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THE BIOSYNTHESIS OF JASMONIC ACID: A PHYSIOLOGICAL ROLE FOR PLANT LIPOXYGENASE[1]

Brady A. Vick and Don C. Zimmerman

U. S. Department of Agriculture, ARS
Department of Biochemistry
North Dakota State University
Fargo, North Dakota 58105

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Linolenic acid was converted to a cyclic product, 12-oxo-phytodienoic acid, by lipoxygenase and hydroperoxide cyclase enzymes present in Vicia faba pericarp. Isotope labeling studies in which [U-^{14}C] 12-[^{18}O] oxo-phytodienoic acid was incubated with thin sections of pericarp tissue showed that 12-oxo-phytodienoic acid is a biosynthetic precursor to jasmonic acid, a plant growth regulator which promotes senescence. Key enzymes proposed for this pathway are a reductase enzyme which reduces a double bond in the cyclopentenone ring, and β-oxidation enzymes which remove six carbons from the carboxyl end of the molecule.

Lipoxygenase (EC 1.13.11.12) catalyzes the oxygenation of polyunsaturated fatty acids which contain a cis,cis-1,4-pentadiene system. Although the presence of lipoxygenase in most plants is well established (1), its physiological function has remained an enigma. The product of the enzymic reaction, a polyunsaturated fatty acid hydroperoxide, is a highly reactive species with potentially pernicious effects on cellular components. We reported previously that a wide variety of plants possess a hydroperoxide cyclase enzyme which converts 13-hydroperoxylinolenic acid to an 18-carbon fatty acid containing a cyclopentenone ring (2,3). The product, 3-oxo-2-(2-pentenyl)-4-cyclopentene-octanoic acid, was given the common name 12-oxo-phytodienoic acid.

Recently there has been much interest in the effects of jasmonic acid and methyl jasmonate, natural products isolated from a number of plant sources, on the promotion of plant senescence (4,5,6,7,8). The similarity in structure

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between 12-oxo-PDA[2] and jasmonic acid prompted us to investigate whether 12-oxo-PDA can serve as a biosynthetic precursor to jasmonic acid.

MATERIALS AND METHODS

Chemicals. Linolenic acid was purchased from Nu Chek Prep, Inc.[3], Elysian, MN and soybean lipoxygenase was a product of Sigma Chemical Company, St. Louis, MO. [1,2-^{14}C]Sodium acetate (54 mCi/mmol) was obtained from ICN, Irvine, CA and $^{18}O_2$ gas (>99%) was from Stohler Isotope Chemicals, Waltham, MA. TLC separations were accomplished with Anasil HF precoated chromatography plates obtained from Analabs, New Haven, CT, and with $LKC_{18}F$ reversed phase plates purchased from Whatman Inc., Clifton, NJ. Except where noted, normal phase TLC plates were developed three times in hexane-diethyl ether-acetic acid (50:50:1, v/v/v) and reversed phase plates were developed once in acetonitrile-water-acetic acid (95:5:1, v/v/v). Octadecyl(C_{18}) disposable extraction columns (3-ml) were obtained from J.T. Baker Chemical Company, Phillipsburg, NJ.

Authentic methyl jasmonate for mass spectral comparison was the generous gift of Dr. Gunther Ohloff, Firmenich SA, Geneva, Switzerland. Authentic 3-oxo-2-(2-pentenyl)-cyclopentaneoctanoic acid was synthesized from 6 mg of 12-oxo-PDA by reduction with 20 mg of sodium borohydride in 2 ml of dimethoxyethane for 30 min, followed by oxidation of the resulting hydroxyl group to a carbonyl by reacting it for 48 h with 20 mg of pyridinium dichromate in 2 ml of dimethylformamide. The product, purified by normal phase TLC, retained the proper stereoconfiguration (cis) of the side chains with respect to the ring.

