Abstract

Colneleic acid (9-\{\{1\}'(E),3'(Z)-nonadienyloxy\}-8(E)-nonenoic acid) is produced from linoleic acid by the sequential action of 9-lipoxygenase and divinyl ether synthase.  We demonstrate that a small fraction of the colneleic acid in potato tubers is esterified in phospholipids. This colneleic acid was released by chemical hydrolysis and a phospholipase A\(_2\), but not by a lipase with 1-acyl specificity. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol contain molecular species with nominal masses consistent with identification as palmitoyl, colneleoyl species. Exact mass analysis of its fragments confirmed the identity of palmitoyl, colneleoyl phosphatidylinositol. To our knowledge, this work represents the first identification of a colneleoyl phospholipid.

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Key words: Colneleic acid; Potato; Oxylinip; Phospholipid; Mass spectrometry

1. Introduction

Colneleic acid (9-\{\{1\}'(E),3'(Z)-nonadienyloxy\}-8(E)-nonenoic acid or 9-oxa-8-trans,10-trans,12-cis-linoleic acid) has been detected in both the tubers and the leaves of potato [1,2]. The 9-hydroperoxide of linoleic acid is formed from linoleic acid by a 9-lipoxygenase, and this is converted to colneleic acid by a divinyl ether synthase [1,2]. Colneleic acid levels rise in potato leaves after Phytophthora infestans infection, and colneleic acid is believed to play a role in plant response to pathogen infection [2]. In potato tubers, suppression of the lipoxygenase, Lox1, that forms 9-hydroperoxides alters tuber development, but the role of colneleic acid in tubers is unclear [3].

Synthesis of colneleic acid, like that of other oxylinips, appears to be at least mainly carried out on free fatty acid substrates, although some plant lipoxygenases have the capability to act on glycerolipids as well as free fatty acids [4]. Recently it was discovered that another plant oxygenated fatty acyl species, 12-oxophytodienoic acid, is largely esterified in phospholipids. This colneleic acid was released by chemical hydrolysis and a phospholipase A\(_2\), but not by a lipase with 1-acyl specificity. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol contain molecular species with nominal masses consistent with identification as palmitoyl, colneleoyl species. Exact mass analysis of its fragments confirmed the identity of palmitoyl, colneleoyl phosphatidylinositol. To our knowledge, this work represents the first identification of a colneleoyl phospholipid.

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2. Materials and methods

2.1. Extraction of potato lipids

Potato tubers (Bintje) (750 g) were frozen in liquid nitrogen and ground to a powder. The powder was added to 1.2 l of isopropanol at 75°C, containing 0.01% butylated hydroxytoluene. After 15 min at 75°C, 600 ml of chloroform and 500 ml of water were added. The mixture was agitated for 30 min at room temperature. The potato powder was re-extracted three times with chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. The solvent was pooled and then washed with 1 l of 1 M KCl. The organic phase was filtered and the solvent was evaporated to dryness in a rotary evaporator.

2.2. Isolation of potato lipid PI-enriched fraction (fraction 5)

The lipid (7.1 g) was dissolved in chloroform and applied to a 20 g silica acid column, packed in chloroform. The column was eluted sequentially with the same volume (15 ml) of chloroform or 300 ml of each of the following: (1) chloroform, (2) acetone, (3) chloroform/methanol (95:5, v/v), (4) chloroform/methanol/ether (80:20:5, v/v); (5) chloroform/methanol (50:50, v/v); (6) methanol. This sequence of elution solvents was described by Christie [6]. The solvent was evaporated from fraction 5 with a rotary evaporator. Fractions 1, 2, and 3 were analyzed by thin layer chromatography (TLC) on silica gel in petroleum ether (60–95°C)/diethyl ether/formic acid (6:4:1.5) and detected with iodine vapor. Fractions 4, 5, and 6 were analyzed with chloroform/methanol/acetic acid/water (8:5:1.5:1.0) and detected with iodine vapor.

