Selective Toxic Effects of Polyunsaturated Fatty Acids Derived from *Ulva fasciata* on Red Tide Phytoplankter Species

Mochammad Amin Alamsjah,¹ Keiko Ishibe,¹ Daekyung Kim,² Kenichi Yamaguchi,² Fumito Ishibashi,³ Yuji Fujita,¹ and Tatsuya Oda²,†

¹Graduate School of Science and Technology, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan
²Division of Biochemistry, Faculty of Fisheries, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan
³Division of Marine Life Science and Biochemistry, Faculty of Fisheries, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

Received August 30, 2006; Accepted October 6, 2006; Online Publication, January 7, 2007

[doi:10.1271/bbb.60475]

**α-linolenic acid and linoleic acid isolated from *Ulva fasciata* showed toxic effects on red tide phytoplankters in a concentration-dependent manner.** Among six species tested, raphidophycean flagellate *Heterosigma akashiwo* was the most susceptible to these fatty acids, and 50% lethal concentrations (LC₅₀) of α-linolenic acid and linoleic acid were estimated to be 0.58 and 1.91 μg/ml respectively, whereas dinoflagellate *Gymnodinium impudicum* and *Heterocapsa circularisquama* were highly resistant and no significant toxic effects were observed up to 1,000 μg/ml. Both fatty acids were less toxic to fish (devil stinger), zooplankters (brine shrimp and rotifer), and mammalian cell lines (U937, HeLa, Vero, and CHO cells) than *H. akashiwo*.

**Key words:** α-linolenic acid; linoleic acid; *Ulva fasciata*; red tide phytoplankter

Harmful algal blooms (HABs) due to *Chattonella marina* and *Heterosigma akashiwo* often cause mass mortality of fish and various marine living organisms, including some aquacultured fish species, that eventually can lead not only to tremendous economic loss but also to environmental pollution.¹² In addition to fish-killing red tide phytoplankters, *Gymnodinium* sp. and *Alexandrium* sp. are known to cause neurotoxic shellfish poisoning and paralytic shellfish poisoning, which frequently cause human illness and even death.³⁴ Therefore, it is urgently important to establish a strategy for prevention and mitigation of the impact of HABs. In spite of considerable efforts to manage HABs with chemical, physical, and biological agents, the methods proposed so far have not yet been attempted at a large scale because of the high cost and the potential for disastrous environmental consequences.⁴¹

Macroalgae are distributed widely in coastal regions and are indigenous to the marine environment. Some macroalgae are known to produce algicidal compounds against some species of red tide phytoplankters. Kakisawa et al.⁵ identified an allelopathic substance from the brown algae *Cladosiphon okamuranus*, and identified it as octadeca-6,9,12,15-tetraenoic acid, which is capable of inhibiting the growth of *Chattonella antiqua*, *Chattonella marina*, *Heterosigma akashiwo*, *Gymnodinium nagasakiense*, and *Gymnodinium sanguineum*. Chiang et al. also identified α-linolenic, oleic, linoleic, and palmitic acid from *Botryococcus braunii* Kutzin (chlorophyceae) as allelopathic substances.⁶ We screened 37 species of macroalga, including 10 chlorophyta, 13 phaeophyta, and 14 rhodophyta for algicidal agents against *H. akashiwo*, and reported that hexadeca-4,7,10,13-tetraenoic acid, octadeca-6,9,12,15-tetraenoic acid, and α-linolenic acid isolated from *Ulva fasciata* (chlorophyceae) showed the strongest algicidal activity.⁷ In addition to these agents, we found recently that *U. fasciata* contains a certain level of linoleic acid. Furthermore, algicidal activity of linoleic acid on *H. akashiwo* has also been reported.⁸ These findings suggest that fatty acid-related compounds are common algicidal agents derived from macroalgae. However, there is only limited information on the precise mechanism and the specificity of the algicidal activity of these fatty acids and their effects on other living organisms including fish. Hence, in this study, we investigated the

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¹ To whom correspondence should be addressed. Fax: +81-95-819-2799; E-mail: t-oda@net.nagasaki-u.ac.jp

Abbreviations: PUFA, polyunsaturated fatty acid; LC₅₀, 50% lethal concentration; HAB, harmful algal bloom; ESM, Erd-Schreiber modified; FBS, fetal bovine serum; MEM, minimal essential medium
effects of α-linolenic acid and linoleic acid on various red tide phytoplankters, zooplankters, fish, and several mammalian cell lines.

Extraction, isolation, and structural determination of algicidal compounds, e.g. α-linolenic acid (C18:3, cis-9,12,15) and linoleic acid (C18:2, cis-9,12), from *U. fasciata* were conducted as described previously.\(^7\) Since these fatty acids are commercially available and our previous study confirmed that these commercially obtained fatty acids showed algicidal activities toward *H. akashiwo* comparable to those of naturally isolated fatty acids,\(^7\) in this study, we used α-linolenic and linoleic acids obtained from Tokyo Kasei Kogyo (Tokyo), and Wako Pure Chemical Industries (Osaka) respectively. The α-linolenic acid and linoleic acid were dissolved in methanol (10 mg/ml) and used as the stock solution. Other chemicals were of the highest grade commercially available.