Growth Conditions. Vicia faba L. cv. Windsor seeds were sown in pots and covered with 3 cm of soil. The seeds were placed in a growth chamber at 25°C with a 16-h light, 8-h dark cycle. Pods were harvested when they reached 4 to 6 cm and weighed 1.5 to 3.0 g. Flax (Linum usitatissimum L. cv. Linnott) was either grown in the field or in pots in a greenhouse with supplemental lighting.

Determination of Vicia faba Lipoxygenase Positional Specificity. The 13:9 positional specificity with linolenic acid was determined at pH 6.0 by a previously reported method (9) with a lipoxygenase preparation partially purified on a 2.6 x 33 cm Sephadex G-200 column. The enzyme preparation was free of hydroperoxide isomerase or hydroperoxide cyclase activity.

Synthesis of 12-oxo-PDA from Linolenic Acid and an Extract of Vicia faba Pericarp. Vicia faba pericarp (3.1 g) was homogenized in a mortar and pestle with 10 ml of 50 mM K-phosphate buffer (pH 6.5) containing 0.1% Triton X-100, and then was centrifuged at 12,000g. Three ml of the extract and 6 ml of an 8 mM linolenic acid solution prepared according to Surrey (10) were added to 120 ml of 50 mM K-phosphate buffer (pH 7.0). After 1 h the mixture was adjusted to pH 4 with 1 M citric acid, passed through an octadecyl(C_{18}) extraction column, and the products were eluted with diethyl ether. The concentrated products were separated by reversed phase TLC, eluted with diethyl ether, esterified with diazomethane, and subjected to GC-MS analysis.

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[2] Abbreviations: 12-oxo-PDA, 12-oxo-cis,cis-10,15-phytodienoic acid; TLC, thin layer chromatography; GC-MS, gas chromatography-mass spectrometry.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Preparation of [U-^{14}C]Linolenic Acid. Uniformly labeled linolenic acid was prepared according to a method adapted from Dybing and Craig (11). Eight flax embryos at 14 days after flowering were placed in a covered 1.8-cm dish lined with filter paper, which was moistened with 50 mM K-phospahte buffer (pH 6.0) containing 1 µg/l biotin. A solution of 10 µCi of [1,2-^{14}C]sodium acetate in 25 µl of the buffer-biotin solution was applied to the embryos, and after 4 h of incubation the embryos were rinsed with water and homogenized in a mortar and pestle with diethyl ether-methanol solvent (95:5, v/v). After centrifugation, the solvent was removed under a stream of N_2 and the lipid material was saponified for 45 min at 80°C in 2 ml of 1 N KOH in 95% ethanol. Five ml of water was added, the solution was acidified to pH 3 with concentrated HCl, and the fatty acids were extracted into diethyl ether. The concentrated ether extract was applied to reversed phase TLC plates, developed twice in the chromatography solvent, and the purified linolenic acid was eluted from the plate with diethyl ether. The specific activity was estimated to be 0.4 mCi/mmol.

Preparation of Isotope-labeled 12-Oxo-PDA. A solution (100 µl) of 2 µCi (1.4 mg) of [U-^{14}C]linolenic acid in 95% ethanol was added to 24 ml of distilled water. When $^{18}O_2$ was to be incorporated into 12-oxo-PDA, the reactants were placed in a culture tube containing $^{18}O_2$ gas and sealed with a septum. The oxygenation reaction was initiated by the introduction of 1.28 ml of soybean lipoxygenase solution at a concentration of 1 mg/ml in 10 mM borate buffer (pH 9). After 20 min at 22°C, the pH was adjusted to 7 with 4 ml of 0.2 M K-phosphate (pH 7.0). The cyclization reaction was begun by allowing the 13-hydroperoxylinolenic acid formed to react for 30 min with 0.64 ml of a flaxseed acetone powder extract (1 g in 10 ml of 50 mM K-phosphate buffer, pH 7). The reaction mixture was acidified with 0.2 ml of 1 M citric acid, passed through an octadecyl extraction column, and the lipid products were eluted with 2 ml of diethyl ether. Purification of [^{14}C]12-oxo-PDA was accomplished by reversed phase TLC, then by normal phase chromatography, and finally by rechromatography on the reversed phase plates. A pure product with approximately 350,000 dpm (125 µg) could usually be obtained by this method.

