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The biochemical origin of pentenol emissions from wounded leaves

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Abstract

Large releases of 1-penten-3-ol (pentenol) and 1-penten-3-one (pentenone) were recently observed from a variety of leaves subjected to freeze–thaw damage in the presence of oxygen. In order to understand the biochemical origins of these volatiles, soybean leaf extracts were used to determine if the formation of pentenol and pentenone can be explained by known O_2-dependent lipoxygenase (LOX) reactions. Enzymatic formation of these C5 volatiles was found to be dependent on α-linolenic acid or the 13(*S*)-hydroperoxide of α-linolenic acid [13(*S*)-HPOT] and blocked by LOX inhibitors. Five soybean leaf LOX isozyme genes (VLXA, VLXB, VLXC, VLXD, and VLXE) were then expressed in *Escherichia coli* and used in in vitro incubations with 13(*S*)-HPOT to test for volatile formation. Each of the LOX isozymes catalyzed the formation of low levels of pentenol, but not pentenone. It therefore seems likely that the C5,13-cleavage activity of LOX is the direct source of abundant pentenol and the indirect source of pentenone observed upon leaf wounding.

Keywords: *Glycine max*; Leguminosae; Soybean; Pentenol; Pentenone; Lipoxygenase; Volatile organic compound; Gas chromatography; Reduction gas detector

1. Introduction

On-line proton-transfer-reaction mass spectrometry was used recently to observe the kinetics of formation of volatile organic compounds (VOCs) following mechanical leaf wounding (Fall et al., 1999). Typical wound VOCs from leaves included 3(*Z*)-hexenal, which is formed within a few seconds of a wound, its isomerization product 2(*E*)-hexenal, and the alcohol and ester metabolites of these aldehydes. These are all well-known products of leaf wounding initiated by oxygen-dependent lipoxygenase (LOX) reactions (Gardner, 1991; Hatanaka, 1993). In subsequent work, we observed a different pattern of VOCs was observed from a variety of leaves, wounded by freeze–thaw damage (Fall et al., 2001). In this case, 3(*Z*)-hexenal release occurred only when oxygen was admitted to the system, and its release was accompanied by comparable amounts of 1-penten-3-ol (pentenol) and 1-penten-3-one, (pentenone). These same C5 VOCs were shown by Gardner and coworkers (Salch et al., 1995; Gardner et al., 1996) to arise from the LOX-dependent C-5,13-cleavage of the 13*(S)*-hydroperoxide of α-linolenic acid, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid [13(*S*)-HPOT], in soybean seed extracts. The abundant LOX in soybean seeds catalyzes both the peroxidation of α-linolenic acid and, under anaerobic conditions, the C-5,13-homolytic cleavage of 13(*S*)-HPOT. This cleavage gives rise to 13-oxo-9(*Z*),11(*E*)-tridecadienoic acid (13-OTA) and a putative pentene radical. Pentenol is proposed to be a product of the pentene radical. Pentenone was not detected as a direct product of the C-5,13-cleavage catalyzed by soybean seed LOX, but was formed when exogenous soybean alcohol dehydrogenase and NAD^+ were added in vitro (Gardner et al., 1996).

Abbreviations: ADH, alcohol dehydrogenase; DTT, dithiothreitol; GC, gas chromatography; 13(*S*)-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; LOX, lipoxygenase; 13-OTA, 13-oxo-9(*Z*),11(*E*)-tridecadienoic acid; PG, *n*-propyl gallate; PVPP, polyvinylpolypyrrolidone; RGD, reduction gas detection; SHAM, salicylhydroxamic acid; VLX, vegetative lipoxygenase; VOC, volatile organic compound

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Since LOX activity is abundant in green leaves (Sekiya et al., 1983), it is likely that C5 wound VOC formation may occur by mechanisms similar to those described for soybean seed LOX isozymes (Salch et al., 1995; Gardner et al., 1996). A goal of the work presented here was to test this hypothesis in a leaf system. Leaves of soybean (*Glycine max*) were chosen as an enzyme source because, in addition to containing relatively high LOX activity (Sekiya et al., 1983), several soybean leaf LOX isozymes have been cloned and characterized (Grayburn et al., 1991; Fischer et al., 1999; Fuller et al., 2001). Experiments are presented demonstrating that pentenol and pentenone are products of LOX-dependent reactions in soybean leaves.

