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RESEARCH ARTICLE

Monogalactosyl diacylglycerol is a substrate for lipoxygenase: its implications for oxylipin formation directly from lipids

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Oxylipins are formed from fatty acids in plants in response to various stresses, and exert their roles as signal molecules or direct defense molecules. Oxylipins are usually found in free forms; however, oxylipins esterified in galactolipids have been identified recently, which implicates an alternative biosynthetic pathway to form oxylipins without a lipid-hydrolyzing step. In order to get insight into the alternative pathway, we examined catalytic nature of soybean seed lipoxygenase-1 on galactolipids. The lipoxygenase showed no activity on the galactolipids; however, significant activity was observed when deoxycholic acid was added into the reaction mixture. HPLC-mass spectrometry analyses showed that either or both the acyl moieties were oxygenated by the lipoxygenase in regio specific manner. Accordingly, we concluded that galactolipids could serve as substrates for lipoxygenases, from which direct oxygenation of galactolipids in an oxylipin metabolism under stress condition in plants was proposed.

Keywords: *Glycine max*; lipoxygenase; oxylipin; monogalactosyldiacylglycerol; LC-MS/MS analysis

Introduction

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are a family of non-heme iron containing dioxygenases that catalyze the insertion of molecular oxygen into polyunsaturated fatty acids containing (1Z,4Z)-pentadiene moieties (Andreou and Feussner 2009). The products are fatty acid hydroperoxides with 1-hydroperoxy-(2E,4Z)-pentadiene moieties. The reaction is usually regio- and stereospecific, and two types of lipoxygenases are known in plants. One inserts molecular oxygen at the C13-position of linoleic and linolenic acids to form corresponding 13S-hydroperoxides. The other oxygenates C9-position to form 9S-hydroperoxides (Andreou and Feussner 2009). In mammals, carbon 20 fatty acids are common substrates, and 5-, 12-, and 15-lipoxygenases named depending on the positions of oxygen insertion into arachidonic acids have been extensively studied (Ivanov et al. 2010). Lipoxygenases are widely distributed in biological organisms ranging from prokaryotes to animals and to higher plants.

The compounds derived from fatty acids through oxygenation reactions, mostly caused by lipoxygenases, are cumulatively called oxylipins. In mammalian tissues, lipoxygenases are involved in the initial steps of the biosynthesis of physiologically active compounds such as leukotrienes and lipoxins (Haeggström et al. 2010). In plants, lipoxygenases are also involved in formation of physiologically active compounds, such as jasmonates or green leaf volatiles

(Matsui 2006; Wasternack 2007). In both the cases, the bioactive oxylipins are in their free forms, and lipoxygenases prefer free fatty acids as substrates. Therefore, it has been widely accepted that lipid-hydrolyzing steps catalyzed by phospholipases, galactolipases, or triacylglycerol lipases to form free fatty acids are one of crucial steps to control the amounts of oxylipins in nature. On the contrary, a number of studies provide evidence that certain lipoxygenases have abilities to oxygenate esterified fatty acids in triacylglycerols, phospholipids, or cholesterol esters (Andreou and Feussner 2009). Esterified fatty acids in galactolipids might be also substrates for lipoxygenases because it was reported that chloroplast membrane was oxygenated by a lipoxygenase (Maccarrone et al. 1994).

Galactolipids, namely, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerols (DGDG), are mostly found in thylakoid membranes in chloroplasts. They are rich source of polyunsaturated fatty acids and more than 50% of linolenic acid is found in galactolipids. From this, it is implicated that early steps of oxylipin biosynthesis take place in chloroplasts. In fact, most plants have lipoxygenases in chloroplasts, and the subsequent enzymes involved in formation of oxylipins, such as allene oxide synthases or hydroperoxide lyases, are also found in chloroplasts.

Recently, accumulating studies indicate that plants form oxylipins esterified in galactolipids,

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such as arabidopsides or linolipins, and that the galactolipid oxylipins are involved in defense responses against various stresses (Kourtchenko et al. 2007; Chechetkin et al. 2009). Even though it is feasible to think that these galactolipid oxylipins are formed through direct oxygenation of MGDG and subsequent modifications without liberation of the acyl moiety from MGDG, there has been no evidence to support this biosynthetic pathway. In this study, we performed a model, *in vitro*, study on the reaction of soybean seed lipoxygenase-1 on MGDG, and found that the lipoxygenase catalyzed direct oxygenation on MGDG. A possible implication of this direct oxygenation in oxylipin formation related to defense responses is discussed.

