

## Preparation of pure lipid hydroperoxides<sup>[S]</sup>

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**Abstract** Increasing evidence of lipid peroxidation in food deterioration and pathophysiology of diseases have revealed the need for a pure lipid hydroperoxide (LOOH) reference as an authentic standard for quantification and as a compound for biological studies in this field. Generally, LOOH is prepared from photo- or enzymatically oxidized lipids; however, separating LOOH from other oxidation products and preparing pure LOOH is difficult. Early studies showed the usability of reaction between hydroperoxide and vinyl ether for preparation of pure LOOH. Because the reactivity of vinyl ether with LOOHs other than fatty acid hydroperoxides has never been reported, here, we employed the reaction for preparation of a wide variety of pure LOOHs. Phospholipid, cholesteryl ester, triacylglycerol, or fatty acid was photo- or enzymatically oxidized; the resultant crude sample containing hydroperoxide was allowed to react with a vinyl ether [2-methoxypropene (MxP)]. Liquid chromatography (LC) and mass spectrometry confirmed that MxP selectively reacts with LOOH, yielding a stable MxP adduct (perketal). The lipophilic perketal was eluted at a position away from that of intact LOOH and identified and isolated by LC. Upon treatment with acid, perketal released the original LOOH, which was finally purified by LC. Using our optimized purification procedures, for instance, we produced 75 mg of pure phosphatidylcholine hydroperoxide (>99%) from 100 mg of phosphatidylcholine. Our developed method expands the concept of the perketal method, which provides pure LOOH references. The LOOHs prepared by the perketal method would be used as “gold standards” in LOOH methodology.—Ibusuki, D., K. Nakagawa, A. Asai, S. Oikawa, Y. Masuda, T. Suzuki, and T. Miyazawa. Preparation of pure lipid hydroperoxides. *J. Lipid Res.* 2008. 49: 2668–2677.

**Supplementary key words** oxidative stress • lipid peroxidation • lipid hydroperoxide standard • 2-methoxypropene

Because lipid peroxidation is involved in food deterioration (1) and pathophysiology of human diseases (2, 3), there has been a great interest in the accurate measure-

ment of lipid hydroperoxide (LOOH) concentration. This can be performed by several quantitative methods (4–11), and the most sensitive and reliable one is chemiluminescence detection-liquid chromatography (CL-LC) (9–11). Since there is no approved LOOH calibrator (“gold standard”) available, it is impossible to compare the LOOH levels from various laboratories around the world.

Currently, researchers prepare their own in-house reference LOOH [i.e., lipids (e.g., phospholipids, cholesterol, triacylglycerols, and fatty acids)] are subjected to photo- (12), free radical (13), or enzymatic oxidation (14, 15). The resultant crude or partly purified LOOH is generally used as a calibrator. However, these references are neither officially approved nor do they correspond to each other, particularly with regard to their purity. As frequently mentioned by LOOH researchers, this problem is mainly caused by the difficulty in distinguishing and isolating LOOH from other oxidation products such as hydroxides (13). Therefore, efficient purification of a wide variety of LOOHs is the key to the development of the gold standard not only for the accurate quantification of LOOH but also for the evaluation of its biological functions.

A few previous studies (16–21) have reported that some vinyl ether compounds [i.e., 2-methoxypropene (MxP)] react with organic hydroperoxides to yield perketals. These perketals, upon treatment with acid, release the original hydroperoxides. For instance, by using these reactions, Porter et al. (19) succeeded in purifying fatty acid methyl

Abbreviations: ChL, cholesteryl linoleate; CL, chemiluminescence; ESI, electrospray ionization; LA, linoleic acid; LAMe, methyl linoleate; LC, liquid chromatography; LLL, trilinoleoylglycerol; LOOH, lipid hydroperoxide; LOX-1, lipoxygenase-1; MS, mass spectrometry; MxP, 2-methoxypropene; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PLPCOOH, 1-palmitoyl-2-hydroperoxyoctadecadienoyl-*sn*-glycero-3-phosphocholine; PLPCOOMxP, MxP adduct of PLPCOOH; PLPE, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine; PLPS, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoserine; PPTS, pyridinium *p*-toluenesulfonate; RB, rose bengal; TIC, total ion chromatogram; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; XIC, extracted ion chromatogram.

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ester hydroperoxides. The reactivity of MxP with LOOHs other than fatty acid methyl ester hydroperoxides has never been reported; but these studies (16–21) indicated that the reaction may be useful for the preparation of a wide variety of pure LOOHs (Fig. 1A).

In the present study, we optimized the reaction between MxP and the hydroperoxides of phospholipids, cholesterol esters, triacylglycerols, fatty acids, and fatty acid methyl esters and developed a purification method of authentic LOOHs by photo- or enzymatic oxidation of lipids followed by derivatization with MxP and liquid chromatography (LC) isolation.

## MATERIALS AND METHODS

### Materials

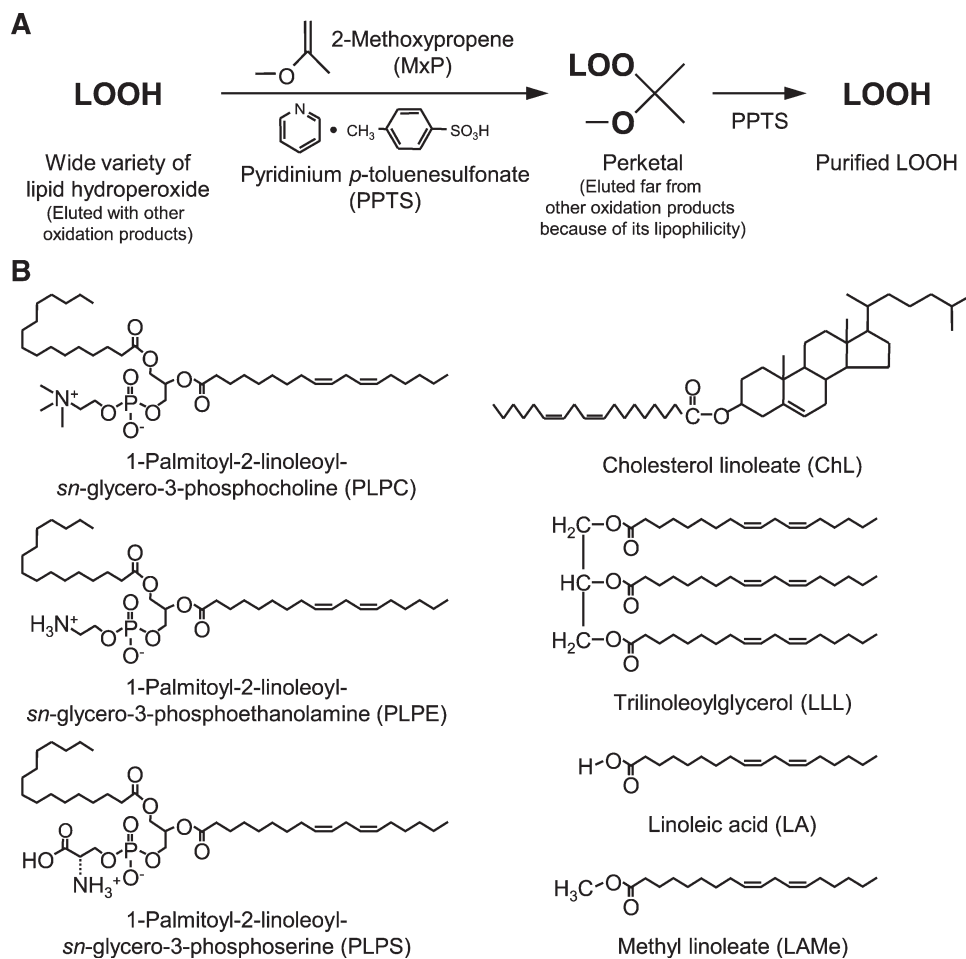
We purchased 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), trilinoleoylglycerol (LLL), linoleic acid (LA), methyl linoleate (LAME), and pyridinium *p*-toluenesulfonate (PPTS) from Sigma (St. Louis, MO). We obtained 1-palmitoyl-2-

