Kasetsart J. (Nat. Sci.) 38 : 72 - 77 (2004)

Arachidonic Acid Conversion by Lipoxygenase in the Brown Alga, *Laminaria angustata*

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ABSTRACT

Arachidonic metabolism with the lipoxygenase to form its hydroperoxides (eicosanoids) has been commonly known in animal. Recently, we reported that eicosanoids, 12(*S*)- and 15(*S*)-hydroperoxyeicosatetraenoic acid were the intermediate product of major aldehyde flavor formation [3(*Z*)- and 2(*E*)- nonenal and *n*-hexanal] in an edible brown alga, *Laminaria angustata* via lipoxygenase (LOX) and hydroperoxide lyase pathway. Here another eicosanoides have been found after enzymatic formation and identified as 11-, 9-, 8- hydroperoxyeicosatetraenoic acid by HPLC and GC/GC-MS. They are believed to be the intermediate of physiological active compounds involving in chemical defense and sex pheromone. These represented the mechanism of positional selectivity of LOX in this marine alga.

Key words: Brown algae, *Laminaria angustata*, Lipoxygenase, 11-Hydroperoxyeicosatetraenoic acid, 9-Hydroperoxyeicosatetraenoic acid, 8-Hydroperoxyeicosatetraenoic acid.

INTRODUCTION

Algae have been used as food for Asian people since ancient time. Their habitats were commonly found in sea, fresh water, snow, and soil. They are the important mass producer that supply the energy to all living organism in the sea. From our previous study, algae could produce several compounds like physiological active in higher plant. Recently we could find that marine algae have two systems to produce the compounds that are believed to be the physiologically bioactive compounds (*n*-hexanal and 2(*E*), 3(*Z*)-nonenal) via C18 and C20 cascade of unsaturated fatty acid (Boonprab, 2003; Boonprab *et al.*, 2003abc). In contrast to higher plant, hydroperoxy-unsaturated fatty acids, especially hydroperoxy-linoleic acid or linolenic acid, have been found to be the intermediate compound of physiological bioactive that involved in chemical defense or wound etc., such as jasmonic acid, *n*-hexanal, 2(*E*)- and 3(*Z*)-nonenal, 2(*E*) and 3(*Z*)-hexenal (Blée, 1998). It could be formed by lipoxygenase (LOX) which is in the family of dioxygenases that can esterify unsaturated fatty acids containing (1*Z*, 4*Z*)-pentadiene structure to produce their hydroperoxide derivatives containing conjugated diene structure (Kuhn, 2000). Recently, it was found that hydroperoxy-arachidonic acid or eicosapentanoic acid such as 11-hydroperoxy arachidonic acid or eicosapentadecaenoic acid might be the intermediate compound of C10 aldehyde (2,4-

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decadienal or 2,4 decatrienal) (Pohnert, 2002; Akakabe *et al.*, 2003). Since hydroperoxy-unsaturated fatty acid could be formed via lipoxygenase reaction, its product will show the mechanism of LOX (Kuhn, 2000) as well as the pathway that might produce the physiologically bioactive compounds. In this report, investigation into eicosaniods (hydroperoxy-arachidonic acid) was carried out, we could find three physiologically bioactive eicosanoids which were believed to be involved in chemical defense and a male gamete attraction in marine brown alga *L. angustata* for the first time.

MATERIALS AND METHODS

Plant material

L. angustata was harvested at Charatsunai beach, Hokkaido (northern part of Japan) facing the Pacific ocean. Fronds were kept at 4°C immediately after harvest, and temperature was maintained during shipping. They were kept at –80°C as coarse powder prepared by liquid nitrogen. The whole powder was mixed and a sample (20 g) was taken for an experiment in order to avoid individual differences.

Chemicals

Arachidonic acid (ARA) 90% purity, glutathione (GSH) and glutathione peroxidase (GPx) were purchased from Sigma Chemical Co., USA. 11(*R,S*)-Hydroxyeicosatetraenoic acid [11 (*R,S*) HETE], 9 (*R,S*) HETE, 8 (*R,S*) HETE were obtained from Cayman Chemical Co., USA. Other chemicals were obtained from Wako Chemical Co., Japan. All solvents used in experiments were purified by distillation.