2. Results and discussion

Experiments were designed to test for the enzymatic production of pentenol and pentenone from α-linolenic acid or 13(*S*)-HPOT in soybean leaf extracts. Pentenol and pentenone were measured directly, without extraction, using a sensitive headspace gas chromatography (GC) method developed for analysis of the C5 alcohol, methylbutenol (Fisher et al., 2000). As shown in Fig. 1, a dialyzed 30–60% ammonium sulfate fraction of a crude soybean leaf extract catalyzed both the α-linolenic acid- and 13(*S*)-HPOT-dependent production of pentenol and pentenone. Both a boiled ammonium sulfate fraction and a protease-treated fraction failed to catalyze the production of pentenol and pentenone (data not shown), indicative of the enzymatic synthesis of these C5 VOCs. The ammonium sulfate fraction also catalyzed

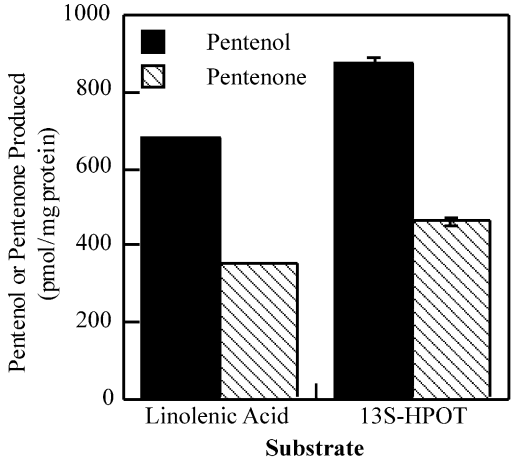

Fig. 1. Production of pentenol and pentenone from 5 mM α-linolenic acid or 1 mM 13(*S*)-HPOT as catalyzed by a dialyzed 30–60% ammonium sulfate fraction from soybean leaves. Each 500-μl reaction contained 3.8 mg protein (α-linolenic acid samples) or 3.1 mg protein [13(*S*)-HPOT samples]. Reactions mixtures were incubated 10 min at 30 °C and analyzed by the GC assay. Reported values are the average of two separate reactions (α-linolenic acid samples) or the average ±1 S.D. for three separate reactions [13(*S*)-HPOT samples].

the production of the C13 cleavage product, 13-oxo-9(*Z*),11(*E*)-tridecadienoic acid (13-OTA), from 13(*S*)-HPOT. The specific activity for 13-OTA formation was 11 nmol min^{-1} (mg protein)$^{-1}$, which is comparable to the specific activity for 13-OTA formation of 19 nmol min^{-1} (mg protein)$^{-1}$ determined by Gardner and coworkers (Salch et al., 1995) for a crude LOX preparation from soybean seed.

To determine if the enzymatic activity in soybean leaves giving rise to pentenol and pentenone was indeed LOX, several inhibitors of LOX were tested for their effect on enzymatic pentenol and pentenone production from 13(*S*)-HPOT. These included salicylhydroxamic acid (SHAM), *n*-propyl gallate (PG), and H_2O_2 (Mitsuda et al., 1967; Vick, 1993). As summarized in Table 1, all three LOX inhibitors effectively blocked the production of pentenol and pentenone from 13(*S*)-HPOT catalyzed by the dialyzed ammonium sulfate fraction. Although these inhibitors are not completely specific for LOX (e.g. Gardner, 1991), taken together the results in Table 1 are consistent with a role for this enzyme in C5 volatile production.

To further confirm the participation of LOX in the formation of C5 wound VOCs, the full-length VLXB, VLXC, VLXD, and VLXE soybean leaf LOX isozymes (Fuller et al., 2001) and a Maltose Binding Domain fusion of the full-length VLXA isozyme (Fischer et al., 1999) were expressed in *Escherichia coli* and analyzed in vitro for both LOX activity and C5 volatile production from 13(*S*)-HPOT. These five isozymes are termed vegetative lipoxygenases (VLXs), as they are present in soybean vegetative tissue (i.e., leaves). The VLX isozymes share a high degree of sequence similarity (Fischer et al., 1999), but differ significantly with respect to regulation, substrate preference, pH profile, and reaction products (Fuller et al., 2001). The appearance of a protein band by SDS-PAGE, at the approximate molecular weight for the appropriate VLX construct, was detected for each of the five expressed isozymes (data not shown). LOX activity with α-linolenic acid