linoleoyl-*sn*-glycero-3-phosphoethanolamine (PLPE) and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoserine (PLPS) from Avanti Polar Lipids (Alabaster, AL). Cholesteryl linoleate (ChL) was obtained from ICN Biomedicals Inc. (Aurora, OH). All other reagents were of analytical grade.

### LOOH preparation

In order to prepare LOOH, the lipid (PLPC, PLPE, PLPS, ChL, LLL, LA, or LAME; Fig. 1B) was subjected to three different types of oxidations [rose bengal (RB)-catalyzed photo-oxidation, ultraviolet (UV) photo-oxidation, and lipoxygenase-catalyzed oxidation].

For RB-catalyzed photo-oxidation, PLPC, PLPE, PLPS, LA, or LAME (100 mg) was dissolved in 5 ml of methanol, whereas ChL or LLL (100 mg) was dissolved in 5 ml of chloroform/methanol (1:1, v/v). RB (CHROMA, Tokyo, Japan) was added to these samples at a concentration of 0.1 mg/ml. The samples were exposed to oxygen gas for 10 s and photo-oxidized for 3–24 h at 4°C (ice-cold conditions). A 100 W incandescent lamp (Matsushita Electric Industrial Co., Osaka, Japan) was positioned vertically 10 cm above the sample. Subsequently, to remove RB, the resultant sample of PLPC, PLPE, LAME, ChL, or LLL was loaded onto a Sep-Pak Plus QMA column (Waters, Milford, MA). The column was eluted with 5 ml of methanol for PLPC, PLPE, and LAME



**Fig. 1.** A: Protocols investigated for the purification of lipid hydroperoxide (LOOH) using 2-methoxypropene (MxP). B: Chemical structures of lipids [1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (PLPE), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoserine (PLPS), cholesteryl linoleate (ChL), trilinoleoylglycerol (LLL), linoleic acid (LA), and methyl linoleate (LAME)].

samples or with 5 ml of chloroform/methanol (1:1, v/v) for ChL and LLL samples. The eluent was collected, evaporated, and redissolved in 20 ml of dichloromethane. The PLPS and LA sample solutions were diluted with 5 ml of water and then loaded onto a flush column filled with 20 g of COSMOSIL 75C<sub>18</sub>-PREP ODS (Nacalai Tesque, Kyoto, Japan). The column was washed with 100 ml of methanol/water (1:1, v/v) to remove RB and eluted with 50 ml of methanol. The eluent was evaporated and redissolved in 20 ml of dichloromethane.

For UV photo-oxidation, 100 mg of PLPC, PLPE, PLPS, ChL, LLL, LA, or LAMe was placed in a test tube and exposed to oxygen gas for 10 s. The tube was capped, and then photo-oxidized using a 15 W UV GL-15 lamp (radiation frequency, 253 nm; Toshiba Electronics Co., Tokyo, Japan) at 20°C (room temperature) for 3–24 h. The light source was held vertically at 30 cm above the test tube. The resultant lipid was dissolved in 20 ml of dichloromethane.

For lipoxygenase-catalyzed oxidation, a solution of PLPC, PLPE, PLPS, LLL, LA, or LAMe (100 mg/5 ml of ethanol) was mixed with 170 ml of 50 mM borate buffer (pH 9.0) containing soybean lipoxygenase-1 (LOX-1,  $1.25 \times 10^6$  units; SERVA Electrophoresis, Heidelberg, Germany) and sodium deoxycholate (375 mg). The mixture was incubated at 20–40°C for 3–24 h in the presence of oxygen. ChL (100 mg) was suspended in 5 ml of isopropanol and subjected to LOX-1-oxidation. The lipid moiety was then extracted by adding 30 ml of 0.2 M HCl and 60 ml of diethylether. After shaking the solution, the diethylether layer was collected, dried, and dissolved in 20 ml of dichloromethane.

A portion (1  $\mu$ l) of the dichloromethane sample was subjected to LC combined with UV, chemiluminescence (CL), and mass spectrometry (MS) (LC-UV/CL/MS) (22, 23) to evaluate LOOH formation, as described below.

### Purification of LOOH by using MxP

We confirmed LOOH formation in the dichloromethane sample allowed the sample to react with MxP in order to obtain perketal, and subsequently, LOOH was regenerated from the perketal as follows.

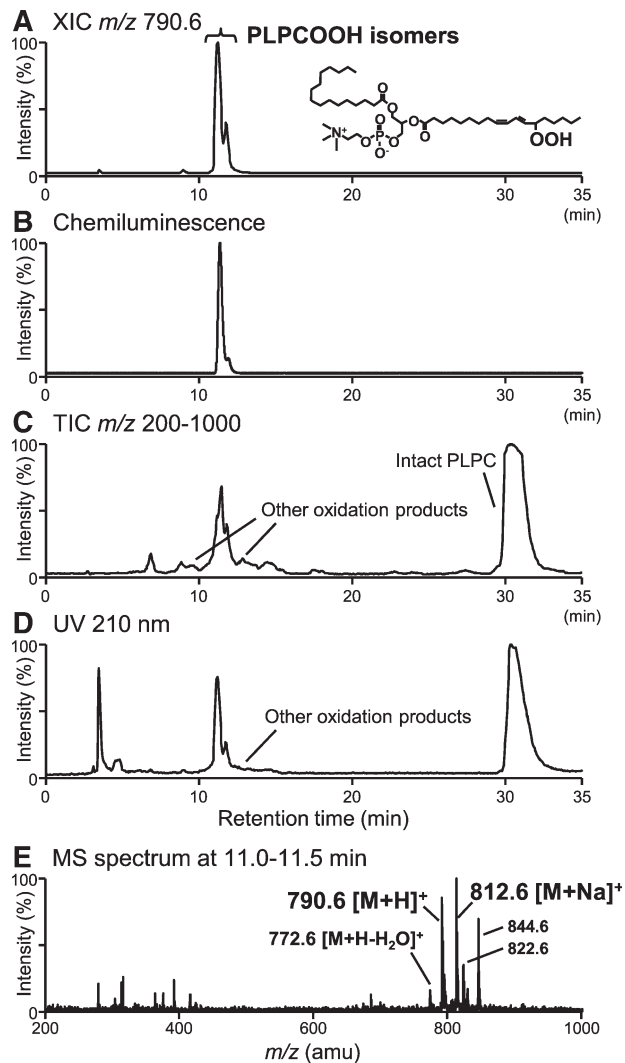
For the reaction with MxP, the dichloromethane sample (20 ml) was mixed with PPTS (2–10 mg/4 ml of dichloromethane). To the sample mixture, 1–5 g (approximately 1.3–6.7 ml) of MxP (Wako, Osaka, Japan) was added. The sample (total approximately 25–31 ml) was vortexed for 1 min and kept standing for 0.5–6 h at 4–20°C. After confirming the perketal formation by LC-UV/CL/MS, a portion (1 ml) of the sample mixture was subjected to semipreparative LC, and the perketal fraction was collected as described below. This isolation procedure was repeated 25–31 times. The collected perketal fractions were combined and evaporated to dryness.