Reaction of Isotope-labeled 12-Oxo-PDA with the Pericarp of Vicia faba. Approximately 125 µg of labeled 12-oxo-PDA was dispersed by sonication into 400 µl of 1 mM dithiothreitol and 0.5 M sucrose in 50 mM K-phosphate buffer (pH 7.0). The solution was placed in a 2.5-cm dish and nine 0.5-mm transverse sections of Vicia faba pericarp with seeds removed were added. After 2.5 h at 22°C the solution and sections were extracted by the method of Bligh and Dyer (12). The concentrated extract was partially purified by reversed phase TLC. The radioactive area of the plate, determined by autoradiography, was removed and eluted with diethyl ether. After a second partial purification by normal phase chromatography, the products were eluted from the plate and esterified with diazomethane. The products were analyzed by gas chromatography on line with a Packard Model 894 gas proportional counter or with a mass spectrometer. For gas chromatography with the gas proportional counter, a 1-m glass column (2 mm i.d.) packed with 3% SP-2100 on 80/100 mesh Supelcoport was temperature programmed from 130 to 210°C at 5°C per min. Mass spectrometry was accomplished with a Hewlett-Packard 5992 GC-MS with a 12-m SP-2100 fused silica capillary column (0.2 mm i.d.) which was temperature programmed from 110 to 210°C at 5°C per min.

RESULTS AND DISCUSSION

A regiospecificity study of partially pure lipoxygenase from Vicia faba pericarp showed that 92% of the product was 13-hydroperoxylinolenic acid and 8% was the 9-hydroperoxide isomer. When linolenic acid was reacted with a crude extract of pericarp, one of the products identified by GC-MS was

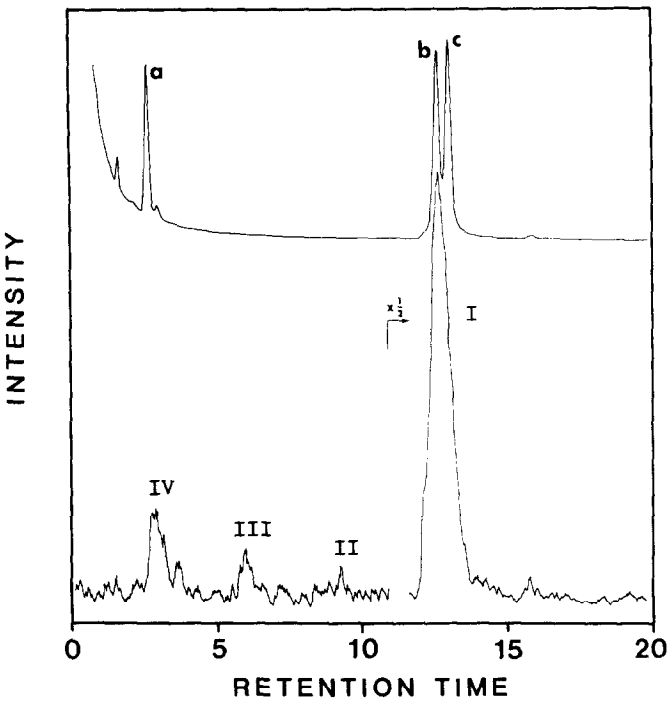

Figure 1. Upper: gas chromatography of authentic reference compounds; (a) methyl jasmonate, (b) methyl 3-oxo-2-(2-pentenyl)-cyclopentaneoctanoic acid, and (c) methyl 12-oxo-PDA. Lower: ^{14}C detection by gas chromatography of the methyl esters of the products which resulted when [U-^{14}C]12-oxo-PDA was incubated with thin slices of Vicia faba pericarp.

12-oxo-PDA. The mass spectrum, with characteristic fragments at m/z 306 $[M]^{+}$, 275, 238, and 206, was identical to that reported by Zimmerman and Feng (3). The presence of 12-oxo-PDA as a reaction product confirmed the presence of a hydroperoxide cyclase enzyme in Vicia faba.

