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Toxic Effect of Free Polyenoic Acids: A Fat-Soluble Marine Toxin

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Abstract

A fat-soluble toxic fraction was separated from the digestive gland of the poisonous Okhotsk scallop. Extraction and chromatography steps were monitored for toxicity of the fraction using mouse and fish tests. The major toxic components were found to be the polyenoic acids in the form of free fatty acids. A similar toxicity of the free fatty acids obtained by the hydrolysis of the triacylglycerols of the toxic and nontoxic scallops, and by hydrolysis of sardine oil, supported this idea. In bioassay with pure unsaturated acids, oleic (18:1) acid was negligibly toxic, linoleic (18:2) and 4, 7, 10, 13, 16, 19–docosahexaenoic (22:6) acids were mildly toxic, and linolenic (18:3), arachidonic (20:4), and 5, 8, 11, 14, 17-eicosapentaenoic (20:5) acids were more highly toxic. Conversion of the acids to methyl esters eliminated the toxicity. This supports the belief in the toxicity of the free fatty acids. Apparently, free highly unsaturated fatty acids in marine organisms are one kind of marine toxin.

Introduction

A peculiar type of food poisoning has often been reported to follow the eating of scallops harvested in some districts of Hokkaido Islands and the northern part of Honshu, Japan. The symptons of the victims are mainly gastrointestinal, such as diarrhea, nausea, vomiting and abdominal pain, and the paralytic symptoms which are usual in food poisoning by toxic scallops have been lacking[1]. Toxicity studies using mouse assay on leftover food and on shellfish from the area have indicated the presence of unknown fat-soluble toxin in the digestive gland of the scallops[2,3].

In the fall of 1980, food poisoning associated with the eating of scallops occurred near Monbetsu on the Okhotsk Coast of Hokkaido Island. It was concluded that this was diarrhetic poisoning by the fat-soluble toxin, and the harvesting of the scallops was officially prohibited.

In this study, the lipids were extracted from the digestive gland of the Okhotsk scallop, and the toxic fraction was separated by chromatography. Finally, the toxic components were characterized as free polyenoic acids. This was confirmed by a similar toxicity of the free polyenoic acids prepared from a common fish oil and a vegetable oil, and by the detoxification of the free fatty acids through esterification.

The toxicity of 18:2[4–6], 18:3[4,5] and 20:4[4] acids to aquatic organisms, including brine shrimp[4], yellow fever mosquito larvae[5], and killifish[6], has been reported.

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In this study, the toxicity of the highly unsaturated fatty acids common in aquatic organisms, such as 20:5 and 22:6, was investigated. They provided the most reasonable choice of toxins released from aquatic organisms.

In our country, cases of diarrhetic poisoning caused by the fat-soluble toxin of scallops, mussels and other shellfish are reported several times annually[7]. Since the effect of the diarrhetic toxin is not as fatal as that of paralytic toxin, some of the less severe cases might be overlooked. Other diarrhetic toxins besides the free polyenoic fatty acids could possibly also be present in some of the poisonous shellfish.

Materials and Methods

Extraction for toxicity test

About 200 g of the digestive gland was extracted three times by homogenization with three 50 ml portions of acetone for 2 min in a stainless steel homogenizer at room temperature. The combined extracts were freed from acetone under reduced pressure in a rotary evaporator and the residual aqueous liquid was extracted with diethyl ether. Removal of the ether by distillation left a viscous residue used for the bioassay.

Mouse assay

The procedures described in the previous paper[3] were employed. A known amount of the extract was suspended in 1% Tween 60–physiological salt solution up to a certain volume. Aliquots (0.5–1.0 ml) of this solution or its serially diluted solution were given intraperitoneally to three male mice of ddY strain weighing 16–20 g each. The minimum amount of toxin required to kill a mouse within 24 hr was defined as one mouse unit (Mu).

Fish assay

The procedures described in the previous paper[6] were employed with some modification. Killifish, *Oryzias laptipes*, 300–350 mg in weight and 3.0–3.5 cm in length were used in the test fish. They were not fed for one day before they were used in the test. The test containers were 500 ml beakers, in which 300 ml of water provided a depth of about 10 cm and was kept at 20°C. Methanol/acetone (1:1 v/v; 2 ml) containing a known amount of the extract was added to the beaker and stirred vigorously. The test solution containing only 2 ml of the solvent served as a control solution. For each test five fish were introduced to the solution in the beakers. Their behaviors were observed continously, any fish which died during the test was immediately removed from the solution, and the time was recorded.

