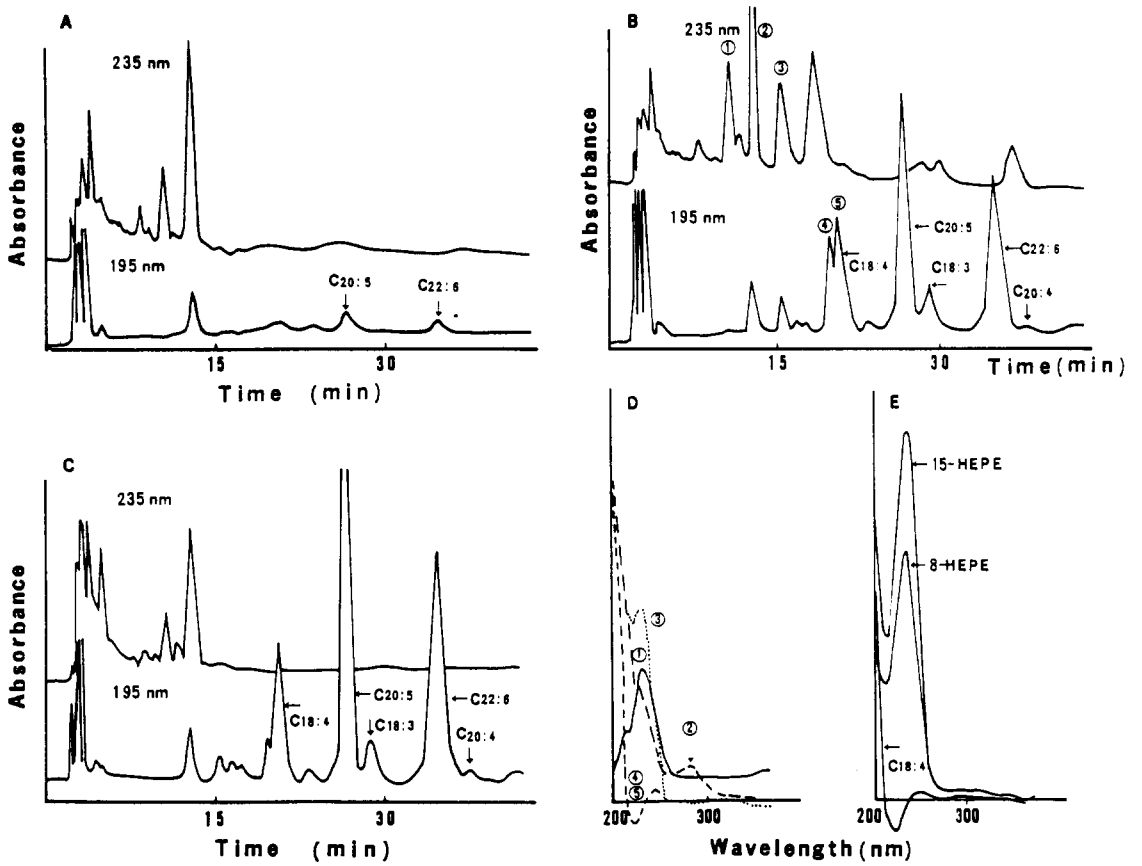

Figure 3. HPLC and UV spectra of PUFA and its metabolites extracted from fresh and frozen oyster under various treatments. (A-C) HPLC spectra: A, nontreated; B, treated with 4% acetate at 37 °C for 3 h; C, treated with PBS at 37 °C for 3 h. (D,E) UV spectra: D, UV spectra from 200 to 370 nm of peaks 1–5 illustrated in HPLC spectra B; E, UV spectra from 200 to 370 nm of authentic 8-HEPE, 15-HEPE, and $C_{18:4}$.

To investigate PUFA formation depending on ambient temperature in oysters with and without acetate, live oysters were collected in the same place, Tohoku district, in summer and winter. The weights of whole oysters and digestive glands used are shown in Table 3. Summer oysters were bigger in weight than winter oysters, but no difference in the digestive gland weight was observed. The soft portion surrounding the digestive gland of summer oysters was filled with eggs. Concentrations of PUFA in summer and winter oysters are listed in Table 4. In both

Table 1. Concentrations[a] of DHA, EPA, and Conjugated Diene Compounds of EPA in Fresh and Frozen Oysters

treatment	no. of samples	DHA (mg/g)	EPA (mg/g)	conjugated diene compds[b] (μg/g)
non	6	0.064 ± 0.030[a]	0.091 ± 0.052[a]	4.430 ± 2.271[a]
4% acetate	6	0.562 ● 0.151[b]	0.980 ● 0.303[b]	6.285 ± 2.114[a]
PBS	6	0.589 ± 0.138[b]	1.141 ± 0.360[b]	3.777 ± 1.281[a]

[a] Values are mean ± SD of six samples. Superscripts in different letters in same column are significantly different at $p < 0.01$. The same superscripts in the same column are not significantly different. [b] Values are represented as relative to 15-HEPE.