For regeneration of LOOH, the isolated perketal was dissolved in 25 ml of chloroform/methanol (1:1, v/v). The solution was then mixed with PPTS [1–20 mg/5 ml of chloroform/methanol (1:1, v/v)] and incubated for 3–24 h at 4–37°C. After the regeneration of LOOH was ascertained by LC-UV/CL/MS, the LOOH was finally purified by semipreparative LC. The structure and purity of the obtained LOOH was evaluated by LC-UV/CL/MS. In addition, <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity Plus-600 spectrometer (Palo Alto, CA) at 600 MHz for <sup>1</sup>H NMR and at 150 MHz for <sup>13</sup>C NMR using CDCl<sub>3</sub> as a solvent.

### LC-UV/CL/MS for LOOH and perketal analysis

LOOH and its MxP adduct (perketal) were analyzed and their concentrations were determined by reversed-phase LC-UV/CL/MS. We used an ODS column (COSMOSIL 5C<sub>18</sub>-MS-II, 5  $\mu$ m,

4.6  $\times$  250 mm; Nacalai Tesque, Kyoto, Japan) fitted with its pre-column (4.6  $\times$  10 mm). The column was eluted with methanol/water (100:5, v/v) containing 5 mM ammonium acetate for PLPC, PLPE, and PLPS samples; methanol/ethanol (100:30, v/v) for the ChL sample; methanol/ethanol (100:10, v/v) for the LLL sample; methanol/water (100:30, v/v) containing 0.1% acetic acid for the LA sample; and methanol/water (100:20, v/v) for the LAMe sample. The flow rate was adjusted to 1 ml/min and column temperature was maintained at 40°C. After the column eluent was monitored with a UV detector (UV-970; JASCO, Tokyo, Japan) at 210 nm, the eluent was divided into two portions. One portion (0.99 ml/min) was mixed with hydroperoxide-specific CL reagent (a mixture of cytochrome c and luminol in 50 mM borate buffer, pH 10.0) (10, 11) and introduced into a CL detector (825-CL;



**Fig. 2.** Preparation of 1-palmitoyl-2-hydroperoxyoctadecadienoyl-*sn*-glycero-3-phosphocholine (PLPCOOH). PLPC [100 mg/5 ml of methanol, containing 0.5 mg of rose bengal (RB)] was photo-oxidized for 8 h at 4°C, and analyzed by liquid chromatography ultraviolet (LC-UV)/chemiluminescence (CL)/mass spectrometry (MS). A: Extracted ion chromatogram (XIC) of PLPCOOH ( $m/z$  790.6 [M+H]<sup>+</sup>) and its representative isomer [1-palmitoyl-2-(13-hydroperoxy-9Z,11E-octadecadienoyl)-*sn*-glycero-3-phosphocholine]. B: CL chromatogram. C: Total ion chromatogram (TIC,  $m/z$  200–1000). D: UV chromatogram (at 210 nm). E: MS spectrum at 11.0–11.5 min.

JASCO). The flow rate of the CL reagent was set at 0.5 ml/min. The second portion (0.01 ml/min) was introduced into a Mariner electrospray ionization (ESI) time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). The MS parameters were positive-ion measurement mode (spray voltage, 3000 V; nozzle potential, 100 V; nozzle temperature, 140°C; flow rate of nebulizer gas, 0.75 l/min; and that of spray gas, 2.0 l/min), but were negative-ion mode for the PLPS sample. Full-scan mass spectra were recorded over a range of  $m/z$  200–1300 in a scan time of 3 s.

### Semipreparative LC for perketal and LOOH isolation

Perketal and LOOH (regenerated from perketal) were isolated using semipreparative LC by using an ODS column (COSMOSIL 5C<sub>18</sub>-MS-II, 5  $\mu$ m, 20  $\times$  250 mm; Nacalai Tesque) fitted with its precolumn (10  $\times$  50 mm). The mobile phases used were the same as those used for LC-UV/CL/MS, as described above. The flow rate was 10 ml/min and column temperature was maintained at 40°C. After the column eluent was monitored with a UV detector at 210 and 234 nm, the fraction of perketal or LOOH was collected.

### Structural determination of PLPCOOH isomers

1-Palmitoyl-2-hydroperoxyoctadecadienoyl-*sn*-glycero-3-phosphocholine (PLPCOOH, about 0.8 mg), purified from RB-photo-oxidation or LOX-1 oxidation sample, was dissolved in 1 ml of methanol. To the solution, 2 mg of sodium borohydride (NaBH<sub>4</sub>) was added and mixed for 30 min at room temperature. To the reaction mixture, 0.1 ml of 4 M HCl, 2 ml of chloroform and 1 ml of water were added to remove remaining NaBH<sub>4</sub> (24).