When thin transverse sections of Vicia faba pericarp were incubated with [U-^{14}C]12-oxo-PDA, four radioactive metabolites could be separated as their methyl esters by gas chromatography and detected with a gas proportional counter (Fig. 1). The predominant product, metabolite I, had a retention time equal to that of authentic methyl 3-oxo-2-(2-pentenyl)-cyclopentaneoctanoate, while the retention time of metabolite IV corresponded to that of authentic methyl jasmonate.

In another experiment [U-^{14}C]12-[^{18}O]oxo-PDA was used as the substrate, and the metabolites were analyzed by GC-MS. Mass spectra for metabolites I,

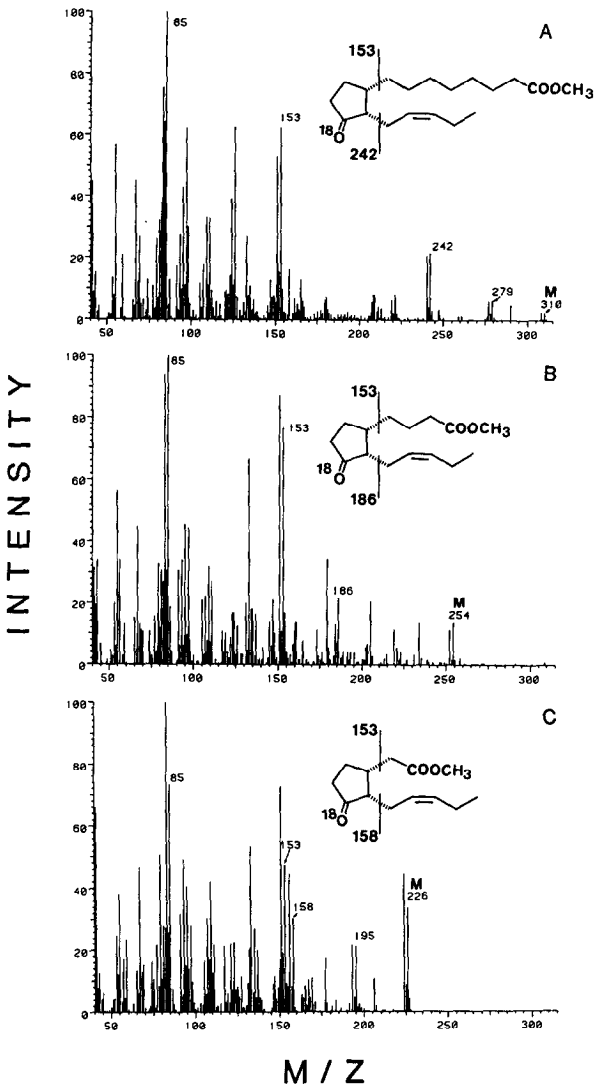

Figure 2. Mass spectra of the methyl esters of metabolites I, III, and IV which resulted when 12-[^{18}O]oxo-PDA was incubated with thin slices of Vicia faba pericarp. (A) Metabolite I, methyl 3-oxo-2-(2-pentenyl)-cyclopentaneoctanoate, (B) metabolite III, methyl 3-oxo-2-(2-pentenyl)-cyclopentanebutanoate, and (C) metabolite IV, methyl 3-oxo-2-(2-pentenyl)-cyclopentaneacetate (methyl jasmonate).

III, and IV are shown in Figure 2. The results show that the ^{18}O atom of 12-oxo-PDA was incorporated into all three metabolites. Characteristic mass fragments at m/z 310[M]$^{+}$, 279, 242, 153, and 85 for metabolite I showed clearly that it resulted from the reduction of the ring double bond of 12-oxo-PDA. Metabolite I was identified as 3-oxo-2-(2-pentenyl)-cyclopentaneoctanoic acid. The mass spectrum of authentic methyl 3-oxo-2-(2-pentenyl)-

cyclopentaneoctanoate showed the same fragmentation pattern (2 mass units lower) at m/z 308$[M]^{+}$, 277, 240, 151, and 83.