Table 2. PUFA Concentrations of Oysters before and after Freezing

treatment	no. of samples	before	after[a]
non	6	0.043 ± 0.016	0.143 ± 0.086[b]
4% acetate	6	1.856 ± 0.383	1.765 ± 0.764

[a] Samples were frozen at −70 °C for a month. Statistical significance was analyzed using Student's t-test. [b] $p < 0.05$.

Table 3. Weight of Live Oysters at Various Seasons

sample	summer (n = 12)	winter (n = 18)
whole (g)	28.13 ± 9.20	16.77 ± 4.48[a]
digestive gland (g)	1.75 ± 0.41	1.87 ± 0.54

[a] Significances of data between two seasons were analyzed according to Student's t-test. $p < 0.01$.

Table 4. Concentrations[a] of Free Polyunsaturated Fatty Acids in Live Oyster with and without Acetate Treatment

	nontreated		acetate treated	
fatty acid	summer (n = 12)	winter (n = 8)	summer (n = 12)	winter (n = 8)
18:3	ND	0.003 ± 0.007	0.242 ± 0.257	0.091 ± 0.048[b]
18:4	0.006 ± 0.009	0.001 ± 0.004	0.201 ± 0.103	0.115 ± 0.035[c]
20:4	ND	ND	0.108 ± 0.197	0.053 ± 0.021
20:5	0.018 ± 0.014	0.009 ± 0.011	1.082 ± 0.502	0.725 ± 0.113[c]
22:6	0.014 ± 0.012	0.003 ± 0.005[b]	0.664 ± 0.313	0.441 ± 0.061[c]
total	0.040 ± 0.025	0.018 ± 0.018[c]	2.298 ± 1.325	1.426 ± 0.240[c]

[a] mg/g wet wt. Significances of data between two seasons were analyzed according to Student's t-test. [b] $p < 0.01$. [c] $p < 0.05$.

Table 5. Concentrations[a] of DHA, EPA, and Conjugated Diene Compounds of EPA in Live Oysters

treatment	no. of samples	DHA (mg/g)	EPA (mg/g)	conjugated diene compds[b] (μg/g)
non	7	0.003 ± 0.005[a]	0.007 ± 0.011[a]	1.416 ± 2.114[a]
4% acetate	7	0.447 ± 0.063[b]	0.736 ● 0.118[b]	12.966 ± 8.504[b]
PBS	7	0.013 ± 0.010[a]	0.037 ± 0.043[a]	2.691 ± 3.754[a]

[a] Values are mean ± SD of seven samples. Superscripts in different letters in the same column are significantly different at $p < 0.01$. The same superscripts in the same column are not significantly different. [b] Values are represented as relative to 15-HEPE.

samples, PUFA concentration without treatment was lower than that with treatment. Total concentrations of PUFA in summer samples were significantly higher ($p < 0.05$) than that in winter in the nontreated and treated samples. Table 5 shows the concentrations of EPA, DHA, and diene compounds of EPA in live oyster digestive glands treated with acetate and PBS. Acetate treatment revealed an increase in the concentrations of these three compounds, but PBS treatment did not bring any change. Values of EPA conjugated diene compounds between the two season oysters are shown in Table 6. These compounds in digestive gland treated with acetate increased in both seasons, but no significant difference was observed between the two seasons.

Table 7 shows the mouse toxicity in lipid extracted from digestive glands of summer and winter oysters. In both

Table 6. Concentration[a] of EPA Conjugated Diene Compounds in Digestive Glands of Live Oysters with and without Acetate Treatment

treatment	summer (n = 12)	winter (n = 8)
non	1.828 ± 1.680	1.270 ± 2.000
4% acetate	12.481 ± 8.324	13.418 ± 7.976

[a] Values are mean (μg/g) ± SD. Values are represented as relative to 15-HEPE.

Table 7. Mouse Toxicity in Lipophilic Fraction of Live Oysters with and without Acetate Treatment

treatment	pH	toxicity (MU/g)[a]
summer		
non	5.4	<0.5
4% acetate	2.5	1.0
4% acetate (pH adjustment)	4.2	1.0
winter		
non	4.8	<0.5
4% acetate	2.9	<0.5
PBS	4.0	<0.5
blank		
4% acetate	3.8	<0.5

[a] Mouse lethal units/g wet wt.

the treated and nontreated winter oysters, no toxicity was observed; only the summer samples with acetate treatment showed high toxicity (1.0 MU/g). Adjustment of pH did not change the toxicity effect in mice but only delayed the dying time. When 1 mL of the treated summer sample was orally administered to each of five mice, all died within 25 min after the injection. To confirm which lipid classes were associated with mouse lethal toxicity, 1 mL of each of the samples of the four lipid classes isolated by TLC (Figure 1) was injected peritoneally into four mice. All mice injected with fraction 2 containing FFA including PUFA, their oxidative metabolites, MG, and Chol died, but all mice injected with other fractions survived for 24 h. Since the mice died in such a short time, diarrhea was not observed; however, significant anatomical changes, such as reddish edematous in the intestine, were observed in all of the dead mice. Also noted were an enlargement of the stomach and reddish edematous in the pyloric region and intestine.