Extraction and fractionation of lipids

Four hundred grams of the digestive gland were extracted with chloroform/methanol (1:2 v/v) by the Bligh and Dyer method[8], using a stainless steel homogenizer. The lipid extracts were separated by liquid chromatography on silicic acid. The neutral and polar lipids were eluted with chloroform and methanol,

respectively. The neutral lipids were fractionated by column chromatography on silicic acid using diethyl ether/hexane for development. The free fatty acid fraction obtained was refined by preparative thin-layer chromatography (TLC) on Silica Gel (Merck and Co.) using diethyl ether/hexane/acetic acid (50:50:1 v/v/v) for development. Triacylglycerols, free fatty acids and other fractions were separated by these procedures.

Preparation of unsaturated acids

Unsaturated acids of purities above 95% and peroxide value (POV) below 10 were prepared using original materials as follow: safflower oil for 18:2, linseed oil for 18:3, pig liver oil for 20:4, and pollack liver oil for 20:5 and 22:6 acids. Methyl esters prepared from the original materials were fractionated by chromatography on silver nitrate-impregnated silicic acid columns[9]. The purified methyl esters were hydrolyzed and the recovered free fatty acids were refined by silicic acid-column chromatography using diethyl ether/hexane for development. The purities of the unsaturated acids were examined by gas-liquid chromatography (GLC) of the methyl esters.

GLC analysis

GLC of the methyl esters was done with a Shimadzu 7A instrument (Shimadzu Seisakusho Co.) equipped with a dual FID detector and glass capillary column (50m×0.3 mm) coated with SP–2300 (a cyanosilicone; Supelco Inc.). The temperature was 175°C at the column and 220°C at the detector and sample inlet, and the carrier gas was H_2. Peak areas were obtained by a Shimadzu R1A integrator.

POV determination

The POV of the lipid samples was determined by the colorimetric iodine method[10] with small amounts of the samples, for example 40 mg.

Nuclear magnetic Resonance (NMR)

A JEOL FX–200 spectrometer (Nippon Denshi Co.) in the Fourier transform mode at 199.50 MHz (^1H) and 25.00 MHz (^{13}C) was used to obtain ^1H and ^{13}C NMR spectra of the sample in $CDCl_3$.

Results and Discussion

About 20 Kg of the digestive gland of the toxic Okhotsk scallops, *Patinopecten yessoensis*, stored below –20°C for few months, was used in this study. As expected, the acetone extract of the digestive gland showed a high toxicity, above 0.5 Mu but below 1.0 Mu per 1 g digestive gland. The official controlled level in this country for the diarrhetic poisoning is 0.05 Mu per 1 g shucked scallop.

The chloroform-methanol extraction of the digestive glands of the toxic and nontoxic scallops gave total lipid recoveries of 2.9% and 7.5% (wet weight), respectively. The nontoxic scallops were obtained at Hakodate in the spring of

Table 1. *Comparison of toxicity of scallop lipids to fish*

Sample	Added[b] mg	Time to kill[c] min	No. of fish
A. Toxic scallop			
Neutral lipids (NL)	57	109±61	3
Polar lipids	24	395±42	3
Nontriacyl glycerol fraction of NL	47	58± 8	5
Free fatty acid fraction of NL[a]	29	110±21	4
Fatty acids from triacylglycerols[a]	25	114±23	3
B. Nontoxic scallop			
Fatty acids from triacylglycerols[a]	27	76±24	3
C. References			
Fatty acids from soybean oil	24	594±48	3
Fatty acids from sardine oil[a]	28	88±38	5

[a] The compositions of fatty acids are shown in Table 2.
[b] Each sample was added to 300 ml water for the test.
[c] Mean±standard deviation.

