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BIOSYNTHESIS OF 12-OXO-10,15(Z)-PHYTODIENOIC ACID: IDENTIFICATION OF AN ALLENE OXIDE CYCLASE*

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Incubation of 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid with corn (Zea mays L.) hydroperoxide dehydrase led to the formation of an unstable allene oxide derivative, 12,13(S)-epoxy-9(Z),11,15(Z)-octadecatrienoic acid. Further conversion of the allene oxide yielded two major products, i.e. α-ketol 12-oxo-13-hydroxy-9(Z),15(Z)-octadecadienoic acid, and 12-oxo-10,15(Z)-phytodienoic acid (12-oxo-PDA). 12-Oxo-PDA was formed from allene oxide by two different pathways, i.e. spontaneous chemical cyclization, leading to racemic 12-oxo-PDA, and enzyme-catalyzed cyclization, leading to optically pure 12-oxo-PDA. The allene oxide cyclase, a novel enzyme in the metabolism of oxygenated fatty acids, was partially characterized and found to be a soluble protein with an apparent molecular weight of about 45,000 that specifically catalyzed conversion of allene oxide into 9(S),13(S)-12-oxo-PDA.

In recent work from our laboratory, formation of allene oxide derivatives from unsaturated fatty acid hydroperoxides has been described. Thus, corn (Zea mays, L.) hydroperoxide dehydrase was found to catalyze the conversion of 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid (13(S)-HPOD) into the allene oxide 12,13(S)-epoxy-9(Z),11-octadecadienoic acid (12,13(S)-EOD), which was characterized by chemical and physical methods (1). 9(S)-HPOD underwent an analogous type of transformation into allene oxide 9(S),10-EOD (2). The allene oxides were rapidly hydrolyzed ($t_{1/2}$, 33-34 sec at 0°) in aqueous

*Part no. IV in a series of papers dealing with the chemistry and biochemistry of fatty acid allene oxides.

Abbreviations: 13(S)-HPOD, 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid; 13(S)-HPOT, 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid; 9(S)-HPOD, 9(S)-hydroperoxy-10(E),12(Z)-octadecadienoic acid; 12,13(S)-EOD, 12,13(S)-epoxy-9(Z),11-octadecadienoic acid; 9(S),10-EOD, 9(S),10-epoxy-10,12(Z)-octadecadienoic acid; 12,13(S)-EOT, 12,13(S)-epoxy-9(Z),11,15(Z)-octadecatrienoic acid; 12-oxo-PDA, 12-oxo-10,15(Z)-phytodienoic acid.

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medium into stable α-ketols, which had been isolated in earlier work (3,4). Confirmation of the formation and structures of fatty acid allene oxides was very recently provided by Brash et al., who isolated the methyl esters of a number of allene oxides and characterized them by eg. NMR spectrometry (5).

12-Oxo-10,15(Z)-phytodienoic acid (12-oxo-PDA) and pre-clavulone A are cyclopentenone derivatives which are formed from fatty acid hydroperoxides in plants and lower marine organisms, respectively. Allene oxides have been proposed to serve as the immediate precursors of 12-oxo-PDA and pre-clavulone A (6-10), eg. because of the well established chemical conversion of allene oxides into cyclopentenones (11,12), and because of the fact that formation of 12-oxo-PDA and pre-clavulone A is accompanied by the formation of α-ketols, non-enzymatic hydrolysis products of allene oxides. Direct evidence for the concept of allene oxides serving as precursors of cyclopentenones of plant and marine origin was recently provided by the finding that the allene oxide 12,13(S)-EOD, in the presence of bovine serum albumin, underwent cyclization into a cyclopentenone related to 12-oxo-PDA (9), and by the finding that degradation of the methyl ester of 12,13(S)-EOT in buffer led to formation of the methyl ester of 12-oxo-PDA (5).