*Chattonella marina* (NIES-3) and *Heterosigma akashiwo* (NIES-4) were obtained from the National Institute for Environmental Studies of Japan. *Alexandrium tamarense*, *Alexandrium taylori*, *Gymnodinium impudicum*, and *Heterocapsa circularisquama* were kindly provided by the National Research Institute of Fisheries and the Environment of the Inland Sea, Japan. These flagellates were cultured at 26 °C in sterilized Erd-Schreiber modified (ESM) medium (pH 8.2) under illumination from a fluorescent lamp (30 μmol photons/m²/s) under a cycle of 12 h light and 12 h dark, as described previously.\(^9\) Cells in the exponential growing phase were used throughout the experiments. The numbers of cells were counted with a hemocytometer. To each of phytoplankter cell suspension in 24-well plates (1 ml/well), varying amounts of α-linolenic acid or linoleic acid were added. After 24 h of cultivation under standard culture conditions, the viability of each phytoplankter was determined, as described previously.\(^7\) In this assay, the initial cell densities of *C. marina*, *H. akashiwo*, *A. tamarense*, *A. taylori*, *G. impudicum*, and *H. circularisquama* were 2.0 × 10⁴, 4.0 × 10⁴, 0.9 × 10⁴, 1.0 × 10⁴, 1.0 × 10⁴, and 5.0 × 10⁴ cells/ml respectively.

The eggs of rotifer (*Brachionus plicatilis*) and brine shrimp (*Artemia* sp.) cysts were hatched as described previously.\(^10,11\) The hatched neones were pipetted out and used for the toxicity assay. Ten individuals of a zooplankter species in each well of 24-well plates were incubated with varying amounts of α-linolenic acid or linoleic acid in ESM medium (1 ml/well) at 26 °C under illumination from a fluorescent lamp (30 μmol photons/m²/s) for 24 h. Then the viable individuals that were swimming in each well were counted with a stereomicroscope.

U937 (human histocytic lymphoma) cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). HeLa (human epithelial carcinoma). Vero (african green monkey kidney), and CHO (chinese hamster ovary) cells were cultured in α-minimal essential medium (MEM) supplemented with 10% FBS, 10 μg each of adenosine, guanosine, cytidine, and thymidine per ml of medium, penicillin (100 μg/ml), and streptomycin (100 μg/ml), as described previously.\(^12\) To U937 cells in 96-well plates (final, 2 × 10⁴ cells/well), varying amounts of each fatty acid were added. After 24 h of incubation at 37 °C, the viability of the cells was measured by Alamar blue assay, as described previously.\(^13\) The viabilities of HeLa, Vero, and CHO cells in 96-well plates (final, 2 × 10⁴ cells/well) were examined by MTT assay after 24 h of incubation with varying amounts of each fatty acid at 37 °C, as described previously.\(^14\)

For a fish toxicity test of fatty acids, we used juvenile devil stingers (*Inimicus japonicus*). To each 2 liters of beaker glass with 1 liter of seawater containing five fish (size, 2.0–2.5 cm), varying amounts of each fatty acid were added. After 24 h at 26 °C, the viability of the fish was examined as described previously.\(^15\)

Interestingly, *C. marina* and *H. akashiwo* belonging to raphidophycean flagellates showed higher susceptibility to α-linolenic acid and linoleic acid than the other phytoplankter species tested (Fig. 1), and the LC₅₀ values of α-linolenic acid and linoleic acid to *C. marina* were estimated to be 3.22 and 22.35, and to *H. akashiwo*, 0.58 and 1.91 μg/ml respectively (Table 1). Although α-linolenic acid and linoleic acid showed toxic effects on dinoflagellates *A. tamarense* and *A. taylori* in a concentration-dependent manner, no significant toxic effects of these fatty acids were observed on other species of dinoflagellates, *H. circularisquama* and *G. impudicum*, up to 1,000 μg/ml. The exact reason for the selective toxicity of α-linolenic acid and linoleic acid on raphidophycean flagellates is still unknown, but the common characteristic cell-surface structure of raphidophycean flagellates might be involved. It is generally known that raphidophycean flagellates have a polysaccharide complex structure called glyocalyx on the cell surface instead of a rigid cell wall, and morphological changes are easily induced by chemical and physical treatment.\(^16\) Therefore, the cell-surface structure of raphidophycean flagellates might be influenced by α-linolenic acid and linoleic acid. In fact, α-linolenic acid and linoleic acid caused morphological changes in *C. marina* and *H. akashiwo* before complete lysis (data not shown). The higher toxicity of α-linolenic acid than linoleic acid may be due to a chemical structural feature of α-linolenic acid such as the number of unsaturated double bonds. Although further studies are required to clarify the detailed toxic mechanisms of α-linolenic acid and linoleic acid, our results suggest that these fatty acids are interesting compounds not only as effective mitigation agents, especially on *H. akashiwo*, but also as potent biologically active fatty acids.