1981. The column chromatography of the total lipids gave proportions for the neutral and polar lipids of 84.5% and 15.5% for the toxic scallops, and 90.8% and 9.2% for the nontoxic scallops. In the toxicity test of each fraction, only the neutral lipids from the toxic scallops showed toxicity towards fish (Table 1). The nontriacylglycerol fraction obtained by TLC of the neutral lipids also showed this toxicity. In further TLC separation, only the extracts of the second band above the application line showed the toxicity. The components of this band were characterized as free fatty acid mixtures with infrared spectroscopy, ^{1}H and ^{13}C NMR, and the retention data in GLC of the methyl esters. The column chromatography of the neutral lipids of the toxic scallops gave the following fractions: sterol esters 5.6%, triacylglycerols 58.4%, free fatty acids 16.4%, free sterols 7.3%, and others 12.4%. The neutral lipids showed an acid value of 35.1. After refining with TLC, the free fatty acids from the toxic scallop lipids showed the toxicity to fish as following: 110 min at 97 mg/l, 139 min at 70 mg/l, 230 min at 47 mg/l, and 700 min at 17 mg/l. The free fatty acids obtained by the hydrolysis of the triacylglycerols from the toxic and nontoxic scallop lipids and sardine oil showed nearly the same toxicity when compared with the natural free fatty acids from the toxic scallops. The free fatty acids from soybean oil showed very weak toxicity (Table 1). The compositions of the total fatty acids from the triacylglycerols of the toxic and nontoxic scallops, and sardine oil, and the free fatty acids from the toxic scallops are shown in Table 2. The fatty acids of soybean oil showed the following composition: 16:0 9.9%, 18:0 3.8%, 18:1 n–9 21.5%, 18:1 n–7 1.5%, 18:2 n–6 54.3%, 18:3 n–3 6.6% and others 2.4%. These facts suggest that the polyenoic acids in marine lipids are the components toxic to fish.

Methyl esters prepared from the natural free fatty acids from the toxic scallops, and free acids obtained by hydrolysis of the triacylglycerols of the toxic scallops, were separated into saturated and monoenoic ester fractions, and a polyenoic ester fraction, with silver nitrate-TLC. After hydrolysis, each fraction was subjected to the mouse test. Only the polyenoic acid fractions showed the high toxicity (Table 3). The compositions of each fraction are shown in Table 2.

Table 2. *Fatty acid compositions*[a] *of the samples used for the fish and mouse test*

