Classification of Marine Algae–Derived Compounds by the Mechanism Responsible for Decreasing Lipoyxgenase-Catalyzed Lipid Hydroperoxides

Hideyuki Kurihara¹, Kenta Yachiyama², and Chihiro Morimoto²

Abstract

Lipoyxgenase (LOX)-mediated lipid hydroperoxides (LHPOs) are converted to physiologically active mediators. Thus, decreasing hydroperoxides is important for diminishing various health-related risks. In this study, decreasing compounds of LOX-catalyzed LHPOs were investigated and classified by mechanism using a modified triple LOX-indamine dye formation (IDF) method. The compounds 2,3-dibromo-4,5-dihydroxybenzyl methyl ether (1) and fucoxanthin (2) were isolated from algae as compounds that can decrease hydroperoxides. Along with previously isolated pheophytin a (3), a chlorophyll-related compound, and purchased nor-dihydroguaiaretic acid (4), a well-known lipoxygenase inhibitor, all the compounds were examined to determine the mechanisms responsible for decreasing LHPOs by using a modified triple LOX-IDF method. Compounds 1 and 4 were found to be LOX inhibitors, compound 2 was a decomposer of the produced LHPOs, and compound 3 was both an inhibitor and a decomposer of the produced LHPOs.

Keywords
phenolics, fucoxanthin, pheophytin a, lipoyxgenase, inhibitor, lipid hydroperoxide, decomposer marine natural products, structure, biosynthesis, biological activities, synthetic derivatives and analogs, short communication

Introduction

Lipid hydroperoxides (LHPOs) are generated through chemical and enzymatic processes.¹,² The chemical process is initiated with free radicals and reactive oxygen species (ROS). Free radicals withdraw hydrogen radicals from lipids to generate lipid radicals. Subsequently, molecular oxygen is incorporated to make a peroxy radical, and then the radical is converted to racemic LHPO by withdrawing hydrogen radical from another lipid.³,⁴ ROS-like singlet oxygen is also incorporated into lipids to form various racemic LHPOs.¹,² The enzymatic process is triggered by various lipoyxgenases (LOXs) and an α-dioxynsgens.¹,² They catalyze enantiomeric incorporation of molecular oxygen into lipids at various positions. The produced LHPOs are converted to physiologically active mediators, such as leukotrienes, which are involved in inflammatory diseases.⁴ LHPO-derived products such as aldehydes and polymeric materials show cytotoxic and genotoxic effects.⁵ Lipid peroxidation products exist in human atherosclerotic lesions and act as signaling messengers. Thus, decreasing produced LHPOs is important for diminishing various health risks.

Decreasing LHPOs is achieved by their reduction and conversion to adducts, anti-oxidation of lipids, and/or inhibition of the LOX reaction.²,⁵ LHPOs are converted to lipid hydroxides (LHOs) or other adducts by enzymatic and nonenzymatic reactions. The enzymes glutathione peroxidase⁶ and peroxiredoxin⁵,⁷ catalyze the reduction in LHPOs to their corresponding LHOs while glutathione S-transferase⁸ catalyzes the formation of glutathione adducts. These enzymes help to quench hazardous ROS that is biologically harmful. Additionally, LHPOs are decreased by enzymatic and nonenzymatic conversion to aldehydes, ketones, divinyl ethers, α- and γ-ketols, and carotenoid...

¹Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan
²Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan

Corresponding Author:
Hideyuki Kurihara, Faculty of Fisheries Sciences, Hokkaido University, Minato 3-1-1, Hakodate, Hokkaido 041-8611, Japan.
Email: kuri@fish.bokudai.ac.jp
adducts. Most of these conversion products belong to the oxylipin family and are known as signaling molecules.

LOX inhibitors have been isolated from terrestrial plants and algae. Terrestrial plant-derived inhibitors are classified into various groups such as fatty acids, polyphenols, amides, and polyacetylene. In our previous search for LOX inhibitors derived from algae, algal extracts were screened using a LOX-indamine dye formation (IDF) method. We have isolated two highly unsaturated hydrocarbons, (1,6,9,12,15,18-henicosahexaene and 1,9,12,15,18-henicosapentaene), a chlorophyll-related compound, pheophytin a, and a phlorotannin, fucophoruthol C, and identified them as LOX inhibitors by employing the conventional LOX-IDF method. The compounds obtained by our group were chemically diverse. Therefore, it was important that the compounds identified from the results of the conventional LOX-IDF method were either confirmed as LOX inhibitors or were determined to be involved in the decomposition of the produced LHPOs or indamine dye.