Materials and methods

Materials

Seeds of soybean (*Glycin max*) cv. Yumeyutaka (15 g) were soaked overnight in water, and the seed coat was peeled off. The imbibed seeds were homogenized with 50 ml of 0.1 M sodium acetate, pH 4.5. Proteins were collected with 70% saturated ammonium sulfate, and dissolved in 0.1 M sodium phosphate, pH 6.3. Yumeyutaka has only one isozyme of lipoxygenase (lipoxygenase-1) out of the three found normally in soybean seeds (Pulvera et al. 2006).

Lipids were extracted from clover (*Trifolium repens*) leaves by chloroform/methanol (1/2, v/v). MGDG was purified from the crude lipids with silica gel chromatography (2×50 cm, Wakogel C-300, Wako Pure Chemicals, Osaka, Japan) with a solvent system of chloroform/acetone (9/1 to 2/8, v/v). Rechromatography was carried out to remove any contaminants. Fatty acid composition was determined by GC analysis after transmethylation with 5% HCl in methanol at 80°C. The composition was 96.4% linolenic acid, 2.21% linoleic acid, and 1.36% palmitic acid.

Lipoxygenase activity

Stock solution (20 mM) of MGDG was prepared with 100 mM deoxycholate (Sigma, St. Louis, MO) after sonication with a bath-type sonicator (US-1R; As-one, Osaka, Japan) for 30 sec under a nitrogen atmosphere. Stock solution of linolenic acid (50 mM) was prepared with 2 mg ml⁻¹ of Tween 20 after sonication as above. Lipoxygenase activity was determined polarographically with a Clark type oxygen electrode (YSI 5331, Yellow Springs Instrument, Yellow Springs, OH) at 25°C. In a typical assay with MGDG, 20 µl of 20 mM MGDG dispersed in 100 mM deoxycholic acid was added to 0.2 M sodium borate, pH 9.0, and after 3 min, an appropriate amount of the lipoxygenase was added to start the reaction in total volume of 1.75 ml.

Analysis of products

After reaction was completed, the products were immediately extracted with chloroform. After removing chloroform, the residue was dissolved in 100 µl of acetonitrile for HPLC analysis. Reversed-phase HPLC was carried out with Mightysil RP-18 (5 µm, 4.6×250 mm). The solvent was acetonitrile/water (85/15, v/v) with 0.1% formic acid, and the flow rate was 1 ml min⁻¹ at 40°C. Detection was carried out with a photodiode array detector (SPD-M10A, Shimadzu, Kyoto, Japan). To determine the composition of oxygenated acyl moieties, oxygenated MGDG separated with HPLC was independently fractionated, reduced with triphenylphosphine, and served to alkaline hydrolysis. The composition of hydroxylated linolenic acids was determined with a straight phase HPLC as described previously (Pulvera et al. 2006).

Liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry (LC-FTICR-MS) using a Finnigan LTQ-FT instrument (Thermo Fisher Scientific, Waltham, MA) coupled with an Agilent 1100 system (Agilent, Palo Alto, CA) was used for estimation of molecular formulas and multi-stage MS/MS fragmentation of the oxidized MGDGs. HPLC was carried out with the Mightysil RP-18 with a linear gradient of a solvent system composed of acetonitrile/water (85/15 to 95/5, v/v) with 0.1% formic acid at the flow rate of 0.7 ml min⁻¹ at 40°C. MS analysis was performed in negative ion mode. The ion spray voltage was set at -4.0 kV, and capillary temperature at 300°C. Nitrogen sheath gas and auxiliary gas were set at 40 and 15 arbitrary units. Full MS scan was performed in the range of 100–1500 (*m/z*) at resolution 100,000 (at *m/z* 400). MS/MS fragmentation analyses were carried out at normalized collision energy 35.0% and isolation width 4.0 (*m/z*), and basically obtained by ion trap mode.

Results

Soybean seeds have three lipoxygenases differing in their catalytic properties. In order to simplify, we used a soybean cultivar (cv. Yumeyutaka) that had only lipoxygenase-1. When the crude enzyme solution prepared from the seeds was mixed with MGDG in the absence of detergent, no uptake of O₂ was detected (Figure 1, *inset*). It has been reported that soybean lipoxygenase-1 acts on phosphatidylcholine when appropriate amount of bile salt is included in the reaction mixture (Eskola and Laakso 1983). When deoxycholic acid was added into the reaction mixture, oxygen uptake started immediately, and almost constant rate of the oxygen consumption caused by lipoxygenase reaction was maintained thereafter. The activity changed depending on the concentration of deoxycholic acid, and met its optimum with 6.9 mM of deoxycholic acid (Figure 1).

