Analysis of Novel Hydroperoxides and Other Metabolites of Oleic, Linoleic, and Linolenic Acids by Liquid Chromatography–Mass Spectrometry with Ion Trap MS^n

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ABSTRACT: Linoleate is oxygenated by manganese-lipoxygenase (Mn-LO) to 11S-hydroperoxylinoate and 13R-hydroperoxyoctadeca-9Z,11E-dienoic acid, whereas linoleate diol synthase (LDS) converts linoleate sequentially to 8R-hydroperoxylinoate, through an 8-dioxygenase by insertion of molecular oxygen, and to 7,8S-dihydroxylinoleate, through a hydroperoxide isomerase by intramolecular oxygen transfer. We have used liquid chromatography–mass spectrometry (LC–MS) with an ion trap mass spectrometer to study the MS^n mass spectra of the main metabolites of oleic, linoleic, ω-linolenic, and γ-linolenic acids, which are formed by Mn-LO and by LDS. The enzymes were purified from the culture broth (Mn-LO) and mycelium (LDS) of the fungus Gaeumannomyces graminis. MS^n analysis of hydroperoxides and MS^2 analysis of dihydroxy- and monohydroxy metabolites yielded many fragments with information on the position of oxygenated carbons. Mn-LO oxygenated C-11 and C-13 of 18:2n-6, 18:3n-3, and 18:3n-6 in a ratio of 1:1–3 at high substrate concentrations. 8-Hydroxy-9(10)epoxyeicosatrienoic acid was identified as a novel metabolite of LDS and oleic acid by LC–MS and by gas chromatography–MS. We conclude that LC–MS with MS^n is a convenient tool for detection and identification of hydroperoxy fatty acids and other metabolites of these enzymes.


Mass spectrometry (MS) has been extensively used for analysis of oxygenated metabolites of arachidonic and linoleic

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Abbreviations: APCI, atmospheric pressure chemical ionization; 7,8-DiHODE, 7,8-dihydroxy-9Z,12Z-octadecadienoic acid; 7,8-DiHOME, 7,8-dihomo-9Z,12Z-octadecadienoic acid; 7,8-DiHOTrE, 7,8-dihydroyx-9Z,12Z,15Z-octadecatrienoic acid; ESI, electrospray ionization; GC–MS, gas chromatography–mass spectrometry; HETE, hydroxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; 8-(H/P)ODE, 8R-hydroperoxyxy-9Z,12Z-octadecadienoic acid; 11H-(H/P)ODE, 11S-hydroperoxyxy-9Z,12Z-octadecadienoic acid; 13H-(H/P)ODE, 13S-hydroperoxyxy-9Z,11E-octadecadienoic acid; 8-(H/P)OME, 8R-hydroperoxyxy-9Z,12Z-octadecadienoic acid; 8-(H/P)OTrE(n-6), 11S-hydroperoxyxy-6Z,9Z,12Z-octadecatrienoic acid; 11H-(H/P)OTrE, 11S-hydroperoxyxy-9Z,12Z,15Z-octadecatrienoic acid; 13H-(H/P)OTrE, 13S-hydroperoxyxy-9Z,11E,15Z-octadecatrienoic acid; 13H-(H/P)OTrE(n-6), 13S-hydroperoxyxy-6Z,9Z,11E-octadecatrienoic acid; LC–MS, liquid chromatography–mass spectrometry; LDS, linoleate diol synthase; Mn-LO, manganese lipoxygenase; PGH, prostaglandin H; RP–HPLC, reversed phase–HPLC.

Mass spectrometry (MS) has been extensively used for analysis of oxygenated metabolites of arachidonic and linoleic acids, which are formed by lipoxygenases, prostaglandin H (PGH) synthases, and cytochromes P450. These metabolites are prostaglandins and thromboxanes, leukotrienes, lipoxins, hydroxyeicosatetraenoic acids (HETE), allene oxide, jasmonic acid, regioisomeric epoxides, vicinal diols, and hydroxy fatty acids (1–3). Mass spectrometric analysis of eicosanoids and lipoxins has mainly been performed by gas chromatography (GC)–MS, which provides structural information and can be used to quantify metabolites in minute amounts (4,5).