The present paper is concerned with the mechanism of formation of 12-oxo-PDA and, specifically, with the presence in corn homogenate of a soluble allene oxide cyclase that catalyzes the conversion of the allene oxide 12,13(S)-EOT into optically pure 12-oxo-PDA.

EXPERIMENTAL

[1-^{14}C]13(S)-HPOD and [1-^{14}C]13(S)-HPOT (specific radioactivities, 1.4 kBq/μmol and 6.3 kBq/μmol, respectively) were prepared as described (13). Pronase was purchased from Sigma Chemical Co.

Defatted meal of corn (Bear X8632, Noble Bear Hybrid Corn, Inc., Decatur, IL) was homogenized in 0.1 M potassium phosphate buffer pH 6.7 (1:5, w/v) at 0° with a Polytron. The homogenate was centrifuged at 9,300xg for 15 min and the resulting supernatant was centrifuged at 105,000xg for 60 min. The 105,000xg supernatant was removed and kept on ice. The 105,000xg particle fraction was either used directly, or washed by resuspension in buffer and recentrifugation at 105,000xg. The following enzyme preparations were used for the incubations: 105,000xg supernatant (1.7 mg protein/ml), 105,000xg particle fraction resuspended in buffer (1.0 mg protein/ml), washed 105,000xg particle fraction in buffer (1.0 mg protein/ml), and washed 105,000xg particle fraction resuspended in 105,000xg supernatant (particle protein, 1.0 mg/ml, soluble protein, 1.7 mg/ml). Endogenous 12-oxo-PDA was undetectable (less than 0.2 μM) in the preparations used.

Hydroperoxide dehydrase activity was determined by a spectrophotometric method in which dilutions of enzyme preparations were treated with either 13(S)-HPOD or 13(S)-HPOT in a cuvette at 20°. The initial rate of decrease in absorption at 236 nm was monitored.

Incubations of [1-^{14}C]13(S)-HPOT were carried out at 0°. In trapping experiments, 10-150 μM 13(S)-HPOT was incubated with 10 ml of enzyme preparation (either 105,000xg supernatant or 105,000xg particle fraction) and 1 ml aliquots were removed at 15, 30, 45, and 60 sec and added to 20 ml of methanol. In studies of 12-oxo-PDA formation, 3-150 μM 13(S)-HPOT was incubated with 10 ml of enzyme preparation (washed particle fraction, resuspended in either buffer or 105,000xg super-

natant) for 10 min and added to 30 ml of methanol. When using low concentrations of substrate, several such incubations were pooled in order to produce sufficient amounts of 12-oxo-PDA for identification and for steric analysis. The incubation mixtures were acidified and extracted twice with diethyl ether. The esterified product was subjected to TLC (solvent system, ethyl acetate/toluene (15:85, v/v)).

Steric analysis of 12-oxo-PDA as well as the instrumental methods used were as described in detail (9,14).

RESULTS

Detection of 12,13(S)-epoxy-9(Z),11,15(Z)-octadecatrienoic acid. Corn homogenates were fractionated by differential centrifugation and hydroperoxide dehydrase activity was measured by a spectrophotometric method using 13(S)-HPOD as the substrate (initial concentration, 34 μM). Dehydrase was present in the 105,000xg particle and supernatant fractions, catalyzing the consumption of 1.9 and 0.2 μmol of 13(S)-HPOD/min·mg of protein, respectively.

As expected from recent work (1), the product formed from 13(S)-HPOT by action of hydroperoxide dehydrase was an unstable allene oxide, 12,13(S)-epoxy-9(Z),11,15(Z)-octadecatrienoic acid (12,13(S)-EOT). The structure of this compound was established as described for the related allene oxide 12,13(S)-EOD (1). Thus, incubation of 10-150 μM 13(S)-HPOT at 0° for 10-60 sec with either the 105,000xg particle fraction in buffer or the 105,000xg supernatant followed by treatment with 20 vol of methanol led to the appearance of a trapping product, 12-oxo-13-methoxy-9(Z),15(Z)-octadecadienoic acid. The half-life of 12,13(S)-EOT at 0° and pH 6.7 as determined by the trapping technique (1) was 26 sec (earlier found for 12,13(S)-EOD and 9(S),10-EOD at 0° and pH 7.4, 33 and 34 sec, respectively (1,2)).