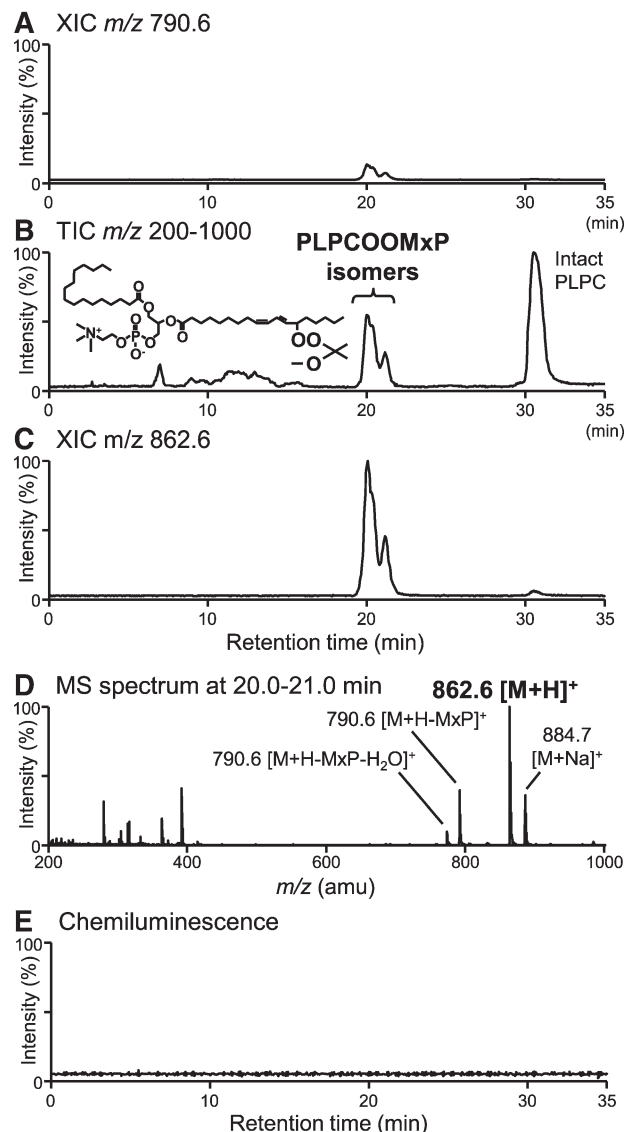
TABLE 1. Optimal oxidation procedure to prepare LOOH

Lipid	Oxidation procedure	Time	Temp	Yielded LOOH <sup>a</sup>
		h	°C	
PLPC	mg			mg
100	RB-photo	8	4	PLPCOOH 30
100	UV-photo	24	20	PLPCOOH 1.7
100	LOX-1	12	40	PLPCOOH 96
PLPE	mg			mg
100	RB-photo	10	4	PLPEOOH 28
100	UV-photo	24	20	PLPEOOH 1.4
100	LOX-1	12	40	PLPEOOH 92
PLPS	mg			mg
100	RB-photo	10	4	PLPSOOH 30
100	UV-photo	24	20	PLPSOOH 1.3
100	LOX-1	12	40	PLPSOOH 94
ChL	mg			mg
100	RB-photo	8	4	ChLOOH 27
100	UV-photo	24	20	ChLOOH 1.1
100	LOX-1	24	40	ChLOOH 6.2
LLL	mg			mg
100	RB-photo	6	4	LLLOOH 31
100	UV-photo	6	20	LLLOOH 35
100	LOX-1	12	40	LLLOOH 9.4
LA	mg			mg
100	RB-photo	8	4	LAOOH 32
100	UV-photo	12	20	LAOOH 35
100	LOX-1	6	20	LAOOH 99
LAMe	mg			mg
100	RB-photo	8	4	LAMeOOH 34
100	UV-photo	12	20	LAMeOOH 36
100	LOX-1	8	20	LAMeOOH 97

LOOH, lipid hydroperoxide; LOX-1, lipoxygenase-1; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PLPCOOH, 1-palmitoyl-2-hydroperoxyoctadecadienoyl-*sn*-glycero-3-phosphocholine; PLPE, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine; RB, rose bengal; UV, ultraviolet.

<sup>a</sup>LC retention times and mass spectrometry (MS) profiles of hydroperoxides are as follows: PLPCOOH, 11.0 min, 790.6 [M+H]<sup>+</sup>; PLPEOOH, 10.5 min, 748.5 [M+H]<sup>+</sup>; PLPSOOH, 8.5 min, 790.4 [M+H]<sup>+</sup>; ChLOOH, 26.5 min, 703.6 [M+Na]<sup>+</sup>; LLLOOH, 23.0 min, 933.7 [M+Na]<sup>+</sup>; LAOOH, 15.5 min, 330.3 [M+NH<sub>4</sub>]<sup>+</sup>; LAMeOOH, 11.5 min, 349.3 [M+Na]<sup>+</sup>.

After centrifugation at 1000 *g* for 5 min at 4°C, the lower chloroform layer was collected and dried under N<sub>2</sub> gas. The dried extract was dissolved in 1 ml of methanol, mixed with 1 ml of 50 mM HCl-Tris [tris(hydroxymethyl)aminomethane] buffer (pH 7.4, containing 1 mg of calcium chloride and 1,500 units of phospholipase A<sub>2</sub> (from honey bee venom; Sigma, St. Louis, MO), and incubated for 2 h at room temperature (25). After centrifugation at 1,000 *g* for 5 min at 4°C, the lower chloroform layer was collected and dried by N<sub>2</sub> gas. The dried residue was redissolved in 1 ml of methanol, and subjected to semipreparative LC for isolation of hydroxy-LA. An ODS column (COSMOSIL 5C<sub>18</sub>-MS-II, 5  $\mu$ m, 20  $\times$  250 mm; Nacalai Tesque, Kyoto, Japan) was eluted with a mixture of methanol/water (100:25, v/v). The flow rate



**Fig. 3.** Conversion of PLPCOOH to MxP adduct of PLPCOOH (PLPCOOMxP) (perketal). RB-catalyzed photo-oxidized PLPC sample (20 ml) was mixed with pyridinium *p*-toluenesulfonate (PPTS) (5 mg/4 ml of dichloromethane) and 3 g (4 ml) of MxP. The sample was maintained for 3 h at 4°C, and then analyzed by LC-UV/CL/MS. A: XIC of PLPCOOH ( $m/z$  790.6 [M+H]<sup>+</sup>). B: TIC ( $m/z$  200–1000) and structure of PLPCOOMxP (representative MxP adduct of PLPCOOH isomer in Fig. 2). C: XIC of PLPCOOMxP ( $m/z$  862.6 [M+H]<sup>+</sup>). D: MS spectrum at 20.0–21.0 min. E: CL chromatogram.