Next we examined the effects of these fatty acids on zooplankters. As shown in Fig. 1, α-linolenic acid and linoleic acid showed toxic effects on rotifers. Although their LC₅₀ values were estimated to be 53.37 and
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Fig. 1. Toxic Effects of Linoleic Acid (■) and α-Linolenic Acid (▲) on C. marina (A), H. akashiwo (B), A. tamarense (C), A. taylori (D), G. impudicum (E), H. circularisquama (F), B. plicatilis (G), and Artemia sp. (H).

The indicated final concentration of each fatty acid was added to each phytoplankter cell suspension in 24-well plates. After 24 h of cultivation under standard culture conditions, the cell viability in each well was examined as described in the text. For the toxicity test on rotifers (Brachionus plicatilis) and brine shrimp (Artemia sp.), the indicated final concentration of each fatty acid was added to 30 individuals in each well of 24-well plates. After 24 h of incubation under standard culture conditions, the viability of the zooplankter in each well was examined as described in the text. Each point represents an average of triplicate measurements, and each bar indicates the standard deviation.

143.71 µg/ml respectively, these values were 70–90 times higher than those of H. akashiwo. Furthermore, no toxic effects of the fatty acids were observed on another zooplankter brine shrimp up to 1,000 µg/ml.

When it comes to the actual application of these fatty acids as mitigation agents to HABs in the field, harmful effects on other living marine organisms, especially on fish are an important issue. Hence, we examined the effects of α-linolenic acid and linoleic acid on juvenile devil stinger (Inimicus japonicus). During 24 h of observation, there were no dead individuals under the experimental conditions. These data are in agreement with an observation by De Lara-Isassi et al., who reported that extracts of U. fasciata were not toxic to fish. Thus, α-linolenic acid and linoleic acid from

Table 1. The LC50 Values of Linoleic Acid and α-Linolenic Acid on Various Species after 24 h of Treatment

<table>
<thead>
<tr>
<th>Species</th>
<th>Linoleic acid LC50 (µg/ml)</th>
<th>α-Linolenic acid LC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoplankter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. tamarense</td>
<td>98.40</td>
<td>66.06</td>
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<tr>
<td>A. taylori</td>
<td>72.47</td>
<td>35.30</td>
</tr>
<tr>
<td>C. marina</td>
<td>22.35</td>
<td>3.22</td>
</tr>
<tr>
<td>H. akashiwo</td>
<td>&gt; 1,000</td>
<td>&gt; 1,000</td>
</tr>
<tr>
<td>H. circularisquama</td>
<td>&gt; 1,000</td>
<td>&gt; 1,000</td>
</tr>
<tr>
<td>G. impudicum</td>
<td>&gt; 1,000</td>
<td>&gt; 1,000</td>
</tr>
<tr>
<td>Zooplankter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. plicatilis</td>
<td>143.71</td>
<td>53.37</td>
</tr>
<tr>
<td>Artemia sp.</td>
<td>&gt; 1,000</td>
<td>&gt; 1,000</td>
</tr>
<tr>
<td>Mammalian cell lines</td>
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<td></td>
</tr>
<tr>
<td>U937</td>
<td>&gt; 1,000</td>
<td>&gt; 1,000</td>
</tr>
<tr>
<td>HeLa</td>
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</tr>
<tr>
<td>Vero</td>
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<tr>
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</tr>
<tr>
<td>I. japonicus</td>
<td>&gt; 1,000</td>
<td>&gt; 1,000</td>
</tr>
</tbody>
</table>

Fig. 2. Cytotoxic Effects of Linoleic Acid (■) and α-Linolenic Acid (▲) on U937 (A), HeLa (B), Vero (C), and CHO (D) Cells.

After 24 h of incubation, the viability of U937 cells was examined by Alamar blue assay, and the viabilities of other cell lines (HeLa, Vero, and CHO) were examined by MTT assay, as described in the text. Each point represents an average of triplicate measurements, and each bar indicates the standard deviation.

U. fasciata can be useful mitigation agents to remove harmful red tide phytoplankters without causing toxic effects on fish.

To gain insight into the question of the mammalian toxicity of α-linolenic acid and linoleic acid, cytotoxicity assays for several mammalian cell lines were conducted. As shown in Fig. 2, neither fatty acid showed significant toxicity on U937, HeLa, Vero, or CHO cells.
In conclusion, we found that α-linolenic acid and linoleic acid isolated from *Ulva fasciata* as algicidal compounds show highly selective toxicity to raphidophycean flagellate *H. akashiwo*. Since these polyunsaturated fatty acids were less toxic to fish, zooplankters, and several mammalian cell lines, it is suggested that both α-linolenic acid and linoleic acid are useful mitigation agents, especially on *H. akashiwo*, without causing detrimental effects on surrounding marine organisms.

Acknowledgments

This study was partly supported by the Nagasaki Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence and the Japan Science and Technology Agency.

References