Crude enzyme preparation

L. angustata was crushed into fine powder under liquid nitrogen with a mortar and pestle, and subsequently ground with a Maxim homogenizer (Nihonseiki Ltd., Japan). The powder was transferred into a glass bottle containing three volumes (v/w) of 0.1 M borate borax buffer pH 9.0, containing 2% Polyclar VT. The suspension was homogenized using a Polytron mixer to break down polysaccharides, then filtered through six layers of cheesecloth. The filtrate was centrifuged at 3076 g for 15 min at 4°C to remove debris. The pH of the supernatant was readjusted to 6.9 on ice using cool 0.1 M HCl or NaOH solution.

Analyses of hydroxy-arachidonic acid for structure

Crude enzyme (60 ml) was incubated with 36 nmole of arachidonic acid in the presence of 2 unit/ml of GSH-Pxd and 6 mM of GSH to gain hydroxy-arachidonic acid. They then were extracted using a Sep-pek C_{18} silica cartridge (Waters Corporation, Milford, Massachusetts, USA) (Lehmann *et al.*, 1992). The acids were eluted by methanol, and following evaporation of methanol, the resultant yellow oil was re-dissolved in diethyl ether and washed with saturated NaCl. The diethyl ether layer was dried over Na_2SO_4 at –10°C for 12 hrs. The diethyl ether soluble was removed, and the residue was subjected to preparative thin layer chromatography essentially as described elsewhere (Boonprab *et al*., 2003a). Hydroxyeicosatetraenoic acid(s) [HETE(s)] were separated using a solvent system of *n*-hexane: diethyl ether:CH_3COOH, 1:1:0.001(v/v/v). The spots at R_f 0.13-0.2 were scraped off, and HETE(s) were extracted from the gel with diethyl ether 2 ml each for five times. After removal of ether, the residue was re-dissolved in diethyl ether (5 ml) and washed with saturated NaCl solution. The ether layer was dried over Na_2SO_4 at –10°C for 12 hrs , then ether was removed and the residue was reconstituted in ethanol (1 ml). The concentrations of HETE(s) were estimated by reading absorbance at 234 nm using an extinction coefficient of 23200 M^{-1} cm^{-1} (Graff *et al.*, 1990). Aliquots were methylated with ethereal diazomethane for the straight phase HPLC analyses, using a Zorbax-

SIL 250 x 4.6 mm column (Du Pont company), eluted with *n*-hexane:isopropanol:CH_3COOH, 987:12:1 (v/v/v) at a flow rate of 0.5 ml/min. Detection was performed with a photodiode array detector (Shimadzu, LC-10ADvp, Japan) at absorbance of 234, 210 and 270 nm.

Mass spectrum analyses of hydroxy-arachidonic acid

For mass spectrum analysis, the procedure developed by Lehmann *et al.* (1992) was followed. After purification of the HETE(s), they were methylated with ethereal diazomethane. The sample was re-dissolved in ethanol (800 ml), and platinum oxide (6 mg) was added. Hydrogen gas was bubbled through a sample solution for 90 min, with platinum oxide being removed by filtration. The solvent was removed under stream of nitrogen, to which they then added bis-(trimethylsilyl)-trifluoroacetamide solution (200 ml, Sigma-Aldrich). The mixture was incubated for 60 min at 60°C with constant stirring under argon. After cooling to room temperature, the reagents were removed under a gentle stream of nitrogen. The sample was re-dissolved in *n*-hexane (40 ml) and subjected to GC-MS analysis. GC-MS (GCMS-QP5050A, Shimadzu, Japan) was equipped with a fused silica capillary column (60 m × 0.25 mm) coated with DB-WAX 0.25 mm film thickness using helium as a carrier gas. The column temperature was programmed to increase from 150°C to 200°C at 5°C/min. Sample injection was carried out with a split ratio of 1/50. The ionization energy was 70 eV.