TABLE 2. Optimal MxP reaction condition to prepare perketal

Sample	Added PPTS	Added MxP	Total reaction volume	Time	Temp	Yielded perketal <sup>c</sup>	Perketal after LC isolation
	mg/4 ml of dichloromethane	g (ml)	ml	h	°C	mg	mg
PLPC	(RB-photo, <sup>a</sup> 30 mg PLPCOOH <sup>b</sup> )	5 3 (4.0)	28	3	4	PLPCOOMxP 30	28
	(UV-photo, 1.7 mg PLPCOOH)	2 1 (1.3)	25	2	4	PLPCOOMxP 1.6	1.5
	(LOX-1, 96 mg PLPCOOH)	5 3 (4.0)	28	3	4	PLPCOOMxP 96	92
PLPE	(RB-photo, 28 mg PLPEOOH)	5 3 (4.0)	28	3	4	PLPEOOMxP 27	22
	(UV-photo, 1.4 mg PLPEOOH)	2 1 (1.3)	25	2	4	PLPEOOMxP 1.3	1.0
	(LOX-1, 92 mg PLPEOOH)	5 3 (4.0)	28	3	4	PLPEOOMxP 90	86
PLPS	(RB-photo, 30 mg PLPSOOH)	5 3 (4.0)	28	3	4	PLPSOOMxP 27	24
	(UV-photo, 1.3 mg PLPSOOH)	2 1 (1.3)	25	2	4	PLPSOOMxP 1.2	1.0
	(LOX-1, 94 mg PLPSOOH)	5 3 (4.0)	28	3	4	PLPSOOMxP 88	83
ChL	(RB-photo, 27 mg ChLOOH)	5 5 (6.7)	31	3	20	ChLOOMxP 26	20
	(UV-photo, 1.1 mg ChLOOH)	2 2 (2.7)	27	3	20	ChLOOMxP 1.0	0.9
	(LOX-1, 6.2 mg ChLOOH)	2 2 (2.7)	27	3	20	ChLOOMxP 5.9	5.3
LLL	(RB-photo, 31 mg LLLOOH)	10 5 (6.7)	31	3	20	LLLOOMxP 30	27
	(UV-photo, 35 mg LLLOOH)	10 5 (6.7)	31	3	20	LLLOOMxP 33	32
	(LOX-1, 9.4 mg LLLOOH)	5 3 (4.0)	28	3	20	LLLOOMxP 9.3	8.2
LA	(RB-photo, 32 mg LAOOH)	2 1 (1.3)	25	1	4	LAOOMxP 30	24
	(UV-photo, 35 mg LAOOH)	2 1 (1.3)	25	1	4	LAOOMxP 32	26
	(LOX-1, 99 mg LAOOH)	5 2 (2.7)	27	1	4	LAOOMxP 95	85
LAMe	(RB-photo, 34 mg LAMeOOH)	2 1 (1.3)	25	1	4	LAMeOOMxP 34	30
	(UV-photo, 36 mg LAMeOOH)	2 1 (1.3)	25	1	4	LAMeOOMxP 36	33
	(LOX-1, 97 mg LAMeOOH)	5 2 (2.7)	27	1	4	LAMeOOMxP 97	93

<sup>a</sup> Oxidation procedure.

<sup>b</sup> LOOH concentration in sample.

<sup>c</sup> LC retention times and MS profiles of perketal are as follows: PLPCOOMxP, 20.0 min, 862.6 [M+H]<sup>+</sup>; PLPEOOMxP, 18.5 min, 820.6 [M+NH<sub>4</sub>]<sup>+</sup>; PLPSOOMxP, 15.5 min, 862.5 [M-H]<sup>-</sup>; ChLOOMxP, 51.5 min, 775.6 [M+Na]<sup>+</sup>; LLLOOMxP, 45.0 min, 1005.8 [M+Na]<sup>+</sup>; LAOOMxP, 49.5 min, 402.3 [M+NH<sub>4</sub>]<sup>+</sup>; LAMeOOMxP, 33.5 min, 416.3 [M+NH<sub>4</sub>]<sup>+</sup>.

was 10 ml/min and the column temperature was at 40°C. The isolated hydroxy-LA was dried, dissolved in 1 ml of hexane (containing 4 μmol trimethylsilyl diazomethane), vortexed, and stored for 1 h at room temperature (26, 27). To the solution, 0.1 g of acetic acid and 1 ml of water were added to terminate the reaction. After that, the upper hexane layer (hydroxy-LAMe fraction) was analyzed by normal phase LC with a silica column (CAPCELL PAK SILICA SG120, 5 μm, 4.6 × 250 mm; Shiseido, Tokyo, Japan). A mixture of hexane/diethylether/water (87:13:0.1, v/v/v) was used as eluent at the flow rate of 1 ml/min. The column temperature was at 40°C. Hydroxy-LAMe isomers were detected by UV detector at 234 nm, and identified by their retention times (based on Refs. 28,29).

### Stability testing

The obtained hydroperoxides of PLPC, PLPE, PLPS, LA, and LAMe (1.5 mg) were dissolved in 1 ml of methanol, while the hydroperoxides of ChL and LLL (1.5 mg) were dissolved in 1 ml of chloroform/methanol (1:1, v/v). These solutions were stored under nitrogen atmosphere at -30°C for 12 months and analyzed by LC-UV/CL/MS. Similarly, the stability of perketal was evaluated.

### Statistics

The data are expressed as mean ± SD and analyzed using Student's *t*-test. Differences were considered significant at *P* < 0.05.

## RESULTS

### LOOH formation during photo- and enzymatic oxidation

We initially analyzed the RB-catalyzed photo-oxidation of PLPC (12). After the reaction, PLPCOOH (*m/z* 790.6 [M+H]<sup>+</sup>) was formed as the major oxidation product

(Fig. 2A) and also confirmed by CL detection (Fig. 2B). The PLPCOOH possessed four structural isomers of hydroperoxyoctadecadienoyl residues: 13-hydroperoxy-9*Z*,11*E*-octadecadienoate (retention time, 11.0 min), 9-hydroperoxy-10*E*,12*Z*-octadecadienoate (11.0 min), 13-hydroperoxy-9*E*,11*E*-octadecadienoate (11.5 min), and 9-hydroperoxy-10*E*,12*E*-octadecadienoate (11.5 min). Similarly, LOOH formation was verified when PLPC, PLPE, PLPS, ChL, LLL, LA, and LAMe were subjected to RB-catalyzed photo-, UV photo-, and LOX-1-catalyzed oxidation. Optimal conditions required for the maximum yield of LOOH are summarized in Table 1.

In the case of all samples, LOOH was coeluted with other oxidation products, particularly in photo-oxidation (as shown in the total ion chromatogram, UV chromatogram, and MS spectrum of PLPCOOH, Figs. 2C–E). This fact indicates that distinguishing and separating LOOH from other hydroperoxides is difficult.

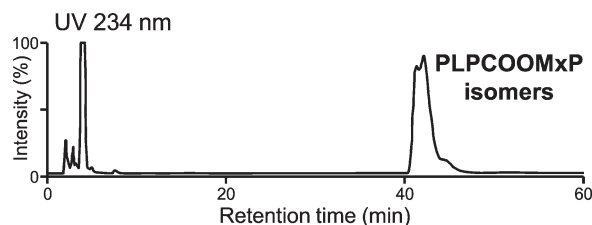
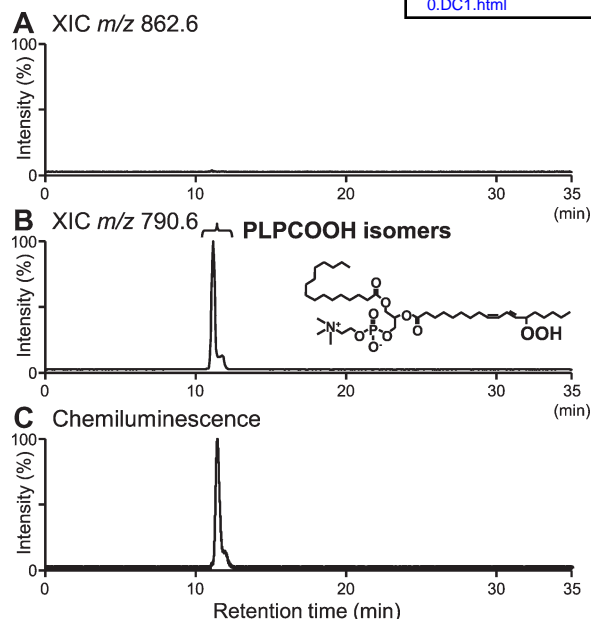


Fig. 4. PLPCOOMxP isolation. PLPCOOMxP sample obtained in Fig. 3 was isolated by semipreparative LC monitored by UV absorption at 234 nm.



