Determination of Free Fatty Acids in Marine Phytoplankton Causing Red Tides by Fluorometric High-Performance Liquid Chromatography

Toshiyuki Suzuki[a,*] and Yukihiko Matsuyama[b]

[a]Tohoku National Fisheries Research Institute, Shiogama, Miyagi 985, Japan
and [b]Nansei National Fisheries Research Institute, Saeki-gun, Hiroshima 739-04, Japan

ABSTRACT: The proportion and content of free fatty acids (FFA) in four species of causative phytoplankton of red tides, cultured in axenic conditions, were obtained by fluorometric high-performance liquid chromatography. Twelve FFA in phytoplankton were identified. Raphidophyte flagellates, *Chattonella antiqua* and *Heterosigma akashiwo*, contained highly unsaturated fatty acids and 16:0 acid as the predominant FFA. Major FFA in diatoms, *Skeletonema costatum* and *Chaetoceros didymum*, were 14:0, 16:0, 16:1, and 20:5 acids.
JAOCS 72, 1211–1214 (1995).

KEY WORDS: 9-Anthryldiazomethane, axenic, *Chaetoceros didymum*, *Chattonella antiqua*, free fatty acid, *Heterosigma akashiwo*, HPLC, phytoplankton, *Skeletonema costatum*.

In 1972, a red tide bloom of the raphidophyte flagellate *Chattonella antiqua* killed 7100 million yen worth of caged yellowtail fish in the Seto Island Sea of Japan (1). Another raphidophyte, *Heterosiama akashiwo*, has killed caged fish in various parts of the world (2).

In experimental assay systems, free fatty acids (FFA) destroy red blood cells and show hemolytic activity (3) that has been provisionally termed "hemolysin." FFA, especially the highly unsaturated fatty acids (HUFA) contained in these raphidophyte flagellates, have been implicated as one of the causative substances which damage the epithelial tissues of the fish gills (1–4). However, there are few detailed reports concerning the proportion and content of FFA in phytoplankton responsible for red tides.

In the present study, the proportion and content of FFA in four species of causative phytoplankton of red tides, *C. antiqua*, *H. akashiwo*, *Skeletonema costatum*, and *Chaetoceros didymum*, cultured in axenic conditions were determined by fluorometric high-performance liquid chromatography (HPLC).

EXPERIMENTAL PROCEDURES

FFA standards. Myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), α-linolenic acid (18:3), and arachidic acid (20:0) were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Palmitoleic acid (16:1), *cis*-6,9,12,15-octadecatetraenoic acid (18:4), and arachidonic acid (20:4n-6) were purchased from Sigma Chemical Co. (St. Louis, MO). *cis*-5,8,11,14,17-Eicosapentaenoic acid (20:5) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (22:6) were supplied by Dr. Y. Itabashi of Hokkaido University (Hakodate, Japan). These FFA were used for peak identification and determination of peak area of FFA obtained from phytoplankton. 9-Anthryldiazomethane (ADAM) was obtained from Funakoshi Pharmacy Co. (Tokyo, Japan).

Algal cultures. Phytoplankton was isolated at the following localities—*C. didymum* and *H. akashiwo*, Hiroshima bay, *C. antiqua* and *S. costatum*, Osaka bay. A clonal axenic culture was established using the micropipette isolation method (5) and/or antibiotic treatment (6). All strains were cultured in modified SWM-3 medium (7,8) for 9–12 d at 22°C under 100 μ Em-2s-1 of cool-white fluorescent illumination on a 12-h light/12-h dark cycle. A sterility test was carried out by the method of Provasoli *et al.* (9) at the end of each culture experiment. Cells were harvested by centrifugation (>3000 rpm × 10 min) and stored at a temperature below −20°C until analysis.

Extraction of FFA and derivatization. The concentrated samples (30–50 mg wet weight: 4.8×10^5 cells for *C. antiqua*, 2.9×10^7 cells for *H. akashiwo*, 1.8×10^7 cells for *S. costatum*, and 2.0×10^7 cells for *C. didymum*) were suspended in 1 mL of chloroform/methanol (2:1, vol/vol) and sonicated (3 × 5 min). The combined extracts were evaporated to dryness under reduced pressure and dissolved in 2 mL of 80% methanol. FFA were extracted three times with 2 mL of *n*-hexane. The combined extracts were dissolved in 3 mL of *n*-hexane after evaporation. An aliquot (300 μL for *S. costatum*, *C. antiqua*, and *H. akashiwo*, and 75 μL for *C. didymum*) of 3 mL *n*-hexane solution was put in a colored vial and dried under nitrogen. The residue was esterified in 100 μL of 0.1% ADAM/methanol solution for 1 h in the dark at ambient temperature (10), and 10 μL of the solution was directly injected into the HPLC.