The mass spectrum of metabolite III indicated the loss of four carbons from the carboxyl chain of metabolite I. It was identified as 3-oxo-2-(2-pentenyl)-cyclopentanebutanoic acid. The mass spectrum of metabolite IV showed that six carbons had been lost from metabolite I. The mass fragments, m/z 226$[M]^{+}$, 195, 158, 153, and 85, were analogous (2 mass units higher) to those of authentic methyl jasmonate, m/z 224, 193, 156, 151, and 83. Therefore, IV was characterized as 3-oxo-2-(2-pentenyl)-cyclopentaneacetic acid (jasmonic acid). We were unable to detect the presence of metabolite II in the experiment with $^{18}O_2$, but in other experiments that used 12-[^{16}O]oxo-PDA as the substrate we were able to detect its presence by the mass fragments at m/z 280$[M]^{+}$, 249, 212, 151, and 83. Metabolite II was characterized as 3-oxo-2-(2-pentenyl)-cyclopentanehexanoic acid.

The proposed enzymic pathway for the synthesis of jasmonic acid from linolenic acid is shown in Figure 3. There were two isomers present for each metabolite in the GC-MS analysis of I, II, and III. These isomers correspond to the cis and trans configurations of the side chain with respect to the ring. We believe that the cis form is the natural stereoisomer and that the trans form results from isomerization during purification or in the injector during gas chromatography (13). The natural form of jasmonic acid (IV) is most likely cis. The projections shown in Figure 3 refer to the relative stereochemistry of the side chains, and not to the absolute configurations R or S, which are not known.

Each spectrum in Figure 2 shows the presence of approximately equimolar ^{16}O in the metabolites, even though unreacted 12-[^{18}O]oxo-PDA showed no exchange and contained >90% ^{18}O (spectrum not shown). Although ^{16}O exchange could have occurred during the enzymic reduction step, a more likely explanation is that the ^{16}O metabolites were synthesized from 12-[^{16}O]oxo-PDA which arose enzymically from linolenic acid and O_2 as a wound response when the

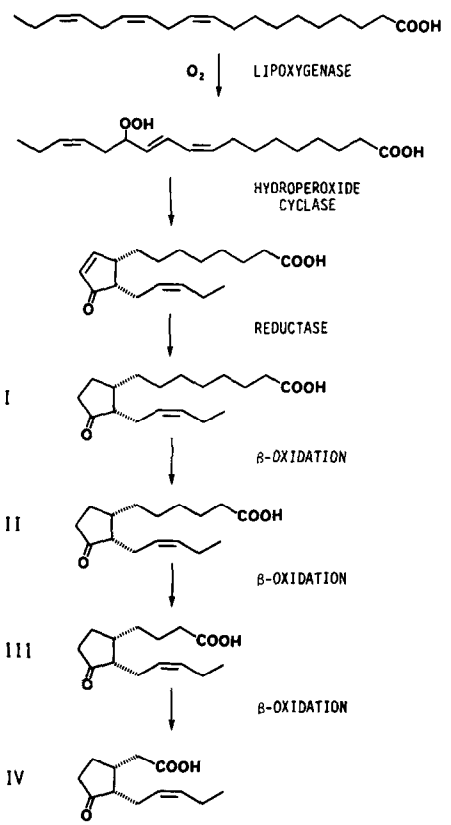

Figure 3. The proposed biosynthesis of jasmonic acid from linolenic acid. The projections shown here represent the cis and trans configurations of the side chains with respect to the ring rather than the R or S configurations, which are not known.

Vicia faba sections were sliced. This type of wound response has been shown in corn seedlings (14).

It is noteworthy that the physiological role of plant lipoxygenase has remained elusive despite the generous attention it has received over the past 50 years. The results presented here suggest that lipoxygenase plays a key role in metabolic regulation by initiating the reactions leading to jasmonic acid, a compound known to have growth regulating properties.

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