**Fig. 5.** PLPCOOH regeneration. PLPCOOMxP isolated in Fig. 4 (25 ml) was mixed with PPTS [10 mg/5 ml of chloroform/methanol (1:1)], maintained for 6 h at 4°C, and the sample was then analyzed by LC-UV/CL/MS. A: XIC of PLPCOOMxP ( $m/z$  862.6  $[M+H]^+$ ). B: XIC of PLPCOOH ( $m/z$  790.6  $[M+H]^+$ ) and its representative isomer (regenerated PLPCOOMxP isomer). C: CL chromatogram.

### Reaction of LOOH with MxP

The photo- and enzymatically oxidized lipid samples were treated with MxP. After treating the RB-catalyzed photo-oxidized PLPC sample with MxP, PLPCOOH isomers were no longer detectable in the sample (Fig. 3A).

Moreover, perketals (PLPCOOMxP isomers,  $m/z$  862.6  $[M+H]^+$ ) were detected at around 20.0 min (Figs. 3B–D). These isomers were CL-negative (Fig. 3E), indicating the absence of a hydroperoxide group.

In the case of other samples, MxP also reacted almost completely with LOOH to yield perketals. The optimal conditions required to obtain perketals with good efficiency are described in Table 2. Due to the lipophilic nature of perketal, it was eluted at a position away from that of the intact LOOH, and therefore easily isolated by semi-preparative LC (refer to the isolation of PLPCOOMxP peak in Fig. 4).

### Regeneration of intact LOOH from perketal

To regenerate LOOH, each isolated perketal was treated with acid (i.e., PPTS). For example, after treatment of the PLPCOOMxP with PPTS, they were no longer detectable, and PLPCOOH isomers were regenerated as compounds corresponding to clear peaks in MS and CL chromatograms (Fig. 5). Other perketals were also efficiently converted to original hydroperoxides under optimized conditions (Table 3).

### Final purification of LOOH

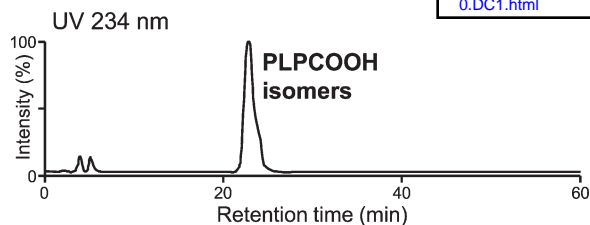
Finally, the regenerated LOOH was purified by semipreparative LC. An example (isolation of PLPCOOH peak) is shown in Fig. 6. The obtained PLPCOOH was a pure mixture of isomers, as judged from the LC-UV/CL/MS data (Fig. 7) and NMR spectra (data not shown). The composition of hydroperoxyoctadecadienyl residues of the obtained PLPCOOH was 13-hydroperoxy-9Z,11E-octadecadienoate

TABLE 3. Optimal LOOH regeneration condition from perketal

Isolated perketal	Added PPTS	Total reaction volume	Time	Temp	Yielded LOOH <sup>b</sup>	LOOH after LC isolation	Purity of isolated LOOH		
	<i>mg/5 ml of chloroform/methanol (1:1)</i>	<i>ml</i>	<i>h</i>	<i>°C</i>	<i>mg</i>	<i>mg</i>	<i>%</i>		
PLPCOOMxP	(RB-photo, 28 mg)	10	30	6	4	PLPCOOH	27	16	98
	(UV-photo, 1.5 mg)	10	30	2	4	PLPCOOH	1.4	0.9	95
	(LOX-1, 92 mg)	10	30	12	4	PLPCOOH	90	75	>99
PLPEOOMxP	(RB-photo, 22 mg)	10	30	6	4	PLPEOOH	20	12	98
	(UV-photo, 1.0 mg)	10	30	2	4	PLPEOOH	0.9	0.6	94
	(LOX-1, 86 mg)	10	30	12	4	PLPEOOH	80	65	98
PLPSOOMxP	(RB-photo, 24 mg)	10	30	6	4	PLPSOOH	22	14	98
	(UV-photo, 1.0 mg)	10	30	2	4	PLPSOOH	0.9	0.6	93
	(LOX-1, 83 mg)	10	30	12	4	PLPSOOH	78	60	98
ChLOOMxP	(RB-photo, 20 mg)	10	30	6	20	ChLOOH	18	11	98
	(UV-photo, 0.9 mg)	10	30	2	20	ChLOOH	0.8	0.5	95
	(LOX-1, 5.3 mg)	10	30	2	20	ChLOOH	4.8	2.3	95
LLLOOMxP	(RB-photo, 27 mg)	10	30	6	4	LLLOOH	26	19	95
	(UV-photo, 32 mg)	10	30	6	4	LLLOOH	30	21	98
	(LOX-1, 8.2 mg)	10	30	6	4	LLLOOH	7.6	5.0	98
LAOOMxP	(RB-photo, 24 mg)	5	30	1	4	LAOOH	22	14	95
	(UV-photo, 26 mg)	5	30	1	4	LAOOH	24	16	95
	(LOX-1, 85 mg)	5	30	6	4	LAOOH	81	57	98
LAMeOOMxP	(RB-photo, 30 mg)	5	30	1	4	LAMeOOH	28	19	95
	(UV-photo, 33 mg)	5	30	1	4	LAMeOOH	31	20	97
	(LOX-1, 93 mg)	5	30	6	4	LAMeOOH	90	78	>99