RESULTS AND DISCUSSION

Enzymatic formation of eicosanoids from arachidonic acid

Crude enzyme preparation (60 ml) was prepared in order to perform the reaction with arachidonic acid (36 nmole) in the presence of glutathione peroxidase (2 unit/ml) and glutathione (6 mM) at 4°C for 30 min. Hydroperoxide isomers formed during the reaction were trapped by glutathione peroxidase to form their corresponding hydroxide isomers. They were then concentrated and purified by Sep-pak C18 and preparative thin layer chromatography (TLC) and re-dissolved in ethanol. The profiles of eicosanoids solution were analyzed by following their conjugated diene structure at λmax 231-234 nm. The yield of eicosanoid was estimated using extinction coefficient of 23200 M^{-1} cm^{-1} (Graff *et al.*, 1990) at 234 nm to be 11.89 mM in 1 ml ethanol. After purification, eicosanoids was further identified by straight phase HPLC, five unknown isomers, compounds I, II, III, IV and V with the typical of conjugated diene chromophore at the λmax 235, 235, 235, 231 and 235 nm in the ratio of 29.65 : 62.76 : 3.45 : 0.69 : 3.45 were found, respectively (Figure 1). Based on Kuhn (2000), these are thought to be formed by lipoxygenases that inserted oxygen molecule at a specific position of arachidonic acid.

Identification of eicosanoid from arachidonic acid

Compounds I and II have been identified as 12(*S*) hydroperoxyeicosatetraenoic acid and 15(*S*)-hydroperoxyeicosatetraenoic acid (Boonprab *et al.*, 2003ac). Compound III, IV and V were identified by co-injection with the authentic standard on straight phase HPLC. They were shown as 11-, 9-, 8- hydroxyeicosatetraenoic acids, respectively (Figure 1). These evidences were confirmed by their mass spectrum with the prominent peaks and the common fragment pattern at m/z 399(M-15), 383(M-31), 367(M-47). The characteristic of the ion peaks of the methyl ester-trimethylsilyl ether derivatives of compound III, IV and V showed their mass spectrum at m/z 229 and 287, 257 and 259, and 245 and 271, respectively (Boeynaems *et al.*, 1980). These evidences supported that compound III, IV and V were formed from 11-, 9-, 8-hydroperoxyeicosatetraenoic acid, respectively.

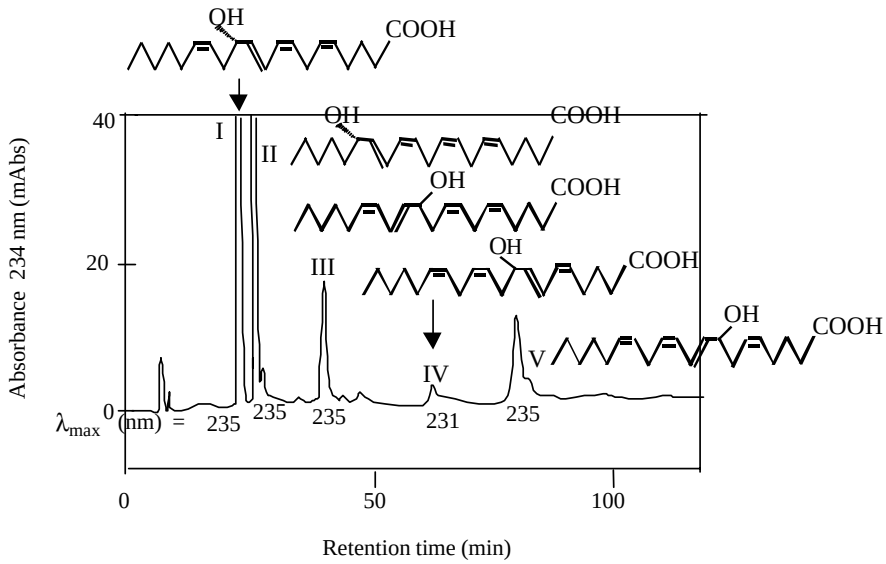

Figure 1 Straight phase HPLC analysis of arachidonic acid metabolites.