HPLC. HPLC separation was carried out with a Hitachi (Tokyo, Japan) L-6200/L-6000 gradient system equipped

*To whom correspondence should be addressed at Tohoku National Fisheries Research Institute, 3-27-5 Shinhama, Shiogama, Miyagi 985, Japan.

with a Develosil ODS-5 column (250 × 4.6 mm i.d.; Nomura Chemical, Seto, Japan) with a gradient of solvent A (acetonitrile/methanol/water, 8:1:1, vol/vol/vol) and solvent B (methanol) at ambient temperature and a flow rate of 1.1 mL/min (11). The following linear gradient was used: segment 1, 100% A for 20 min; segment 2, initial conditions changed to 100% B over 30 min; and segment 3, 100% B for 50 min. The peaks of fluorescent derivatives were monitored with a Hitachi F-1050 spectrofluorometer. The excitation and emission wavelengths were set at 365 and 412 nm, respectively. Eluate containing 14:0 and 16:1 was collected from the outlet of the fluoromonitor and rechromatographed on a Capcell Pak CN SG 120 column (250 × 4.6 mm i.d.; Shiseido, Tokyo, Japan) with acetonitrile/water (7:3, vol/vol) as mobile phase at ambient temperature. The flow rate was kept at 1.1 mL/min. Excitation and emission wavelengths of the fluoromonitor were the same as those for the other HPLC.

RESULTS AND DISCUSSION

Figure 1 shows the chromatogram of FFA as ADAM derivatives obtained from *H. akashiwo* on Develosil ODS-5 column. FFA identifications were carried out by comparing relative retention times (RRT) of samples with those obtained from commercial standards (Table 1). Although peaks of 16:1 and 14:0 completely overlapped, partial separation of 22:6 and 18:3, as well as fair separation of other prominent FFA, were obtained. An unknown peak with an RRT (0.42) shorter than that of 18:4 (RRT 0.52) was observed. An unidentified peak also was eluted in the HPLC of *S. costatum* fatty acids, but with a difference in RRT (0.47). From the RRT, the unknown peaks are estimated to those of the 16:4 and 16:3 frequently observed in phytoplankton (12–17) because the ADAM derivatives of FFA elute in the order of increasing carbon number and decreasing number of double bonds in reversed-phase HPLC (11). Linearity of the respective peak area with increasing concentrations of FFA was confirmed over a wide range of concentrations, showing that reaction of ADAM with each FFA was both quantitative and reproducible (11).

Because 14:0 and 16:1 acids are frequently predominant FFA in several species of phytoplankton, especially diatoms (12–17), resolution of 14:0 and 16:1 as ADAM derivatives was attempted in determining these FFA. Figure 2 shows the HPLC resolution of eluate containing 14:0 and 16:1 collected in the HPLC run in Figure 1. The 14:0 and 16:1 were separated with 1.08 in separation factor (α).

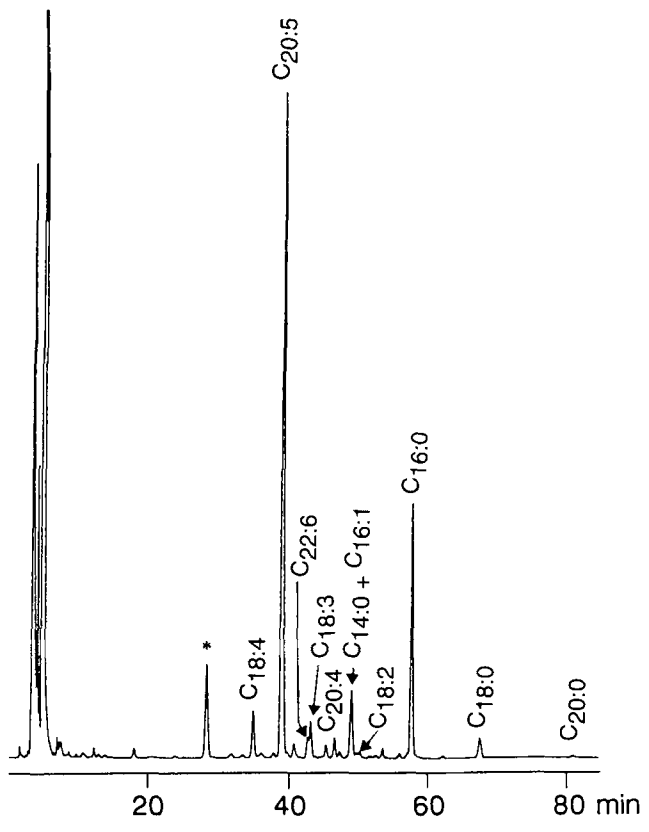

FIG. 1. High-performance liquid chromatographic resolution of the 9-anthrylmethyl esters of free fatty acids obtained from *Heterosigma akashiwo* cultured in axenic conditions. An aliquot (1/100 of 31 mg of phytoplankton) of the extract was injected. The peak of 20:5 corresponds to 746 ng. *Unidentified peak with 0.42 in relative retention time with respect to 18:0. See text for chromatographic conditions.