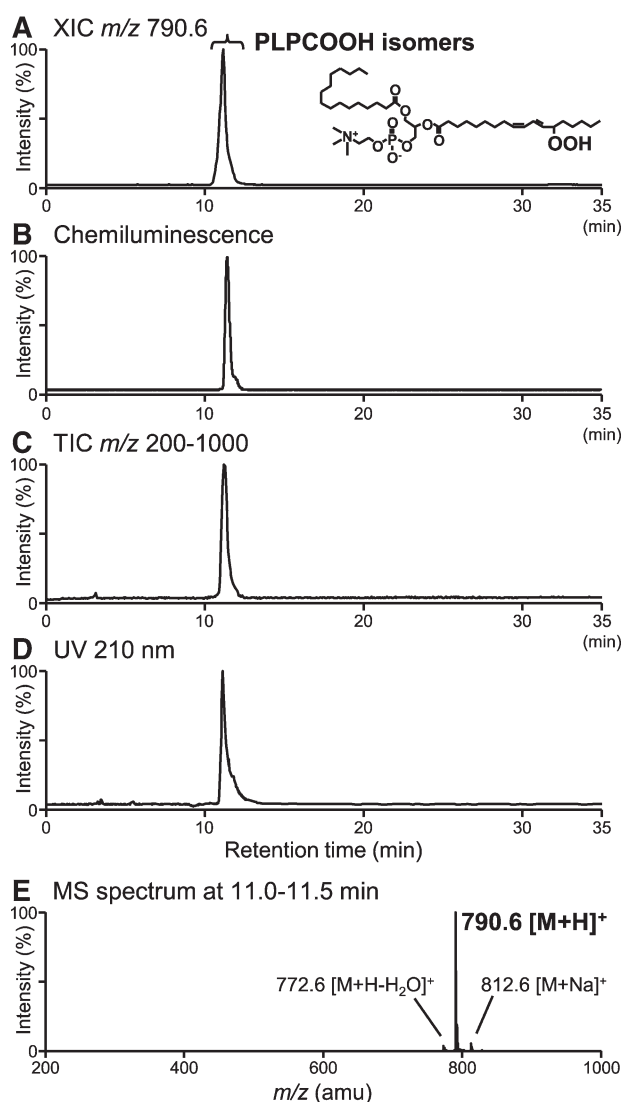
<sup>a</sup> Oxidation procedure.

<sup>b</sup> LC retention times and MS profiles of hydroperoxides are as follows; PLPCOOH, 11.0 min, 790.6  $[M+H]^+$ ; PLPEOOH, 10.5 min, 748.5  $[M+H]^+$ ; PLPSOOH, 8.5 min, 790.4  $[M+H]^+$ ; ChLOOH, 26.5 min, 703.6  $[M+Na]^+$ ; LLLOOH, 23.0 min, 933.7  $[M+Na]^+$ ; LAOOH, 15.5 min, 330.3  $[M+NH_4]^+$ ; LAMeOOH, 11.5 min, 349.3  $[M+Na]^+$ .



**Fig. 6.** PLPCOOH isolation. PLPCOOMxP sample regenerated in Fig. 5 was isolated by semipreparative LC monitored by UV absorption at 234 nm.

(46%), 9-hydroperoxy-10*E*,12*Z*-octadecadienoate (43%), 13-hydroperoxy-9*E*,11*E*-octadecadienoate (7%), and 9-hydroperoxy-10*E*,12*E*-octadecadienoate (4%). For other hydroperoxides of PLPE, PLPS, ChL, LLL, LA, and LAMe,



**Fig. 7.** Purity determination of PLPCOOH. PLPCOOH prepared by the final isolation in Fig. 6 was analyzed by LC-UV/CL/MS. A: XIC of PLPCOOH ( $m/z$  790.6  $[M+H]^+$ ) and its representative isomer. B: CL chromatogram. C: TIC ( $m/z$  200–1000). D: UV chromatogram (absorbance at 210 nm). E: MS spectrum at 11.0–11.5 min.

pure mixtures of hydroperoxide isomers could also be prepared (see supplementary Figs. I–VI).

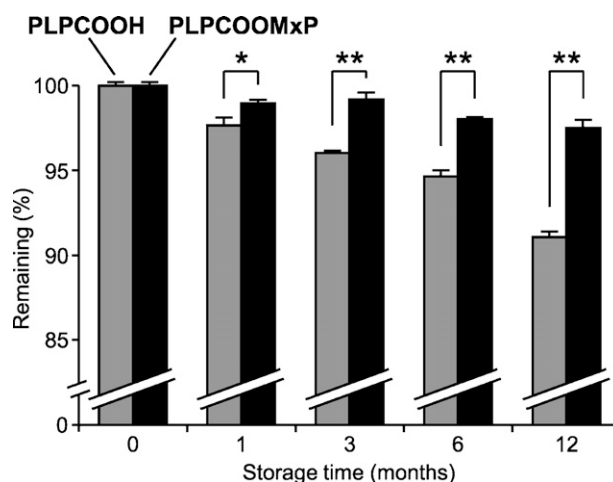
On the other hand, when LOX-1-catalyzed oxidation was conducted instead of photo-oxidation, we obtained an LOOH isomer (e.g., PLPCOOH bearing 13-hydroperoxy-9*Z*,11*E*-octadecadienoate) with high purity and yield.

### Stability of LOOHs and perketals

About 9% of PLPCOOH was decomposed after 12 months during storage at  $-30^{\circ}\text{C}$  (Fig. 8). In contrast, PLPCOOMxP (perketal) was more stable (about 97% remained) than PLPCOOH. The stabilities (measured as % wt remaining) of other hydroperoxides and perketals at 12 months after preparation were as follows: PLPEOOH (63%), PLPEOOMxP (91%); PLPSOOH (50%), PLPSOOMxP (85%); ChLOOH (89%), ChLOOMxP (94%); LLLOOH (86%), LLLOOMxP (95%); LAOOH (40%), LAOOMxP (78%); and LAMeOOH (90%), LAMeOOMxP (98%).

### DISCUSSION

The oxidative modification of lipids, particularly LOOH accumulation, plays a major role in food deterioration (1) and pathophysiology of human diseases, such as atherogenesis, diabetes, and dementia, and aging (2, 3, 30–34). Accurate measurement of LOOH levels is therefore important, but no approved LOOH standard is available yet. In this study, we developed a method for the preparation of the hydroperoxides of PLPC, PLPE, PLPS, ChL, LLL, LA, and LAMe through reaction with MxP.



**Fig. 8.** Stabilities of PLPCOOH and PLPCOOMxP. PLPCOOH in Fig. 6 and PLPCOOMxP in Fig. 4 (1.5 mg/1 ml of methanol) were stored under nitrogen atmosphere at  $-30^{\circ}\text{C}$  for approximately 12 months, and the remaining were analyzed by XIC of PLPCOOH ( $m/z$  790.6  $[M+H]^+$ ) or XIC of PLPCOOMxP ( $m/z$  862.6  $[M+H]^+$ ) on LC-UV/CL/MS compared with XIC ( $m/z$  758.6  $[M+H]^+$ ) of PLPC reference. The data are expressed as mean  $\pm$  SD,  $n = 3$ , and analyzed using Student's *t*-test. Differences were considered significant at \*  $P < 0.05$  or \*\*  $P < 0.01$ .

Over 50 years ago, it was reported that under acidic conditions, some vinyl ethers can react with organic hydroperoxides to form perketals (16, 17). At around 1990, Porter et al. (18, 19) reported the application of vinyl ether (*trans*-2-phenylcyclohexyl 2-propen-2-yl ether) for the synthesis of optically pure hydroperoxides (e.g.,  $\alpha$ -phenethyl hydroperoxide, 2-octyl hydroperoxide, and some fatty acid methyl ester hydroperoxides). The procedures include protecting the racemic hydroperoxides as perketals by using vinyl ether, separating the perketal diastereomers by chromatography, and regenerating the optically pure hydroperoxides from the perketal diastereomers. Baba et al. (21) used MxP for the conversion of LAMeOOH into its perketal. The perketal was utilized for preparation of PLPCOOH (21), but the preparation needed several synthetic steps. In this study, we thought that if MxP reacts efficiently with a variety of LOOHs (refer to the beginning of this article), this reaction can be applied for preparation of pure LOOHs (Fig. 1A).