2.3. Lipase treatment of fraction 5

To release the fatty acyl groups in the 1-position of the phospholipids in fraction 5, 40 mg of lipid was dispersed in 1 ml of 50 mM Tris-HCl, pH 7.5. An aliquot (50 μl) of the lipase solution was added to the lipid dispersion, and the mixture was incubated for 2 h at 37°C. To release the fatty acyl groups in the 2-position of the phospholipids in fraction 5, 40 mg of lipid was dispersed in 1 ml of 50 mM Tris-HCl, pH 8.9. 100 mg of Streptomyces violaceoruber phospholipase A\(_2\) (Sigma-P-8685, 10 U/mg) was dissolved in 1 ml of 50 mM Tris-HCl, pH 8.9. An aliquot (90 μl) of the phospholipase solution was added to the lipid dispersion, and the mixture was incubated for 2 h at 37°C. Each of two reaction mixtures (1-acyl lipase and the phospholipase A\(_2\)) was extracted for 10 min at room temperature with 2 ml of diethyl ether, and re-extracted the same way two more times. The ether was evaporated from the combined extracts of each lipase reaction, and the extracts were re-dissolved in 200 μl acetonitrile.

2.4. Chemical hydrolysis of fraction 5

120 mg of fraction 5 was dissolved in 3 ml of 2 N NaOH in methanol/water (3:1, v/v). Sodium was bubbled through the sample. The solution was heated 1 h at 70°C. 9 ml of water was added, and the pH was lowered to 1 with 4 N HCl. The mixture was extracted three times with 10 ml of diethyl ether, each time for 10 min at room temperature.
temperature. The ether was evaporated, and the residue was dissolved in 100 μL of acetonitrile and filtered.

2.5. Extraction of potato tubers and addition of standard for free colneleic acid determination

15-Hydroxy-11,13-eicosadienoic acid (15-HEDE, internal standard) (400 μL of 1.8×10^−8 M) was added to 5 g of potato tuber powder. The powder was extracted with 15 mL of diethyl ether at room temperature for 10 min. The powder was re-extracted with 10 mL of diethyl ether for 10 min at room temperature and the ether fractions were pooled and dried with anhydrous sodium sulfate. The solvent was evaporated to dryness under nitrogen and the extracted compounds were redissolved in 100 μL of acetonitrile. A blank was made with the same protocol omitting the potato powder.

2.6. Colneleic acid determination by HPLC

15-HEDE was used as an internal standard. The analysis was performed with a Hewlett Packard series 1050 high performance liquid chromatography (HPLC) (with diode array detector) on an Inertsil C18 × 4.6 mm 5 ODS-2 column (Chrompack, Middelburg, The Netherlands) with a flow rate of 0.7 mL/min. Solvent A was water (0.01% v/v trifluoroacetic acid); solvent B was acetonitrile (0.01% v/v trifluoroacetic acid). Elution conditions were: 0–61 min, 47% solvent A; 61–63 min, a gradient from 47% A to 20% A; 63–74 min, 20% A; 74–75 min, from 20% A to 0% A; 75–95 min, 100% B. For re-equilibration, the flow was continued from 95 to 100 min with a gradient from 0% A to 47% A, and from 100 to 105 min at 47% A. Identification of colneleic acid was based on its UV spectrum and retention time by comparison with the pure compound synthesized, extracted, purified by preparative HPLC, derivatized, and characterized by gas chromatography-mass spectrometry (MS) analysis according to Rusterucci et al. [7].

2.7. MS/MS precursor ion scanning

Potato extract in chloroform/methanol/300 mM ammonium acetate in water (300:665:35) was analyzed by electrospray ionization tandem MS (ESI-MS/MS), as previously described [8].

2.8. Q-TOF mass spectral analysis

The solvent was evaporated from fraction 5, and fraction 5 was dissolved in chloroform/methanol (2:1, v/v). The sample was diluted so that the final composition of the solvent for mass spectrometry was chloroform/methanol/300 mM ammonium acetate in water (300:665:35). This sample was infused at 30 μL/min into the ESI source of a Micromass Q-Tof-2 ESI (Micromass, Manchester, UK) operated in the negative mode as total number of acyl carbons/total number of double bonds. Note that the spectrum in the range of m/z 845–867 is expanded 20-fold in relation to the rest of the spectrum. The species at 847.6 was analyzed further by MS/MS.