The ultraviolet spectrum of 12,13(S)-EOT showed an absorption band with λ_{max} = 236 nm and was virtually identical to that of 12,13(S)-EOD (1), thus proving the presence in 12,13(S)-EOT of one pair of conjugated double bonds. As was earlier observed for 12,13(S)-EOD (1), stability of 12,13(S)-EOT was significantly enhanced in non-hydroxylic solvents. For example, the half-life of 12,13(S)-EOT in 90% aqueous acetonitrile at 15° was 39 sec (earlier found for 12,13(S)-EOD at 15°, 35 sec). Finally, degradation of 12,13(S)-EOT in aqueous acetonitrile did not lead to the formation of the α-ketol derivative but to two isomeric macrolactones, 12-oxo-9(Z),15(Z)-octadecadien-11-olide and 12-oxo-9(Z),15(Z)-octadecadien-13-olide. These findings were in complete agreement with results recently obtained with 12,13(S)-EOD (15) and 9(S),10-EOD (2).

Detection of an allene oxide cyclase in corn homogenate. In order to study the further transformations of the allene oxide 12,13(S)-EOT, this compound was generated by incubation of [1-^{14}C]13(S)-HPOT with hydroperoxide dehydrase contained in the washed 105,000xg particle fraction. The dehydrase activity in the preparation used was sufficient to catalyze complete conversion of more than 150 μM 13(S)-HPOT into 12,13(S)-EOT in 20 sec at 0°. A total incubation time of 10 min at 0° was used in order to allow complete conversion of the allene oxide into stable end products. Fig. 1A shows a radio-TLC analysis of the esterified product isolated after incubation of

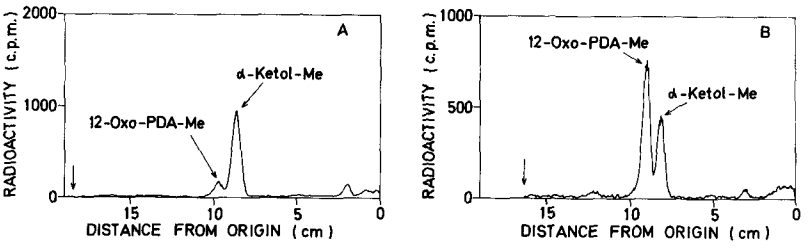

Fig. 1. TLC radiochromatograms of esterified products formed by incubation of 25 μM [1-^{14}C]13(S)-HPOT with (A) washed 105,000xg particle fraction resuspended in 10 ml of buffer, and (B) washed 105,000xg particle fraction resuspended in 10 ml of 105,000xg supernatant. Solvent system, ethyl acetate/toluene, 15:85 (v/v).

[1-^{14}C]13(S)-HPOT with washed 105,000xg particle fraction resuspended in buffer. Two labeled compounds, together forming about 80% of the recovered radioactivity, appeared. The minor compound was identified as methyl 12-oxo-10,15(Z)-phytodienoate (methyl ester of 12-oxo-PDA; R_f = 0.54) and the major one as the α-ketol derivative methyl 12-oxo-13-hydroxy-9(Z),15(Z)-octadecadienoate (R_f = 0.48) by UV and IR spectrometry, GC-MS, and chemical conversions as described in detail (1,9). Interestingly, when 13(S)-HPOT was incubated with the 105,000xg particle fraction resuspended in the 105,000xg supernatant, the generated 12,13(S)-EOT was preferentially converted into 12-oxo-PDA at the expense of α-ketol (Fig. 1B). Formation of 12-oxo-PDA and α-ketol at different concentrations of 13(S)-HPOT (and hence of 12,13(S)-EOT) is shown in Fig. 2. As seen, in the absence of 105,000xg supernatant, the amounts of 12-oxo-PDA produced were modest and were directly proportional to the substrate concentration. Furthermore, 12-oxo-PDA and α-ketol, the non-enzymatically produced hydrolysis product of allene oxide, appeared in a fixed ratio (about 0.14), irrespective