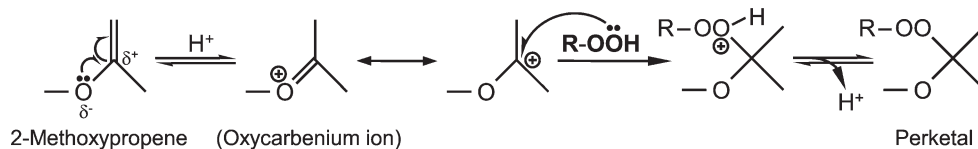
We investigated and optimized the preparation conditions of LOOH (Fig. 2 and Table 1). Under optimal conditions, when the lipid used (PLPC, PLPE, PLPS, ChL, LLL, LA, or LAMe) was subjected to RB-catalyzed photo-, UV photo-, or LOX-1-catalyzed oxidation, the unsaturated fatty acid (linoleoyl) residue was converted to hydroperoxides. In cases of RB-catalyzed photo- and UV photo-oxidation of lipids, the structure of hydroperoxy linoleic residue was characterized as a mixture of 13-hydroperoxy-9*Z*,11*E*-octadecadienoate, 9-hydroperoxy-10*E*,12*Z*-octadecadienoate, 13-hydroperoxy-9*E*,11*E*-octadecadienoate, and 9-hydroperoxy-10*E*,12*E*-octadecadienoate. In LOX-1-catalyzed oxidation, 13-hydroperoxy-9*Z*,11*E*-octadecadienoate was produced predominantly. In addition, we found that RB-catalyzed photo- and UV photo-oxidation of ChL yielded mono-hydroperoxide as well as bis-hydroperoxide. More-

over, these reactions produced mono-, bis-, and tris-hydroperoxides of LLL. These LOOHs were partly decomposed to a wide range of secondary oxidation products, including hydroxides, epoxides, aldehydes, and ketones. These secondary oxidation products were the major obstacle in the isolation of pure LOOH.

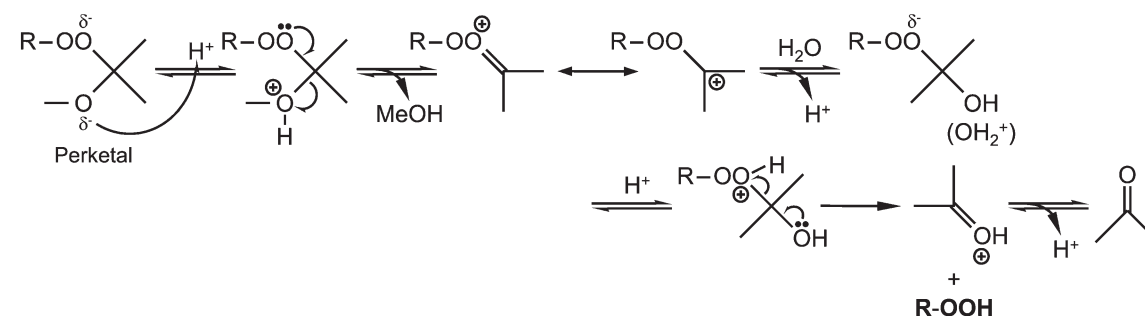
For the reaction of LOOH with MxP, one of the important parameters was the reaction solvent. We found that dichloromethane, chloroform, or acetonitrile could be used as a solvent, but other water-containing solvents inhibited the reaction. Reaction temperature is also important since high temperatures (above 30°C) caused the formation of unknown products. Based on these results, we optimized the reaction conditions (Fig. 3 and Table 2). Under optimal conditions, LOOH was almost completely converted (above 90%) to perketal within a short time period of less than 3 h. The lipophilic perketal was eluted in a position apart from that of intact LOOH, and thereby the perketal could be identified and isolated by semipreparative LC (Fig. 4). No decomposition products of perketal were detected, indicating the stability of perketal. In addition, because MxP did not react with the secondary oxidation products, the isolation of perketal could be performed effectively. To reveal this point, we selected RB-photo-oxidized PLPC as an example (the example has an advantage to show that MxP reacts selectively to LOOHs but not to other oxidation secondary products derived from the RB-photo-oxidation).

A possible reaction scheme for the regeneration of intact LOOH from perketal is shown in Fig. 9. As shown in the scheme, the kind and the concentration of the acid used were important, and these factors were optimized (Fig. 5 and Table 3). In this study, chloroform/methanol (1:1, v/v) was used as the reaction solvent, and methanol or acetonitrile could also be used. Under optimal conditions, perketal

#### A Addition of MxP



#### B Elimination of MxP




**Fig. 9.** Presumed mechanism of the reaction between MxP and hydroperoxide. A: Addition of MxP by nucleophilic addition of hydroperoxide to 2-methoxypropene. B: Elimination of MxP and regeneration of hydroperoxide.

was converted to original LOOH with a high yield (above 90%), and the regenerated LOOH was next subjected to final semipreparative LC purification (Fig. 6).

The obtained LOOHs after photo-oxidation were highly pure (Fig. 7, Table 3), but structural isomers were present (e.g., the prepared PLPCOOH contained mainly 13-hydroperoxy-9Z,11E-octadecadienoate and 9-hydroperoxy-10E,12Z-octadecadienoate). However, the “structural mixture” would be sufficient for use as a standard in most analytical and quantitative experiments. In contrast, when LOX-I-catalyzed oxidation was carried out, a pure LOOH isomer bearing 13-hydroperoxy-9Z,11E-octadecadienoate moiety was obtained. On the other hand, by using the present method, we prepared pure bis-hydroperoxide of ChL. Its structure was speculated based on the following findings: 1) it reacted with two MxP molecules; and 2) when the MxP adduct was subjected to alkaline hydrolysis, the reaction yielded LAOOMxP and perketal of cholesterol hydroperoxide.

With regard to the stability test, perketals were found to be more stable than LOOHs. Although perketal bearing an acidic carboxyl group (PLPSOOMxP or LAOOMxP) tended to degrade to some extent after 12 months storage at  $-30^{\circ}\text{C}$ , such degradation was kept to a minimum by storage at  $-80^{\circ}\text{C}$  for 3 months. We therefore recommended the following procedures: researchers should prepare and store perketals at  $-80^{\circ}\text{C}$  in advance, regenerate LOOH from perketals before the experiment (e.g., quantitative study), and use the pure LOOH as the reference material.

In conclusion, we developed a convenient method for preparing a wide variety of pure LOOH references. We are now synthesizing and using the pure LOOH (e.g., PLPCOOH) as the standard for quantification of phosphatidylcholine hydroperoxide present in the blood plasma of healthy and nonhealthy humans, as well as a model compound to evaluate its biological functions in cell culture studies. We propose to use the MxP procedures developed by us as gold standard in the preparation method for the LOOH assay. 

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