GC–MS has some drawbacks. It requires derivatization, and it is not suitable for direct analysis of many labile metabolites, e.g., hydroperoxy fatty acids. In recent years, liquid chromatography (LC)–MS has become a routine method thanks to robust ionization techniques, e.g., electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) (4,6). Many fatty acid metabolites have already been studied by LC–MS (4,7–13).

In this report we use LC–MS with MS^n to study the fatty acid metabolites of two unique enzymes: linoleate diol synthase (LDS) with ferriheme (15) and linoleic acid 13R-hydroperoxygenase (Mn-LO) with a manganese metal center (16). These enzymes were isolated from mycelium (LDS) and from the culture broth (Mn-LO) of the “take-all” fungus (Gaeumannomyces graminis), which is a devastating pathogen of wheat (14).

LDS converts linoleate sequentially to 8R-hydroperoxylinoleate (8-HPODE) through an 8-dioxygenase by insertion of molecular oxygen (17,18) and to 7,8S-dihydroxylinoleate (7,8-DiHODE) through a hydroperoxide isomerase by intramolecular oxygen transfer (19,20). LDS will also oxygenate oleic and ω-linolenic acids, but not γ-linolenic acid (18). The reaction mechanism of LDS has features in common with PGH synthases, e.g., formation of ferryl intermediates and a tyrosyl radical during catalysis (21).

Mn-LO metabolizes linoleic acid to 11S-HPODE and to 13R-HPODE (16,22). Its oxygenation mechanism thus differs from iron lipoxygenases (22). Mn-LO will also oxidize ω- and γ-linolenic acids but not oleic acid (16). The oxygenation of unsaturated C18 fatty acids by Mn-LO and by LDS is summarized in Table 1. These oxylipins have been characterized mainly by GC–MS (16–18,22).
Wheeler et al. (8) systematically performed low-energy fast atom bombardment tandem MS\(^2\) of monohydroxy substituted oleic, linoleic, linolenic, and arachidonic acids. ESI and tandem MS have also been used for MS\(^2\)-analysis of HETE and dihydroxyeicosatetraenoic acids, epoxieicosatrienoic acids, and certain hydroperoxides of linoleic and arachidonic acids (7,9–12). Structurally informative and abundant product ions were formed by fragmentation processes, which were influenced by the position of the hydroxy and epoxy groups and by the double bonds.

The first aim of the present study was to evaluate LC–MS with MS\(^n\) as a tool for analysis of hydroperoxy-, hydroxy- and dihydroxy-substituted linoleic acids, which are formed by two novel enzymes, Mn-LO and LDS. The interpretation of the mass spectra of the linoleate hydroperoxides was not straightforward without comparison with structurally related compounds. We therefore studied some of the metabolites in deuterated methanol/water and performed a systematic study of different unsaturated C\(_{18}\) fatty acid metabolites, which are formed by Mn-LO and LDS. MS\(^3\) spectra of hydroperoxy fatty acids and MS\(^2\) spectra of mono- and dihydroxy fatty acids were found to provide sufficient information for identification.