Transmit a monoisotopic species at 847.6 and product ions were collected for 5 min of acquisition time to increase signal to noise. Collision gas was the same as for MSI acquisition, and the collision energy was varied during the 5 min from 25 to 45 eV to obtain a good distribution of product ions. Acyl carboxylate ions were observed with maximum yields at 35 V. All TOF spectra were acquired with the daily mass calibration, and collision-induced dissociation spectra were mass corrected with a fragment common to PIs, C6H14O6P1 (297.0375).

3. Results

Crude fractionation of potato extracts by silicic acid column chromatography resulted in six lipid fractions with increasing polarity. TLC analysis demonstrated that fraction 1 contained simple lipids, fraction 2 contained galactolipids, and fraction 3 contained phosphatidic acid. Fractions 4 and 5 were the two major phospholipid fractions. TLC, as well as mass spectrometric analysis, showed that fraction 5, which was eluted with chloroform/methanol (50:50), contained only phospholipids, including PI and some phosphatidylycer-

<table>
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<th>Formula</th>
<th>Theoretical mass for formula</th>
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<th>Width of peak (amu fwhh)</th>
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<td>293.2817</td>
<td>See Fig. 3</td>
<td>0.074 (see Fig. 3)</td>
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Fig. 1. ESI-MS of silicic acid column fraction 5 in the range of m/z 825 to 875. This fraction is enriched in PI, and all of the peaks in this mass range correspond to PI species. The species are labeled as total number of acyl carbons/total number of double bonds.
line (PC) and phosphatidylethanolamine (PE), although fraction 4 was more enriched in PE. The most abundant negatively charged ions produced by electrospray of fraction 5 were PIs. Chemical hydrolysis of 120 mg of fraction 5 released 3.0 nmol of colneleic acid (25 pmol/mg fraction 5), indicating that a small amount of this oxylipin was incorporated in a phospholipid or phospholipids. Treatment of fraction 5 with a 1-acyl-specific lipase did not release detectable colneleic acid, but treatment of 40 mg of fraction 5 with phospholipase A2 released 0.9 nmol colneleic acid (22 pmol/mg fraction 5), indicating that the colneleic acid was esterified to a phospholipid or phospholipids in the 2-position.

To identify phospholipid species containing colneleic acid, lipid profiles of unfraccionated potato extracts were obtained by ESI-MS/MS precursor scanning. Scans for the precursors of headgroup-specific fragments yield spectra of each headgroup class [8,9]. The spectra indicated that several classes of lipids contained minor peaks with masses that corresponded to the masses of a 16:0-colneleyl pair. The phospholipid species detected included positively charged ions of PC at mz 772 and PE at mz 730 and a negatively charged PI ion at mz 847. Because few other components of the potato lipid mixture have masses near the mass of the PI species, this species was chosen for fragment analysis to determine the structure of its acyl components.

To analyze the acyl structure of the PI at mz 847, fraction 5 from the silicic acid chromatography was utilized. Fraction 5 was enriched in PI and a mass spectrum of fraction 5 in the mass range 825–875 showed that the components detected in this mass range corresponded to various PI species (Fig. 1). Fragmentation in the negative mode of the peak at mz 847 with the Micromass Q-Tof produced several fragments (Fig. 2). Exact mass analysis of the fragments indicated that five of the fragments were derived from the PI headgroup [10] (Table 1). No fragments of lipids from other headgroup classes were identified in the material with mz 847. Four fragments were compatible with identification as acyl species. One acyl combination included species at mz 269 and mz 279, which corresponded to heptadecanoic (17:0) acid and linoleic (18:2) acid, respectively. The second acyl combination included species at mz 255 and mz 293. The peak at mz 255 corresponded to palmitic (16:0) acid. Careful analysis of the peak near mz 293 showed that this peak was wider than the other peaks and the mass corresponding to the center of the peak did not correspond exactly to the mass of a reasonable chemical formula for the fragment. Instead, the mass of the center of the peak was between the mass of colneleic acid and that of nonadecadienoic (19:2) acid. Modeling of this peak indicated that this peak contained both species (Fig. 3).