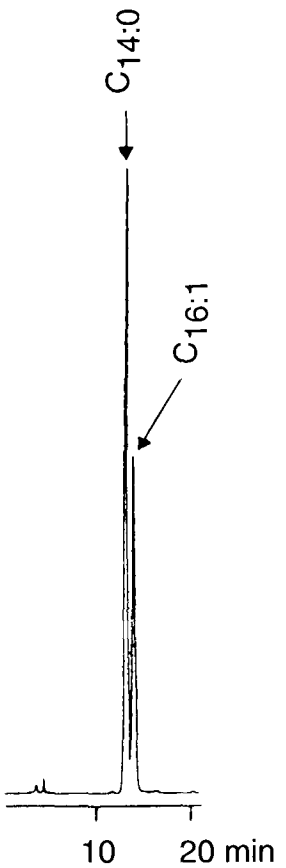

FIG. 2. High-performance liquid chromatographic resolution of the 9-anthrylmethyl esters of 14:0 and 16:1 acids collected from the first high-performance liquid chromatography run in Figure 1 and re-analyzed. See text for chromatographic conditions.

TABLE 1
Proportion (wt%) and Total Cellular Content (pg/cell) of Free Fatty Acids in Four Species of Phytoplankton

		Raphidophytes		Diatoms	
FFA[a]	RRT[b]	*Chattonella antiqua*	*Heterosigma akashiwo*	*Skeletonema costatum*	*Chaetoceros didymum*
Saturated					
$C_{14:0}$	0.72	3.1	4.3	42.9	43.4
$C_{16:0}$	0.85	18.6	19.0	6.7	15.3
$C_{18:0}$	1.00	1.4	2.8	1.1	1.2
$C_{20:0}$	1.20	0.2	0.5	0.1	0.1
Monounsaturated					
$C_{16:1}$	0.72	6.1	2.5	16.4	33.2
$C_{18:1}$	0.84	0.7	ND[c]	1.5	1.4
Polyunsaturated					
$C_{18:2}$	0.74	3.0	0.2	0.8	0.3
$C_{18:3}$	0.64	6.6	3.2	ND	0.4
$C_{18:4}$	0.52	20.2	4.3	0.8	0.2
$C_{20:4}$	0.69	6.1	1.8	1.8	ND
$C_{20:5}$	0.58	31.0	59.6	26.4	4.5
$C_{22:6}$	0.63	3.0	1.8	1.5	ND
Total		100.0	100.0	100.0	100.0
Total cellular FFA contents (pg/cell)		386.7	4.3	1.6	25.5

[a]Free fatty acids (FFA) are expressed as the number of carbon atoms: number of double bonds.
[b]Relative retention time (RRT) with respect to 18:0 acid.
[c]ND, none detected.

The proportion and content of FFA in four species of phytoplankton determined after HPLC separation are given in Table 1. To our knowledge, the FFA proportion of *C. antiqua* and *C. didymum* has not been reported. The high absolute levels of FFA in *C. antiqua,* compared with those of other species, is indicative of larger cell size of this alga (18).

Major FFA in *C. antiqua* were 20:5, 18:4, and 16:0. The FFA composition of *C. antiqua* was characterized by a high proportion of 18:4 in comparison with that of other species. On the other hand, major FFA present in *H. akashiwo* were 20:5 and 16:0. The very high percentage of 20:5 in *H. akashiwo* is noteworthy. The sum of the HUFA (18:4, 20:4, 20:5, and 22:6) in *C. antiqua* and *H. akashiwo* accounted for a 60.3 and 67.5% (wt%) in identified FFA, respectively. These results demonstrate that prominent FFA in *C. antiqua* and *H. akashiwo* isolated from the coastal area of western Japan are HUFA which have been suggested as toxic substances damaging the epithelial tissues of fish gills (1).