Fatty acid	RRT	Scallop							Sardine TG
		Toxic						Nonotoxic TG	
		TG			FFA				
		Total	SM	P	Total	SM	P		
		(Peak area %)							
13:0	0.162	0.1	0.2	0.3	0.1	0.1	0.2	0.1	0.1
14:0	0.232	4.4	10.9	0.9	4.6	12.4	0.2	5.2	6.9
14:1 n–7	0.266	0.1	—	—	0.1	—	—	0.1	—
14:1 n–5	0.279	0.1	0.2	0.1	0.1	0.2	0.1	0.2	0.2
15:0	0.336	0.3	0.7	—	0.6	1.1	—	0.4	0.4
16:0	0.489	15.3	33.2	0.1	13.8	28.1	—	16.5	19.1
16:1 n–11	0.533	0.2	0.3	—	0.2	0.5	—	—	—
16:1 n–9	0.539	0.2	0.3	—	0.3	0.4	0.1	0.6	0.6
16:1 n–7	0.559	12.3	18.1	7.0	10.0	22.1	2.8	12.6	6.3
17:0	0.692	1.0	0.8	1.3	1.0	1.2	1.1	0.9	1.2
17:1 n–8	0.776	0.3	0.4	0.8	0.2	0.5	0.8	0.3	0.3
16:2 n–6	0.918	0.5	—	1.7	0.3	—	1.2	0.5	1.1
18:0	1.000	2.0	4.2	0.1	3.2	4.1	—	2.1	3.0
18:1 n–13	1.052	0.2	0.3	—	0.2	0.2	—	0.2	—
18:1 n–11	1.088	—	0.1	—	0.1	0.1	—	0.1	0.1
18:1 n–9	1.114	4.2	8.1	0.3	3.9	6.2	0.1	4.4	10.3
18:1 n–7	1.145	5.7	10.3	0.5	4.4	7.1	0.2	5.7	3.4
18:2 n–6	1.330	2.1	1.3	2.4	2.0	2.7	1.5	2.1	1.1
19:0	1.419	0.7	0.5	0.6	0.5	0.9	0.3	0.7	0.4
18:3 n–6	1.472	0.2	—	0.4	0.2	—	0.4	0.2	0.2
19:1 n–8	1.586	0.1	0.1	0.2	0.1	—	0.2	—	0.1
18:3 n–3	1.651	1.3	0.6	2.2	1.5	2.1	1.9	1.4	1.0
18:4 n–3	1.830	4.0	0.1	9.1	3.4	0.1	10.2	3.8	2.8
20:0	2.043	0.1	0.2	—	0.1	0.1	0.1	—	0.2
20:1 n–11	2.200	0.7	1.3	—	1.4	1.0	0.1	0.8	2.3
20:1 n–9	2.254	0.4	0.9	—	0.8	0.7	—	0.4	2.0
20:1 n–7	2.328	1.2	2.0	—	1.3	1.4	—	1.1	0.3
20:2 Δ5,11	2.377	0.2	—	—	0.4	—	—	—	—
20:2 Δ5,13	2.427	0.2	—	—	0.2	—	—	0.2	—
20:3 n–9	2.696	0.4	0.5	—	0.5	0.6	0.3	0.3	0.2
20:3 n–6	2.968	0.2	—	0.3	0.2	0.1	—	0.3	0.2
20:4 n–6	3.136	1.1	—	2.0	2.9	0.2	3.7	1.1	1.0
20:3 n–3	3.341	0.2	0.2	—	0.1	0.3	—	0.2	0.1
20:4 n–3	3.676	0.8	—	—	0.5	0.3	1.0	0.8	1.0
20:5 n–3	3.935	26.5	0.8	51.6	20.3	1.0	45.5	25.0	14.4
21:5 n–3	5.533	0.7	—	1.7	—	—	1.5	—	0.4
22:5 n–3	7.972	0.3	—	—	0.6	—	0.6	—	2.3
22:6 n–3	8.426	7.5	—	13.1	14.0	—	23.1	6.6	10.3
others		4.2	3.4	5.0	6.7	4.0	2.8	5.1	6.8

[a] RRT: Relative retention time to 18:0. TG: Triacylglycerols. FFA: Free fatty acids. SM: Saturated and monoenoic acid fraction. P: Polyenoic acid fraction. SM and P of FFA were separated from the total FFA different with that listed in this Table.

Table 3. *Toxicity of natural fatty acids and free fatty acids obtained by hydrolysis of triacylglycerols from toxic scallop to mouse*

Acid fraction	Sample	Wt. for 1 Mu mg
Saturated and monoenoic	Natural free acids	58-29
	Free acids from triacylglycerols	53<
Polyenoic	Natural free acids	5-10
	Free acids from triacylglycerols	10-20

[a] See the text for the procedures of the test. The period for the test is 48 hr. The compositions of fatty acids are shown in Table 2. Three mice are used in each test.

Table 4. *Toxicity of free polyenoic fatty acids to fish and mouse*

Fatty acid	Fish test[a]		Mouse test[c]	
	No. of fish used	Time to kill[b] min	No. of mouse used	Wt. for 1 Mu mg
18:1 n-9	5	700	3	25-50
18:2 n-6	6	254±77	3	12-25
18:3 n-3	7	124±61	3	6-12
20:4 n-6	4	162±53	3	6-12
20:5 n-3	6	160±49	6	6-12
22:6 n-3	6	527±40	5	12-25

[a] Each 50 mg was added to 300 ml water for the test.
[b] Mean±standard deviation.
[c] See the text for the procedures of the test. The periods for the test are 24 hr for 18:1 and 48 hr for others.

Toxicity of pure unsaturated fatty acids by both bioassays is shown in Table 4. In the mouse and fish tests, 18:1 was negligibly toxic, 18:2 and 22:6 were toxic to a small degree, and 18:3, 20:4 and 20:5 were toxic to a higher degree. The toxicity of the free fatty acids in the fish test was found to be approximately parallel to those obtained by the mouse test in this study, though 20:4, 20:5 and 22:6 showed somewhat lower toxicity relative to 18:2 and 18:3 in the fish test than in the mouse test. Therefore, the fish test can be used for the preliminary tests for evaluation of toxicity of free fatty acids.