MATERIALS AND METHODS

Materials. Mn-LO and LDS were purified to homogeneity as described (15,16). The acids 18:1n-9 (99%) and 18:3n-3 (99%) were from Merck (Darmstadt, Germany); 18:2n-6 (99%) and 18:3n-6 (99%) were from Sigma (St. Louis, MO) and Nu-Chek-Prep (Elysian, MN), respectively. The labeled compounds [\(^{1,14}\)C]18:2n-6 (55 Ci/mol) and [\(^{1,14}\)C]18:1 (55 Ci/mol) were from Amersham Corp. (Amersham, United Kingdom). The compounds 8\(^\text{R}\)-HPODE, 8\(^\text{R}\)-HPOTrE, and 7\(^\text{S}\),8\(^\text{S}\)-DiHODE were prepared and characterized as described (18). Compounds 11\(^\text{S}\)-HPODE, 13\(^\text{R}\)-HPODE, 13\(^\text{R}\)-HPOTrE, 11\(^\text{S}\)-HPOTrE(n-6), and 13\(^\text{R}\)-HPOTrE(n-6) were prepared from biosynthesis with Mn-LO as described (16,22). Hydroperoxy fatty acids were reduced to alcohols with NaBH\(_4\) (16). C\(_7\)H\(_9\)O\(_2\)H (99.5%) and H\(_2\)O (99.5%) were from Merck.

**LC–MS analysis.** The pump for HPLC was from Thermo Separation Products (SpectraSYSTEM P2000; San Jose, CA) equipped with a degasser (Uniflow Degasys DG-1310, Tokyo, Japan). The column contained octadecasila cine (SepPak/C\(_{18}\); 23), the metabolites were either separated by thin-layer chromatography (18) or directly analyzed by LC–MS with a reversed-phase–high-performance liquid chromatography (RP–HPLC) column.

**RESULTS**

**General observation and nomenclature.** APCI–MS resulted in fragmentation of the hydroperoxides (M) with three strong signals, [M – 1]\(^+\), [M – 19]\(^+\), and [M – 17]\(^+\), which corresponded to the carboxylate anion of the hydroperoxide ([M – H\(^-\)]\(^+\)), a fragment due to loss of water from the carboxylate anion of the

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**TABLE 1**

Overview of the C\(_{18}\) Fatty Acid Metabolites Formed by Mn-LO and by LDS

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Mn-LO</th>
<th>LDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:0</td>
<td>Not a substrate</td>
<td>Not a substrate</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>Not a substrate</td>
<td>8(^\text{R})-HPOME, 7(^\text{S}),8(^\text{S})-DiHODE, 8-Hydroxy-9(10)epoxy-stearic acid*</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>11(^\text{S})-HPODE, 13(^\text{R})-HPODE</td>
<td>8(^\text{R})-HPODE, 7(^\text{S}),8(^\text{S})-DiHODE</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>11(^\text{S})-HPOTrE(^\text{b}), 13(^\text{R})-HPOTrE</td>
<td>8(^\text{R})-HPOTrE, 7(^\text{S}),8(^\text{S})-DiHOTrE</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>11(^\text{S})-HPOTrE(n-6), 13(^\text{R})-HPOTrE(n-6)</td>
<td>Not a substrate</td>
</tr>
</tbody>
</table>

*Identified in this study.

\(^{b}\)Identified in this study and tentatively in Reference 16. Other metabolites were identified by gas chromatography–mass spectrometry and chemical methods as described (Ref. 16,17). Arachidonic acid is oxygenated neither by LDS nor by Mn-LO (Refs. 16,17). Abbreviations: Mn-LO, manganese lipoxygenase; LDS, linoleate diol synthase; 8\(^\text{R}\)-HPODE, 8\(^\text{R}\)-hydroperoxy-9Z,12Z-octadecadienoic acid; 8\(^\text{R}\)-HPOTrE, 8\(^\text{R}\)-hydroperoxy-9Z,12Z-octadecadienoic acid; 13\(^\text{R}\)-HPODE, 13\(^\text{R}\)-hydroperoxy-9Z,12Z-octadecadienoic acid; 7\(^\text{S}\),8\(^\text{S}\)-DiHOTrE. 11\(^\text{S}\)-HPOTrE, 11\(^\text{S}\)-hydroperoxy-9Z,12Z-octadecadienoic acid; 13\(^\text{R}\)-HPODE, 13\(^\text{R}\)-hydroperoxy-9Z,12Z-octadecadienoic acid; 7\(^\text{S}\),8\(^\text{S}\)-DiHOTrE.

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Lipids, Vol. 33, no. 9 (1998)