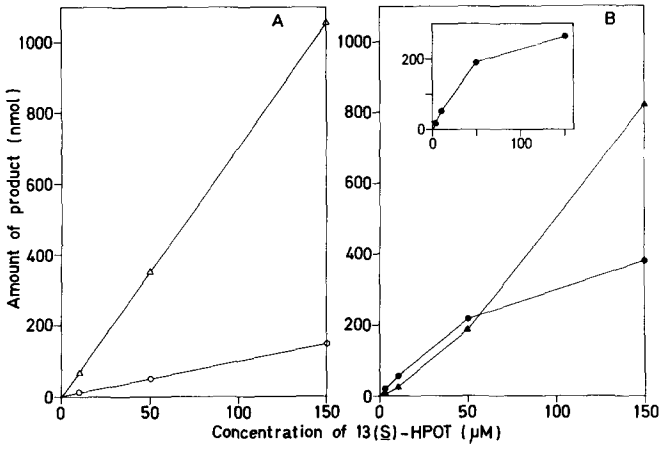

Fig. 2. Amounts of 12-oxo-PDA and α-ketol formed at different concentrations of 13(S)-HPOT. A: 13(S)-HPOT incubated with washed 105,000xg particle fraction in 10 ml of buffer. 12-Oxo-PDA, -○-○-; α-ketol, -△-△-. B: 13(S)-HPOT incubated with washed 105,000xg particle fraction resuspended in 10 ml of 105,000xg supernatant. 12-Oxo-PDA, -●-●-; α-ketol, -▲-▲-. Inset in panel B: amounts of enzymatically formed 12-oxo-PDA calculated from total amounts of 12-oxo-PDA and correction for 12-oxo-PDA formed by a non-enzymatic reaction.

TABLE I

Ratio 12-oxo-PDA/α-ketol and enantiomeric composition of 12-oxo-PDA

Incubation[a]	Concentration of 13(S)-HPOT (μM)	Ratio of 12-oxo-PDA/α-ketol	Enantiomers of 12-oxo-PDA (9S,13S/9R,13R)
A	10	0.17	51:49
A	50	0.14	50:50
A	150	0.14	51:49
B	3	2.88	98: 2
B	10	2.18	97: 3
B	50	1.17	95: 5
B	150	0.46	88:12
C	10	0.16	-
D	10	0.15	-

[a] Incubations consisted of washed 105,000xg particle fraction resuspended in: A, buffer; B, 105,000xg supernatant; C, 105,000xg supernatant heated at 90° for 10 min; D, 105,000xg supernatant preincubated at 37° for 1 h with 8 mg of pronase.

of substrate concentration. In the presence of 105,000xg supernatant, formation of 12-oxo-PDA was enhanced (Fig. 2B). In this case, the ratios 12-oxo-PDA/α-ketol were high at low substrate concentrations and dropped when substrate concentration was increased. These results suggested that, in the absence of 105,000xg supernatant, formation of 12-oxo-PDA occurred by a non-enzymatic, chemical cyclization of the allene oxide, whereas 12-oxo-PDA formation observed in the presence of 105,000xg supernatant occurred by action of a saturable activity, that selectively catalyzed formation of 12-oxo-PDA, as well as by non-enzymatic cyclization.