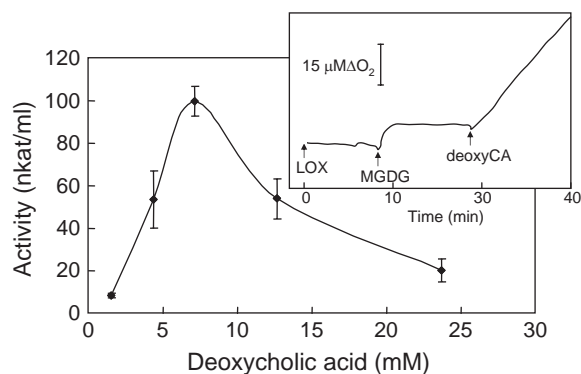


Figure 1. Effect of deoxycholic acid (deoxy CA) on the activity of soybean lipoxygenase-1 on monogalactosyl-diacylglycerol (MGDG). The lipoxygenase activity was determined with an oxygen electrode (mean \pm SE, $n = 3$). Inset: A representative oxygen-consumption curve.

The optimum activity to MGDG was found at pH 9.0 that was similar to the pH optimum of soybean lipoxygenase-1 with free linolenic acid (Axelrod et al. 1981).

For this study, we used MGDG purified from clover (*Trifolium repens*) leaves. Clover is a 18:3 plant, and it has no 16:3 (hexadecatrienoic acid). We found that linolenic acid was most abundant (96.4%) in the clover leaf MGDG, which indicated that 1,2-di-*O*-linolenoyl-3-*O*-(β -D-galactopyranosyl)-*sn*-glycerol (dilinolenoyl MGDG) was most abundant lipid species among MGDG (ca. 93%). This made analysis of oxygenated products easier. The lipoxygenase products were extracted after ca. 50% of MGDG was oxygenated. The products were reduced with triphenylphosphine, then, alkaline-hydrolyzed for HPLC analysis. The analysis showed that 13-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid was most abundant and only trace amount of its 9-isomer could be detected, which indicated that the lipoxygenase inserted molecular oxygen into the acyl moiety of MGDG in a positionally and geometrically specific manner as found with free linolenic acid (Axelrod et al. 1981).

Three major peaks appeared after HPLC separation of the products when absorption at 234 nm originated from the conjugated diene hydroperoxide moiety was monitored (Figure 2). All the three peaks showed absorption spectra peaking at 234 nm. LC-FTICR-MS analysis showed that the first peak (compound A) had m/z 883.51 [$M + HCOO$] $^-$ and m/z 837.51 [$M - H$] $^+$. This corresponded to a molecular formula of $C_{45}H_{74}O_{14}$ (839.06) that could be assigned as 1,2-di-*O*-(13-hydroperoxylinolenoyl)-3-*O*-(β -D-galactopyranosyl)-*sn*-glycerol (MGDG *bis*-hydroperoxide). Multistage MS/MS profiles showed a peak of m/z 291.28 that corresponded to a fragment derived from hydroperoxylated linolenoyl moiety (Figure 3). Taken together, it was feasible to annotate compound A as MGDG *bis*-hydroperoxide.

MS analysis of the twin peaks eluted later (compounds B and C, in the order of elution) showed that both of them had the same m/z 805.04 [$M - H$] $^+$ that corresponded to a molecular formula of $C_{45}H_{74}O_{12}$ (806.52). MS/MS profiles showed prominent three peaks with both the peaks (Figure 3). Among them, the compounds B and C shared the peaks of m/z 291 and 277. They corresponded to the fragments derived from hydroperoxylinolenic acid and linolenic acid, respectively. Therefore, it was assumed that compounds B and C were monohydroperoxylated MGDG. The m/z values of the other peaks were different. The peak of m/z 527.28 was accountable to a fragment that lost linolenoyl moiety, while that of m/z 513.07 to a fragment that lost hydroperoxylated linolenoyl moiety. It was reported that *sn*-2 acyl group was preferentially cleaved from mono-oxygenated lipid species, and that the mono-oxygenated lipid with the oxygenated acyl group at its *sn*-1 position eluted earlier from C18 HPLC column (Nakanishi et al. 2010). Accordingly, compound B was tentatively assigned as 1-*O*-(13-hydroperoxylinolenoyl)-2-*O*-(linolenoyl)-3-*O*-(β -D-galactopyranosyl)-*sn*-glycerol (MGDG 1-*mono*-hydroperoxide), and compound C as 1-*O*-(linolenoyl)-2-*O*-(13-hydroperoxylinolenoyl)-3-*O*-(β -D-galactopyranosyl)-*sn*-glycerol (MGDG 2-*mono*-hydroperoxide).