In the present research, a known bromophenol, 2,3-dibromo-4,5-dihydroxybenzyl methyl ether, and a carotenoid, fucoxanthin, were obtained from the red alga Chondrus pinnulatus and the brown alga Sargassum confusum as compounds that can decrease LHPOs. Additionally, compounds 1 and 2, pheophytin a (3), and nordihydroguaiaretic acid (NDGA, 4), a well-known LOX inhibitor, were examined to determine the detailed mechanisms responsible for decreasing LHPOs by using a modified triple LOX-IDF method.

**Results and Discussion**

**Isolation of LHPO-Decreasing Compounds**

Compounds 1 and 2 were isolated from 2 algae with the guidance of results of LHPO-decreasing activity tests. They were identified as 2,3-dibromo-4,5-dihydroxybenzyl methyl ether and fucoxanthin by comparison of their MS and NMR data (Supplemental Materials) with literature data. Since the report of the first isolation of bromophenol 1 from Rhodomela larix, it has been isolated from many species of red algae. In the course of our search, bromophenol 1 was identified as an α-glucosidase inhibitor. To our knowledge, this is the first report in which bromophenol 1 shows a decreasing effect on LOX-catalyzed LHPOs. Fucoxanthin (2), a unique carotenoid derived from brown algae and diatoms, has been reported to prevent reactive oxygen species-related cell damage.

**Determination of the Mechanism Responsible for Decreasing LOX-Catalyzed LHPO Using a Modified Triple LOX-IDF Method**

All the compounds examined showed the ability to decrease LOX-catalyzed LHPOs. A modified triple LOX-IDF method was used to determine the detailed mechanism responsible for the decrease in LOX-mediated LHPOs. The results are shown in Figure 2. None of the compounds 1–4 affected the decomposition of indamine because they did not decrease the production of indamine dye in experiment III (D_{ExIII} ≈ 0). Compounds 1 and 4 only decreased LHPOs in experiment I, not in experiment II. These results were interpreted that both the compounds only decreased the production of LOX-catalyzed LHPOs without influence on the produced LHPOs. Therefore, the mechanism of compounds 1 and 4 was concluded to involve LOX inhibition with IC_{50} values of 167 and 290 μM under the substrate concentration of 1.25 mM, respectively. Compound 2 decreased LHPOs to the same extent as experiments I and II (D_{ExII} nearly equal to D_{ExIII}). These results were interpreted that compound 2 only decreased the produced LHPOs without influence on production of LOX-catalyzed LHPOs, opposite to compounds 1 and
LOX-catalyzed LHPOs are trigger compounds of biosynthetic pathway cascades of leukotrienes. It is important to decrease the production of LOX-catalyzed LHPOs and also reduce already produced LHPOs for the prevention of leukotriene-related diseases. The LOX-A234 method can screen only for LOX inhibitors, not LHPO decomposers. The conventional LOX-IDF consists of 3 steps. In the first enzyme reaction step, soybean LOX catalyzes the oxidation of linoleic acid to the corresponding LHPO. In the second indamine dye formation step, the produced LHPO is converted to an indamine dye in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-dimethylamino- benzoic acid (DMAB). In the final measurement step, the resulting solution is homogenized by adding sodium dodecyl sulfate (SDS) and measured its absorbance. Thus, the conventional LOX-IDF method can screen for both LOX inhibitors and decomposers of LHPOs, but not distinguish between the inhibitors and the decomposers. Therefore, both the previous methods cannot determine the detailed mechanism(s) of active compounds. However, the novel modified triple LOX-IDF method can screen for and distinguish between the inhibitors and the LHPO, already generated by LOX, decomposers simultaneously. The modified method would become a powerful tool for screening LOX inhibitors and LHPO decomposers (reductants) because the compounds showing decreasing LHPO are rapidly determined decreasing mechanisms.

Figure 2. Determination of the decreasing ratios of compounds 1–4 using a modified triple lipoxygenase-indamine dye formation method. All final concentrations of test compound were 20 μg/mL. The data obtained from the modified triple LOX-IDF method represent the mean ± standard error of the mean. A significant difference between experiments I and II was determined by the Student t test (P < .05, n = 3).

4. Compound 2 was determined to function by decomposing the produced LHPOs. Compound 3 decreased LHPOs in experiments I and II, and its decreasing ratio value (D1a) in experiment I was higher than its decreasing ratio value (D2a) of experiment II. These results were interpreted that compound 3 decreased production of LOX-catalyzed LHPOs, moreover, decreased produced LHPOs. Thus, the mechanism of compound 3 was determined to be a LOX inhibitor and a decomposer of the produced LHPOs.