Further evidence for the formation of 12-oxo-PDA by both enzymatic and non-enzymatic routes was provided by steric analysis of 12-oxo-PDA (Table I). As seen, 12-oxo-PDA formed in the absence of 105,000xg supernatant was racemic, irrespective of the substrate concentration used. On the other hand, 12-oxo-PDA produced in the presence of 105,000xg supernatant was enriched with respect to the 9(S),13(S)-enantiomer. This enrichment was especially pronounced at low substrate concentrations. For example, incubation of 3 μM 13(S)-HPOT led to the formation of essentially optically pure 12-oxo-PDA (ratio between 9(S),13(S)- and 9(R),13(R)-enantiomers, 98:2).

Partial characterization of allene oxide cyclase. As seen in Table I, the stimulatory effect of the 105,000xg supernatant on the formation of 12-oxo-PDA was abolished by heat treatment (90°, 10 min) as well as by treatment with pronase. In further experiments with ammonium sulfate-fractionated supernatant, the fraction precipitated at 30-55% saturation was found to contain 90-95% of the allene oxide cyclase activity, whereas fractions collected at 0-30% and 55-80% saturation contained little or no activity.

In order to further characterize the allene oxide cyclase, ammonium sulfate precipitate of 105,000xg supernatant (30-55% saturation) was subjected to gel filtration (Fig. 3). Aliquots of the fractions were assayed for hydroperoxide dehydrase activity by the spectrophotometric

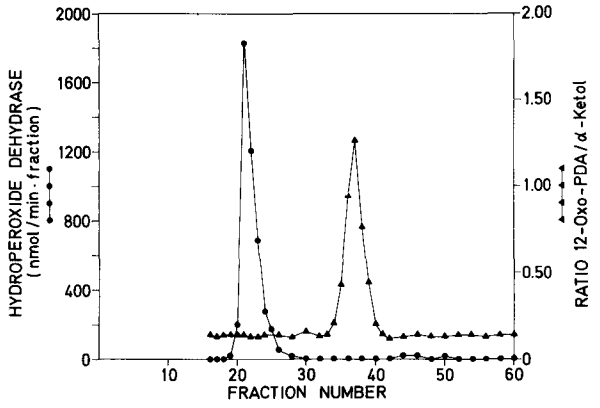

Fig. 3. Separation of hydroperoxide dehydrase and allene oxide cyclase by gel filtration. Ammonium sulfate precipitate obtained from 50 ml of 105,000xg supernatant (30-55% saturation; 40 mg of protein) was applied to a column of AcA 44 (LKB-Produkter AB, Bromma, Sweden; 88 x 2.6 cm). The column was eluted with 0.1 M potassium phosphate buffer pH 6.7 at a flow rate of 0.6 ml/min. Fraction size, 8.6 ml. Hydroperoxide dehydrase, -●-●-; ratio 12-oxo-PDA/α-ketol, -▲-▲-. As described in the text, non-enzymatic cyclization of allene oxide produced 12-oxo-PDA and α-ketol in a ratio of about 0.14:1.

method, and for allene oxide cyclase activity by incubation with [1-^{14}C]13(S)-HPOT in the presence of 105,000xg particle fraction followed by determination of 12-oxo-PDA and α-ketol by radio-TLC. The hydroperoxide dehydrase activity was excluded from the gel, indicating a molecular weight in excess of 130,000. In this context, it may be mentioned that reported molecular weights of cotton and spinach hydroperoxide dehydrases are 250,000 and 220,000, respectively (16,7). The allene oxide cyclase, detected by an increased formation of 12-oxo-PDA relative to the background level caused by non-enzymatic cyclization of allene oxide, appeared with an effluent volume of 292-344 ml (Fig. 3). As expected, 12-oxo-PDA generated by incubation of 13(S)-HPOT with the 105,000xg particle fraction plus fractions containing the allene oxide cyclase was enriched with respect to the 9(S),13(S)-enantiomer (eg., peak fraction, ratio between 9(S),13(S)- and 9(R),13(R)-enantiomers, 95:5), whereas similar incubations using fractions eluted before and after the allene oxide cyclase-containing fractions led to the formation of racemic 12-oxo-PDA. By using appropriate standard proteins to calibrate the column, it was found that the chromatographic behaviour of the cyclase was the same as that of a protein with a molecular weight of about 45,000.