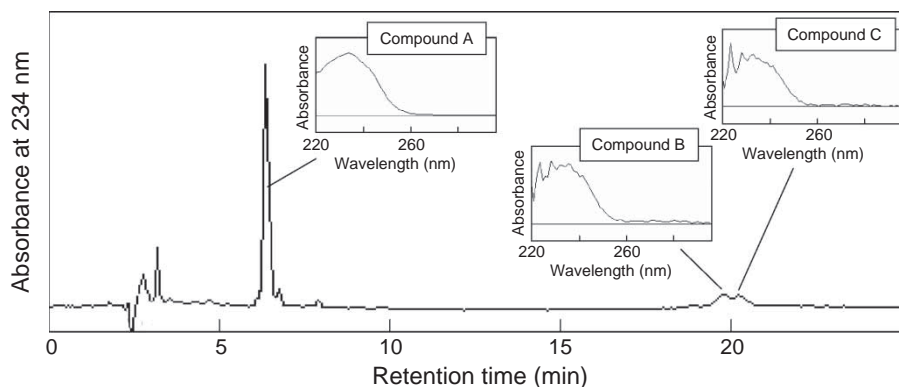


Figure 2. Separation of the oxygenation products with HPLC. After separation with HPLC, three peaks (compounds A to C) could be detected by monitoring A234. The spectrum of each peak is shown in inset.