DISCUSSION

In Japan, oysters pickled with vinegar are popular with many people, in spite of a high incidence of oyster-associated acute gastroenteritis (Sekine et al., 1989). Bacteria (Klontz et al., 1988; Reeve et al., 1989) or viruses (Sekine et al., 1989; Murphy et al., 1979; Gill et al., 1983) have been considered to be causative factors of acute gastroenteritis, accompanied with symptoms such as vomiting and diarrhea. Klontz (1991) reported that illnesses induced by eating raw oysters continue to occur, frequently resulting in hospitalization and occasionally in death, despite warnings.

It is generally considered that an increase of free PUFA is important because they are precursors of oxidized products which cause off-flavors, quality deterioration (Wilson et al., 1976), and toxicity to animals (Oarada et al., 1989; Jurgens et al., 1986; Kanazawa and Ashida, 1991). We prevously reported that peritoneal injection of EPA and its oxidized products induces death in mice (Sajiki and Takahashi, 1992) and diarrhea, as indicated by the loop test using rabbit intestine (Sajiki et al., 1993). The remarkable increase of PUFA observed in oysters treated with acetate at 37 °C for 3 h suggests a close association with poisoning by vinegar-pickled oysters. Poisonous

oysters used in this experiment gave gastrointestinal disorders, such as vomiting, stomachache and diarrhea, to humans within 3 h after eating.

In our mouse toxicity testing, using the live oysters collected in winter and summer, only the acetate-treated summer oysters had high toxicity (1.0 MU/g). This shows summer oysters are more toxic than winter ones. Fractionation of lipid classes by TLC was performed, and the fractions were injected to mice; the results showed that only the fraction containing FFA and their oxidative metabolites, MG and Chol, showed toxicity. Since separation of substances in this fraction was not possible, it was not possible to determine which of them caused the toxicity. Although PUFA concentrations in acetate-treated summer oyster were highest among oysters used in the toxicity test, the level was only about 2.3 mg/g. This level was lower than the authentic EPA level, for which the LD_{50} of peritoneally injected mice was 7.3 mg/g (Sajiki et al., 1992), so it is hard to conclude that only the PUFA cause high toxicity in mice. The sudden death induced by injection of the toxic oyster was similar to the result of our previous experiment in which mice peritoneally injected with autoxidized EPA prepared by shaking authentic EPA at 37 °C died within 1 h (Sajiki et al., 1992). The sudden death observed might be due to the formation of oxidized metabolites of PUFA caused by addition of acetate.

It is well-known that many conjugated diene compounds with maximum absorption at 235 nm are formed when PUFA is autoxidized, and various kinds of conjugated diene compounds of PUFA including EPA and DHA were synthesized via enzymes such as lipoxygenase in fish (German and Creveling, 1990; Josephson et al., 1987; Henderson et al., 1985) and played an important biological role as a hatching factor in the barnacle (Hill et al., 1988; Song et al., 1990). The concentrations of conjugated diene compounds of EPA in acetate-treated oysters were higher than those in nontreated oysters in both seasons. The unknown substances (peaks 3 and 4) possessing maximum absorbance at 235 nm, which formed in the acetate-treated but not in the nontreated oysters, might participate in an expression of toxicity to animals.

Recently, DTX_3, a compound that bound fatty acid with DTX_1, was reported as a highly toxic substance; DTX_3 containing PUFA ($C_{22:6}$) had 10 times higher mouse lethal toxicity than that containing saturated fatty acid ($C_{16:0}$) (Yanagi et al., 1989). These compounds may be also considered as causative toxins, although the question remains whether these toxins are produced in oyster by the treatment with acetate or not.

PUFA in nontreated and treated summer oysters were significantly higher than in winter oysters. These data show that storage time post mortem and ambient temperature are very important factors in the formation of PUFA. These results conformed with the results found for sardine (Tsukuda, 1978). Also, freezing might influence the formation of PUFA.

Further studies concerning the mechanism of PUFA formation in oysters and the cause of poisoning by consumption of raw vinegared oysters are necessary.

ACKNOWLEDGMENT

I express my gratitude to Dr. N. Sanceda of Ochanomizu University for her helpful suggestion and to Mr. K. Taguchi of Kesennuma Fisheries Experiment Station for sending live oysters. Also, I extend my appreciation to Dr. H. Takahashi and Mr. K. Mori of my laboratory for helping with the anatomical study for the mouse toxicity test.

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Received for review July 6, 1993. Revised manuscript received March 28, 1994. Accepted April 15, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, June 1, 1994.