CONCLUDING REMARKS

From the finding of enzymatic formation of several flavor compounds, they were believed to be the bioactive compounds like in higher plants. Here another isomers of hydroperoxy-arachidonic acid could be found. This represented the product specificity of lipoxygenase enzyme in *L. angustata*. The positional selectivity of LOX shows 15-, 12-, 11-, 9-, and 8-hydroperoxyeicosatetraenoic acid (Figure 2). Based on LOX mechanism, which documented by Kuhn (2000), oxygenated mechanism on arachidonic acid structure in *L. angustata* was described. In principle, LOX mechanism composed of three consequently steps which were hydrogen abstraction, radical rearrangement and oxygen insertion. The LOX(s) of algae selected pro-*S* hydrogen removal at C-10 for 12-hydroperoxyeicosatetraenoic acid and C-13 for 15-hydroperoxyeicosatetraenoic acid resulted in pentadienyl radical for regioselectivity and enantioselectivity controlling in hydrogen abstraction step. Then pentadienyl radical of both eicosanoids is rearranged by electron redistribution in direction of the methyl terminus (+2 rearrangement) of arachidonic acid in radical rearrangement step. For the last step, dioxygen was introduced to C-1 of rearranged pentadienyl radical. Here the steps of 11-, 9-, and 8- hydroperoxy eicosanoic acid formation still are not clarified until the configuration of this hydroperoxides will be determined. The understanding of mechanism of LOX will clarify the primary step to produce several kinds of bioactive compounds in brown alga. However, the enantioselectivity of this enzyme should be investigated in near future to describe the characteristic of this enzyme involving the confirmation of their final products such as C10 or C12 aldehyde (Pohnert, 2002; Boonprab, 2003) those could be formed by hydroperoxide lyase reaction. Then the regulation of this pathway will be investigated to take benefit from this pathway in the aspect of agricultural application or flavor production.

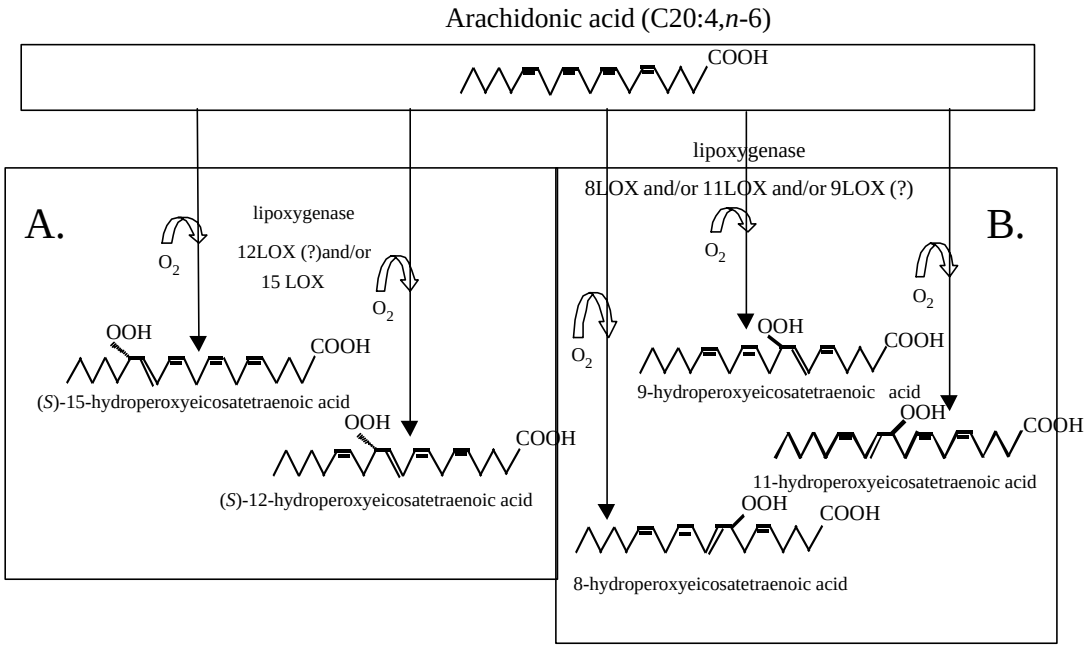

Figure 2 Proposed metabolism of arachidonic acid catalyzed by lipoxygenase (LOX) in brown alga, *L. angustata.* A. has been reported by Boonprab *et al.* (2003ac). B. has been firstly found in *L. angustata* in this report.

ACKNOWLEDGMENTS

This work was performed under the cooperation of Japan and Thai scientists performed by the JSPS-NRCT core university program in association with Kasetsart University (Thailand) and Yamaguchi University (Japan) under the project "development of thermotolerant microbial resources and their applications".

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