Phenolic compounds 1 and 4 possess α-diphenol moieties. These compounds may inhibit enzymes by interacting with certain amino acid residues and/or chelation with a metal ion in the active site. The mechanism of interaction between phenolic compounds and certain amino acid residues may be important for inhibition because fucophlorethol C, a phlorotannin of the w-diphenol type, also showed inhibition against lipoxygenase.

Although fucoxanthin also showed the decreasing effect of LHPOs, the compound was found not to be a LOX inhibitor but rather a decomposer of LHPOs from the results of the modified triple LOX-IDF method. Previous reports also disclosed that it showed no inhibition against LOX from the results of LOX-absorbance at 234 nm (A234) using a generated conjugated diene measurement method. However, the LOX-A234 method cannot be adapted to screen for a decomposer of LHPOs because LHPOs and LHOs possessing a conjugated diene moiety cannot be assorted to each other. Fucoxanthin may act as a direct reductant of lipid hydroperoxides similar to its reducing efficiency toward metal ions.

Pheophytin a (3) acts as both a LOX inhibitor and decomposer of LHPOs. It consists of 2 moieties, a phytol side chain and a porphyrin ring. The former moiety may be involved in the LOX inhibition because of its hydrophobic chain, while the latter moiety may be related to the reductive decomposition of LHPOs.28

Conclusion

Two compounds, bromophenol (1) and fucoxanthin (2), decreased LOX-catalyzed LHPOs were obtained from algae-derived extracts. The mechanisms responsible for the decreases in LOX-mediated LHPOs were determined for the chemically diverse compounds. Bromophenol 1 and a phenol, NDGA (4) were determined as LOX inhibitors. Fucoxanthin (2) was found to be a decomposer of LHPOs but was not an inhibitor. Pheophytin a (3) was identified to be both a LOX inhibitor and a decomposer of LHPOs by the modified triple LOX-IDF method established.

Experimental

General

Soybean LOX (lipoxidase Type I-B), linoleic acid, and MBTH were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), Kanto Chemical Co., Inc. (Tokyo, Japan), and Nacalai Tesque, Inc. (Tokyo, Japan), respectively. Pheophytin a, DMAB, and Tween 20 were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other reagents were purchased from Kanto Chemical Co., Inc. and Wako Pure Chemical Industries. High-performance liquid chromatography (HPLC) was performed using a Shimadzu LC-10ATVP.
Isolation and Identification of Compounds That Decrease LOX-Mediated LHPOs

The red alga *C. pinnulatus* (1340 g, air-dried) and the brown alga *S. confusum* (1070 g, air-dried) were collected from the coast of Hakodate, Japan (41°56′11.5″N, 140°56′56.9″E). They were washed with tap water and independently extracted with methanol (ca. 5 L) at room temperature for several days.

The methanolic extract (36.5 g) of *C. pinnulatus* was separated by organic solvent partitioning to obtain an ethyl acetate soluble fraction (663 mg). The fraction was chromatographed on silica gel (diameter 3.0 cm, height 30 cm) eluted with *n*-hexane:ethyl acetate (1:3, vol/vol) followed by preparative TLC developed with chloroform:methanol (9:1, vol/vol) to obtain a semi-purified fraction. This fraction was finally purified by HPLC eluted with acetonitrile: 0.1% formic acid (6:4, vol/vol) as the eluent to afford compound 1 (1.85 mg).

The methanolic extract (29.6 g) of *S. confusum* was separated into an ethyl acetate soluble fraction (1.30 g) and chromatographed on silica gel (diameter 3.0 cm, height 30 cm) eluted with *n*-hexane:ethyl acetate (1:3, vol/vol) followed by preparative TLC developed with chloroform:methanol (9:1, vol/vol) to obtain a semi-purified fraction. This fraction was finally purified by HPLC eluted with acetonitrile: 0.1% formic acid (6:4, vol/vol) as the eluent to afford compound 2 (35.0 mg).