On the other hand, the data demonstrate that colneleic acid was specifically esterified to the 2-position of the phospholipids. Colneleic acid is an ether lipid species found in plants. To our knowledge, colneleoyl phospholipids have not been identified previously. However, phospholipids containing epoxyeicosatrienoic acids have been identified in the 2-position of PI and PE from rat liver [11]. Recently, Stelmach and coworkers [5] showed that the bulk of 12-oxophytodienoic acid in Arabidopsis rosettes is esterified in 1-(12-oxophytodienoyl),2-(hexadecatrenoyl) monogalactosyldiacylglycerol. Lipase treatment released 1–2 μg of 12-oxophytodienoic acid per g Arabidopsis fresh weight. In wounded leaves, the levels of 12-oxophytodienoyl-containing monogalactosyldiacylglycerol reached 20 μg/g fresh weight. In contrast, only about 2 ng/g potato fresh weight was freed from the phospholipids of silicic acid column fraction 5. Since fraction 5 represented a sizable fraction of total potato phospholipids and since potato tubers contained about 3 μg (10 nmol) colneleic acid per g potato fresh weight, the data suggest that colneleoyl-containing phospholipids accounted for a small fraction of the total colneleic acid pool.

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4. Discussion

This work describes the first identification of a colneleoyl phospholipid in vivo and demonstrates that colneleic acid is esterified to the 2-position of the phospholipids. Colneleic acid is an ether lipid species found in plants. To our knowledge, colneleoyl phospholipids have not been identified previously. However, phospholipids containing epoxyeicosatrienoic acids have been identified in the 2-position of PI and PE from rat liver [11]. Recently, Stelmach and coworkers [5] showed that the bulk of 12-oxophytodienoic acid in Arabidopsis rosettes is esterified in 1-(12-oxophytodienoyl),2-(hexadecatrenoyl) monogalactosyldiacylglycerol. Lipase treatment released 1–2 μg of 12-oxophytodienoic acid per g Arabidopsis fresh weight. In wounded leaves, the levels of 12-oxophytodienoyl-containing monogalactosyldiacylglycerol reached 20 μg/g fresh weight. In contrast, only about 2 ng/g potato fresh weight was freed from the phospholipids of silicic acid column fraction 5. Since fraction 5 represented a sizable fraction of total potato phospholipids and since potato tubers contained about 3 μg (10 nmol) colneleic acid per g potato fresh weight, the data suggest that colneleoyl-containing phospholipids accounted for a small fraction of the total colneleic acid pool.

On the other hand, the data demonstrate that colneleic acid was specifically esterified to the 2-position of the phospholipids in a fraction enriched in PI. In PI, as in other phospholipids synthesized by the plant extra-plastidic pathway, the 2-position is likely to contain nearly all 18-carbon fatty acids, such as linoleic acid [12]. The localization of colneleic acid to the 2-position of the phospholipids implies that the colneleoyl phospholipids were generated enzymatically, but it is unclear whether the colneleic acid was generated from linoleic acid while esterified to the phospholipid or whether colneleic acid was incorporated into the phospholipid fraction after synthesis from free linoleic acid. 9-Lipoxygenase activity in potato tubers apparently occurs in the cytoplasm [4,13,14]. Since PC, PE, and PI are found primarily in extra-plastidic locations, the cytoplasmic location of the lipoxygenase leaves open the possibility that this enzyme might occasionally use an intact phospholipid as a substrate in vivo. Thus, it is possible that colneleoyl phospholipid were ‘accidental’ products of enzymes.
that are promiscuous toward their substrates. On the other hand, colneleoyl phospholipids may play an as yet unknown role in tuber physiology.

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References