The fatty acid composition of phytoplankton is generally regarded as species-specific and is usually regulated by environmental factors (12–14,17), whereas intraspecific diversity (15) and difference in growth phase (16) have been observed to affect fatty acid composition in some phytoplankton. The 14:0 and 16:1 FFA in diatoms, *S. costatum* and *C. didymum*, obtained in this study were particularly abundant compared with fatty acids of raphidophyte flagellates. The high proportions of 14:0, 16:0, 16:1, and 20:5 acids and low percentages of C_{18} FFA series in both diatoms under investigation are in agreement with previous analyses for the total lipids of diatoms (12–17). It has been shown that some minor fatty acids in phytoplankton are derived from bacteria (14,15), and in this connection, Kattner *et al.* (16) suggested that C_{18} acids are not synthesized by *S. costatum*. However, detection of C_{18} acids in *S. costatum* culture in axenic conditions in the present HPLC analyses indicates that C_{18} acids were synthesized by *S. costatum*. None of the species under investigation had significant amounts of 20:0, and elongation in saturated fatty acids above C_{20} was therefore not considered.

Our study shows that the proportion and content of FFA in phytoplankton can be quantitatively measured by HPLC as their ADAM derivatives with a programmed gradient. The method offers a simple and rapid approach to FFA analysis of phytoplankton as it does not require isolation and esterification of FFA by heating the sample under the acidic conditions, in which there is a risk of artifact formation from mineral acids, especially easily oxidizable HUFA. Thus, peaks corresponding to 10 ng of FFA can be detected by the present HPLC-fluorometry technique (11). The peaks of ADAM derivatives of FFA obtained from phytoplankton are readily detected; for example, the peak of 20:5 shown in Figure 1 corresponds to 746 ng. The sensitivity of this HPLC method appears to be sufficient to determine the FFA of phytoplankton.

ACKNOWLEDGMENTS

We express our gratitude to Dr. Y. Itabashi of Hokkaido University for supplying 20:5 and 22:6. We thank Dr. S. Yamochi of Osaka Pre-

fectural Fisheries Experimental for providing *S. costatum*, and Dr. M. Yamaguchi of Nansei National Research Fisheries Institute for providing *C. antiqua*. We also acknowledge Dr. M. Yamasaki for his valuable suggestions.

REFERENCES

1. Okaichi, T., in *Red Tides*, edited by T. Okaichi, D.M. Anderson and T. Nemoto, Elsevier, New York, 1989, pp. 137–142.
2. Hallegraeff, G.M., *Phycologia 32*:79 (1993).
3. Fusetani, N., C. Ozawa and Y. Hashimoto, *Bull. Jpn. Soc. Sci. Fish. 42*:941 (1976).
4. Yamaguchi, K., K. Ogawa, N. Takeda, K. Hashimoto and T. Okaichi, *Ibid. 47*:403 (1981).
5. Iwasaki, H., in *The Isolation and Culture of Unicellular Algae*, Japan Fisheries Resource Conservation Association, Tokyo, 1967, p. 55 (in Japanese).
6. Kondo, K., Y. Seike and Y. Date, *Bull. Plankton Soc. Japan 37*:19 (1990).
7. Chen, L.C.M., T. Edelstein and J. McLachlan, *J. Phycol. 5*:211 (1969).
8. Itoh, K., and I. Imai, in *A Guide for Studies of Red Tide Organisms*, Japan Fisheries Resource Conservation Association, Tokyo, 1987, pp. 122–130 (in Japanese).
9. Provasoli, L., J.J.A. McLaughlin and M.R. Droop, *Arch. Microbiol. 25*:392 (1957).
10. Lee, J.S., T. Yanagi, R. Kenma and T. Yasumoto, *Agric. Biol. Chem. 51*:877 (1987).
11. Suzuki, T., *J. Chromatogr. A677*:301 (1994).
12. Chuecas, L., and J.P. Riley, *J. Mar. Biol. Ass. U.K. 49*:97 (1969).
13. DeMort, C.L., R. Lowry, I. Tinsley and H.K. Phinney, *J. Phycol. 8*:211 (1972).
14. Volkman, J.K., S.W. Jeffrey, P.D. Nichols, G.I. Rogers and C.D. Garland, *J. Exp. Mar. Biol. Ecol. 128*:219 (1989).
15. Shaw, P.M., G.J. Jones, J.D. Smith and R.B. Johns, *Phytochem. 28*:811 (1989).
16. Kattner, G., and U.H. Brockmann, *J. Exp. Mar. Biol. Ecol. 141*:1 (1990).
17. Parrish, C.C., A.S.W. DeFreitas, G. Bodennec, E.J. Macpherson and R.G. Ackman, *Phytochem. 30*:113 (1991).
18. Hara, Y., in *Red Tide Organisms in Japan*, edited by Y. Fukuyo, H. Takano, M. Chihara and K. Matsuoka, Japan Fisheries Resource Conservation Association, Tokyo, 1990, pp. 332–333 (in Japanese with English summary).

[Received September 15, 1994; accepted July 25, 1995]