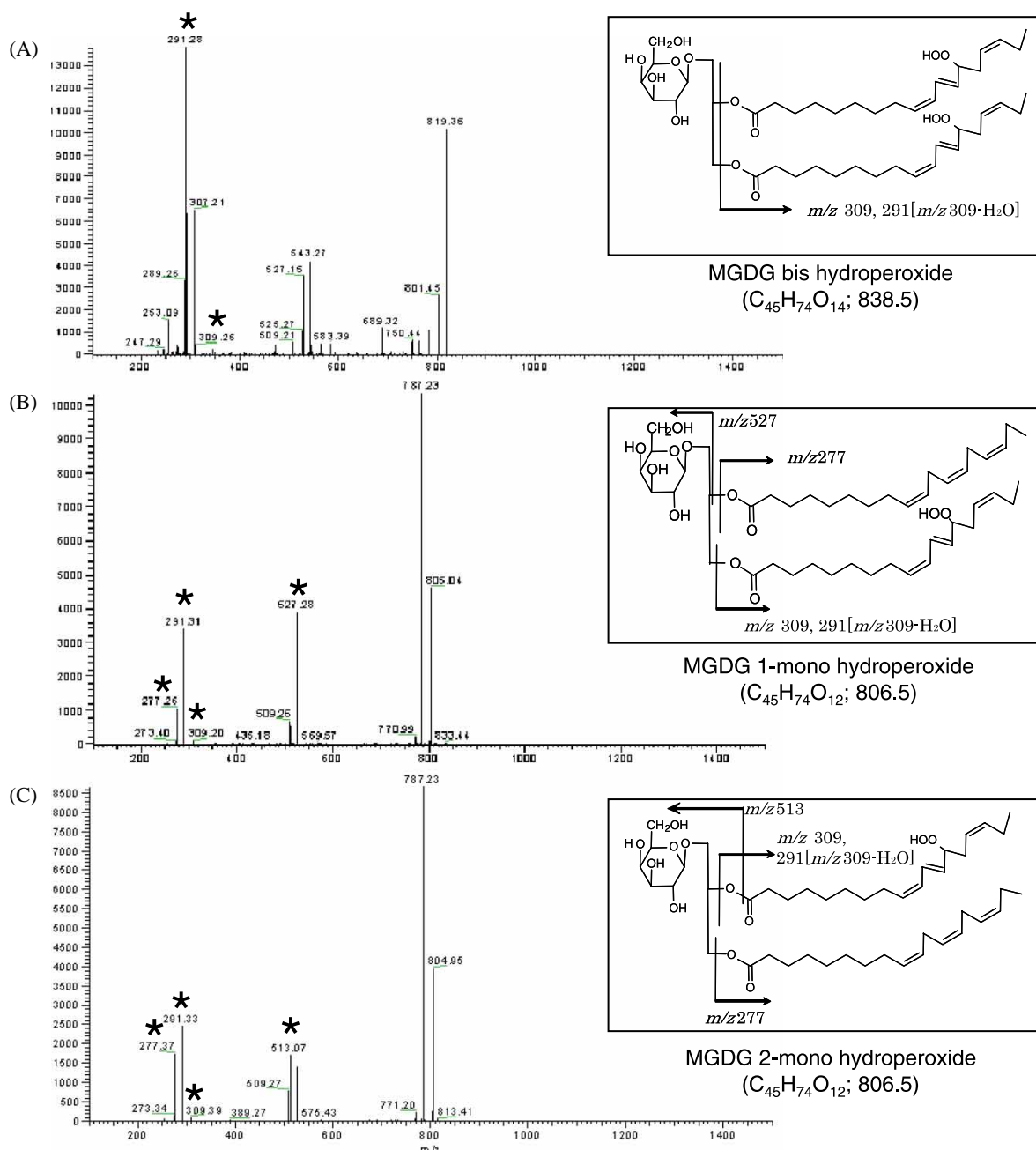


Figure 3. Multistage MS/MS profiles of compounds A, B, and C. The structures tentatively assigned are shown in insets. The MS peaks used for assignment are shown with asterisks (see text for details).

Discussion

It has been widely accepted that plant lipoxygenases act on the free form of fatty acids. This holds true in most cases of their reactions; however, it has been also reported that under a specified reaction condition some lipoxygenases exert their catalytic activities against esterified fatty acids in phospholipids, triacylglycerols, or cholesterol esters (Andreou and Feussner 2009). It was also reported that chloroplast membrane that was rich in galactolipids was efficiently oxygenated by soybean lipoxygenase-2 (Maccarrone et al. 1994). In this study we present clear evidence that MGDG is also a substrate for soybean lipoxygenase-1 when appropriate concentration of deoxycholic acid is

provided. Because of the hydrophobicity of MGDG, it was difficult to prepare aqueous solution of the lipid in the absence of detergents. However, under the optimum reaction condition, mixed micellar aggregates might be formed with MGDG and deoxycholic acid because the reaction solution was transparent. Probably, such mixed micelle structure must be prerequisite for the lipoxygenase to recognize the esterified fatty acids.

13-Hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid was the major acyl moiety in the oxygenated products from MGDG, indicating that a specific oxygen insertion was catalyzed by the enzyme. This suggested that soybean lipoxygenase-1 exerted its normal catalysis with high product specificities even

without free carboxylic acid group. We found almost equal amounts of MGDG *mono*-hydroperoxides, suggesting no preference of oxygenation regarding the *sn*-position of acyl group. The *mono*-hydroperoxides were still substrate, and further oxygenated to yield the *bis*-hydroperoxide.

In nature, several kinds of esterified oxylipins have been reported (Kourtchenko et al. 2007; Chechetkin et al. 2009). Arabidopsides are galactolipids composing 12-oxophytodienoic acid (OPDA) or dinor-phytodienoic acid (dn-OPDA) (Kourtchenko et al. 2007). OPDA and dn-OPDA are the products of allene oxide synthase from hydroperoxides of linolenic acid and hexadecatrienoic acid, respectively. Linolipins are also galactolipids composing divinyl ethers as their acyl groups. Divinyl ethers are formed from fatty acids through consecutive reactions by lipoxygenase and divinyl ether synthase (Chechetkin et al. 2009). At this moment, no clear evidence has been provided if direct lipoxygenase action on galactolipids is involved in the formation of these galactolipid oxylipins because it is still possible that they are formed from free oxylipins through re-esterification to MGDG moieties. However, this study support that the direct oxygenation is feasible. Contents of arabisopsides and linolipins increased under stresses such as mechanical wounding, herbivore attacks, or pathogen attacks, thus, their implications in plant-herbivore and plant-pathogen interactions have been suggested (Kourtchenko et al. 2007; Chechetkin et al. 2009). If this is the case, then, the direct lipoxygenase action on MGDG should be important step to regulate the amounts of galactolipid oxylipins. In order to test this hypothesis, we are now conducting a line of investigations to examine if lipoxygenase reacts MGDG directly in vivo and to examine the mechanism to regulate its reaction on MGDG under biotic stresses in plants.

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