DISCUSSION

The present paper is concerned with the mechanism of formation of the plant cyclopentenone, 12-oxo-10,15(Z)-phytodienoic acid (12-oxo-PDA) from α-linolenic acid 13-hydroperoxide (13(S)-HPOT). Transformation was initiated by hydroperoxide dehydrase-catalyzed conversion of the hydroperoxide into the unstable allene oxide derivative, 12,13(S)-EOT. Formation of 12-oxo-PDA from allene oxide was found to occur by non-enzymatic, chemical cyclization, as well as by an allene oxide cyclase-catalyzed reaction (Fig. 4). In the first

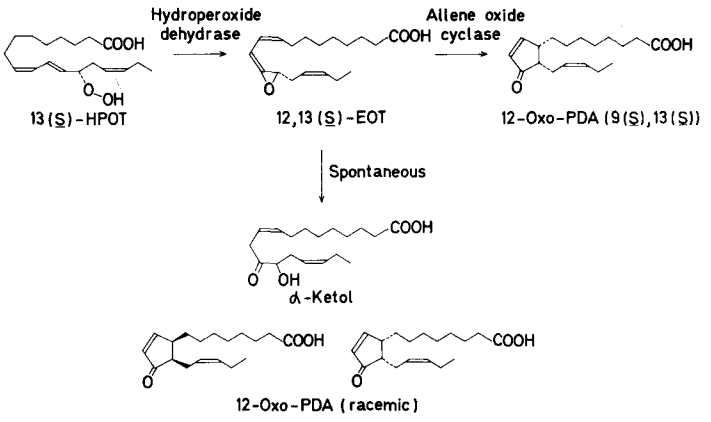

Fig. 4. Formation of 9(S),13(S)-12-oxo-PDA from 13(S)-HPOT by sequential actions of hydroperoxide dehydrase and allene oxide cyclase. Competing non-enzymatic conversion of allene oxide 12,13(S)-EOT into α-ketol and racemic 12-oxo-PDA is also shown.

case, the yield of 12-oxo-PDA relative to α-ketol, a non-enzymatically produced hydrolysis product of 12,13(S)-EOT, was relatively low, and the 12-oxo-PDA formed was racemic. In the second case, 12-oxo-PDA was the only product formed from allene oxide, and consisted of the pure 9(S),13(S)-enantiomer. The allene oxide cyclase activity appeared in the soluble fraction of corn homogenate, was destroyed by heating and proteolytic digestion, was precipitable by ammonium sulfate, and migrated like a protein with a molecular weight of about 45,000 on gel filtration.

In recent studies, 12-oxo-PDA obtained by incubation of 13(S)-HPOT with preparations of corn (14) and flaxseed (17) was found to be only partially optically pure and racemic, respectively. The present work, demonstrating the existence of a plant allene oxide cyclase, suggests that the incomplete optical purity of 12-oxo-PDA observed was due to low (14) or missing (17) allene oxide cyclase activity in the preparations used. It is postulated that the in vivo biosynthesis of 12-oxo-PDA occurs by sequential actions of ω6-lipoxygenase, hydroperoxide dehydrase, and allene oxide cyclase, resulting in the formation of optically pure 12-oxo-PDA. In this context it should be noted that the stereochemistry of 12-oxo-PDA formed in the presence of allene oxide cyclase described in the present paper is the same as that of (+)-7-iso-jasmonic acid, a growth-regulating hormone which is biosynthesized from 12-oxo-PDA in plant tissue (18).

Further studies on the kinetic and molecular properties of the corn allene oxide cyclase are in progress and will be reported later.

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