---

**Figure 3.** The modified triple lipoxygenase-indamine dye formation method. Enzyme, soybean lipoygenase in borate buffer (pH 9.0); substrate, linoleic acid, Tween 20, sodium hydroxide in water; solution A, 3-dimethylaminobenzoic acid (DMAB) in borate buffer (pH 9.0); solution B, 3-methyl-2-benzothiazolinone hydrazone (MBTH) and hemoglobin in phosphate buffer (pH 5.0); SDS, sodium dodecyl sulfate in water. Decreasing ratio (%) values, $D_{\text{ExI}}, D_{\text{ExII}},$ and $D_{\text{ExIII}},$ represent as decreasing ratios (%) in experiments I, II, and III, calculated by equation (1).
Modified Triple LOX-IDF Method

Preparation of Reagent Solution. Solutions A and B and the substrate solution in the LOX-IDF method were prepared as described previously\textsuperscript{15} with slight modification. Solution A contained 20 mM DMAB in 100 mM borate buffer (pH 9.0), and solution B contained 10 mM MBTH and 5.0 mg/mL hemoglobin in 50 mM phosphate buffer (pH 5.0). The substrate solution was prepared from 140 mg linoleic acid and 280 mg Tween 20 in 1 N NaOH (0.60 mL) and water (5.0 mL) and then mixed with a Pasteur pipette. The mixture was made up to 20.0 mL with water. The substrate solution was divided into 2 mL aliquots in plastic vials, flushed with argon gas, and stored at $-30^\circ C$ until use. Test compound was dissolved in methanol at concentration of 1.00 mg/mL.

Experiment I (the Conventional Method)

Experiment I was the conventional method\textsuperscript{15} by the addition of the test compound before pre-incubation. Test compound solution (0.020 mL) was added into solution A (0.910 mL) and 128000 U/mL soybean LOX in 100 mM borate buffer (pH 9.0) (0.020 mL). This solution was preincubated at 27.5°C for 5 min. The LOX reaction was initiated by adding the substrate solution (0.050 mL) under the final test compound concentration of 20 $\mu$g/mL, incubated at 27.5°C for 5 min, and terminated by adding solution B (1.00 mL) to form the colored product. After 5 min, 1% SDS (1.00 mL) was added to the reaction mixture, and it was mixed vigorously. The absorbance at 598 nm was measured and compared with that obtained in the experiment performed by adding methanol instead of adding test compound solution.

Experiment II

Experiment II was the modified method by adding the test compound after the LOX reaction. The experimental procedure was similar to that of experiment I except each test compound was added after the LOX reaction at the same time of adding solution B. The absorbance at 598 nm was measured and compared with that obtained in the experiment performed by adding methanol instead of adding test compound solution.

Experiment III

Experiment III was the modified method by adding the test compound after LOX-IDF formation. The experimental procedure was similar to that of experiment I except each test compound was added after the IDF reaction. The absorbance at 598 nm was measured and compared with that obtained in the experiment performed by adding methanol instead of adding test compound solution.

Estimation of Mechanism

The 3 decreasing (%) values, $D_{ExI}$, $D_{ExII}$, and $D_{ExIII}$, were calculated from the decrease of the absorbance of the indamine dye solution compared with those of the control solutions as equation (1):

$$D_X(\%) = \left\{1 - \left(\frac{A_{598 \text{ with test compound in experiment } X}}{A_{598 \text{ without test compound in experiment } X}}\right)\right\} \times 100$$

The detailed mechanisms responsible for the decreasing LHPOs can be estimated from comparison of the 3 values $D_{ExI}$, $D_{ExII}$, and $D_{ExIII}$ (Figure 3).

Statistical Analyses

The data obtained for the modified triple LOX-IDF method represent the mean $\pm$ standard error of the mean. A significant difference between experiments I and II was determined by the Student $t$ test ($P < .05$).

Acknowledgements

The authors thank Dr Eri Fukushi and Mr Yusuke Takata from the GC-MS and NMR Laboratory, Faculty of Agriculture, Hokkaido University, who acquired the NMR and MS spectra. The authors thank Renee Mosi, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iD

Hideyuki Kurihara https://orcid.org/0000-0002-1013-6089

Supplemental Material

Supplemental material for this article is available online.

References

1. Mosblech A, Feussner I, Heilmann I. Oxylipins: structurally diverse metabolites from fatty acid oxidation. Plant Physiol Biochem. 2009;47(6):511-517. doi:10.1016/j.plaphy.2008.12.011
2. Niki E, Yoshida Y, Saito Y, Noguchi N. Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochem Biophys Res Commun. 2005;338(1):668-676. doi:10.1016/j.bbrc.2005.08.072
3. Frankel EN. Antioxidants in lipid foods and their impact on food quality. Food Chem. 1996;57(1):51-55. doi:10.1016/0308-8146(96)00067-2
4. Bell RL, Young PR, Albert D, et al. The discovery and development of zileuton—an orally active 5-lipoxygenase inhibitor. Int J...