In this study, the pure polyenoic fatty acids used for the bioassay showed POV below 10 and showed a single spot on the TLC plate. POV remained below 10, even after any increase, when the free fatty acid from the sardine oil was left for 5 hr under the same conditions as in the fish test. This shows that the rate of oxidation of the free fatty acids was not fast, though it had a significant influence on the results of the fish test. In turn this shows that autoxidized polyenoic acids in the sample were in a trace amount, and did not have an appreciable influence on the bioassay.

It have often been observed at pearl-culture stations in the Okinawa District that the pearl-oyster larvae show a high mortality when kept in a tank having microalgae. The ichthyotoxicity was attributed to free fatty acids released by

the green alga, *Chaetomorpha minima*[6]. The acids were reported to consist mainly of 16:0 33%, 16:1 12%, 18:4 14%, and 18:2 10%. In that study, authentic samples of saturated acids (C_8-C_{20} even carbon number acids and C_9 acid), and 16:1, 18:1 and 18:2 were tested for their toxicity to killifish. Relatively high toxicity was observed for lower saturated acids (C_9-C_{12}), 16:1 and 18:1. In this study, on the contrary, 18:2 showed higher toxicity to killifish than 18:1. In any event, both 18:1 and 18:2 showed a relatively low toxicity. Marine algae usually contain highly unsaturated fatty acids having more than three double bonds[11,12], exactly those that showed the higher toxicity to the fish in this study. The toxicity of the free fatty acids of *C. minima* to fish may be associated with the more highly unsaturated fatty acids. In the previous study[6], occurrence of such highly unsaturated acids could be detected if GLC analysis was carefully continued after the appearance of 18:2 peak.

In a previous paper, which described the fish assay of microbial toxins[4], the toxicity of free fatty acids to brine shrimp larvae was reported to increase in the following order (LC_{50} μg/ml): 87 (18:1), 3.3 (18:2), 2.4 (18:3), 1.5–2.0 (20:4). In this study, the order of the toxicity to fish was nearly the same, but the difference between the toxicity of 18:2 and 18:3 was more remarkable.

Fish reared by aquaculture often suffer severe mortalities from the fat-soluble toxin released from the plankton, *Hornellia* sp., which causes the "red tide" in southern Japan. This toxin was reported to be free fatty acids, but the structure of the fatty acids was not described[13]. In such cases, the gills of the fish that died became darkly stained by immersion in a 1% trypan blue aqueous solution, although the gills of the reference fish were not stained by the same treatment[14]. In this study, the gills of the killifish that died following the addition of the free polyenoic acids were similarly stained by the same dye. A viscous film was observed on the surface of the gills of the dead fish before the staining. This fact privides evidence for damage to the permeability of the gill epithelia of the fish that die in a similar manner to that described previously[14].

Since highly unsaturated fatty acids such as 20:5 are common components of phytoplankton[15,16], and they showed higher toxicity in this study, the toxin of the "red tide" is presumed to consist of free highly unsaturated fatty acids.

In the official method of testing for shellfish poisons in our country, diarrhetic and paralytic toxins are examined using a mouse by the intraperitoneal injection of diethyl ether-soluble and water-soluble fractions extracted from shellfish, respectively. This method has been used for testing toxicity and the procedures are described in the experimental section of this paper. The appearance of diarrhetic toxicity in shellfish has always been accompanied by a positive indication of the toxicity in the intraperitoneal injection of diethyl ether-soluble fraction in the mouse. The facts indicate that the poisonous material in the mouse test is actually the portion diarrhetically toxic to humans. Since only the free polyenoic acid fraction of the scallops having diarrhetic toxicity showed toxicity in the mouse tests, the free polyenoic acids are likely the toxic component to human. The comparably higher content of free fatty acids in the toxic shellfish also supports this conclusion.

Since the free polyenoic acids showed clear toxicity, but the methyl esters did not, the emergence of the toxic effect requires both the oxidized polyenoic chain

and free carboxylic group. Investigation on the structures of the oxides from free polyenoic acids that possess the toxicity is under further study.

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