Table 1
Enzymatic production of pentenol and pentenone from 1 mM 13(*S*)-HPOT is inhibited by several inhibitors of LOX

Treatment	Pentenol production (% inhibition)	Pentenone production (% inhibition)
None (control)	0	0
H_2O_2	97	99
SHAM	99	99
PG	100	99

Inhibitors (5 mM) were pre-incubated with enzyme for 5 min at room temperature prior to the reaction. A dialyzed 30–60% ammonium sulfate fraction from soybean leaves was used as the enzyme source. Values of % inhibition were determined using the average pentenol or pentenone production of two separate inhibitor reactions and comparing to data presented in Fig. 1.

was not detected in *E. coli* BL21(DE3) control cells (data not shown), consistent with the general lack of LOX in prokaryotes. In contrast, both oxidative LOX activity and pentenol formation from α-linolenic acid was detected in all five VLX constructs, although these two activities varied widely (Fig. 2). In addition, each of the cloned VLX isozymes catalyzed the production of pentenol from 13(*S*)-HPOT, also with a substantial range of activity, but did not produce pentenone (data not shown). No production of pentenol or pentenone from 13(*S*)-HPOT was detected from control cells lacking recombinant LOX.

The observed rates of formation of pentenol from α-linolenic acid by the cloned VLX isozymes in vitro were orders of magnitude lower than oxidative LOX activity (Fig. 2). Comparable results were obtained by Gardner et al. (1996) with a soybean seed LOX preparation, where the rates of C5,13-cleavage of 13(*S*)-HPOT were much lower than for oxidative LOX activity. This suggests that pentenol formation from α-linolenic acid is mainly limited by the cleavage reaction and not the rapid oxidative reaction. The C-5,13-free radical cleavage of 13(*S*)-HPOT by soybean seed LOX isozymes was inhibited by O_2, although the LOX-3 isozyme functioned "anaerobically" even in the presence of O_2 (Salch et al., 1995). Here, pentenol production (and pentenone formation in leaf extracts) was routinely observed in reactions in which no attempts

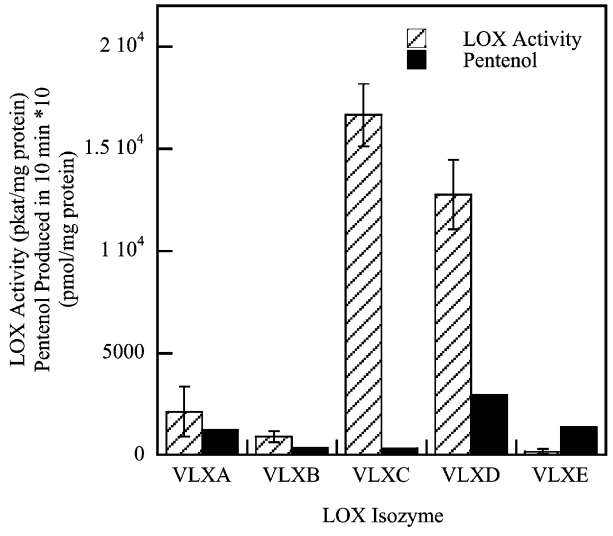

Fig. 2. LOX and pentenol-forming activities of soybean leaf LOX isozymes expressed in *E. coli*. LOX isozymes (VLXA-VLXE) were cloned into bacterial expression vectors and expressed in *E. coli* BL21(DE3)pLysS (Fischer et al., 1999; Fuller et al., 2001). *E. coli* lysates were assayed for LOX activity (photometric assay) and then concentrated to assay for pentenol production (GC assay). Values for LOX activity are averages ±1 S.D. for three separate reactions, using 50 mM BisTris propane (pH 6.0) and 0.5 mM α-linolenic acid in the assay. Values for pentenol production are averages of two separate reactions, using 50 mM Hepes–KOH (pH 7.5) and 1 mM 13(*S*)-HPOT in the assay. LOX, lipoxygenase; VLX, vegetative lipoxygenase; ROOH, hydroperoxide.

were made to eliminate O_2, and, in preliminary experiments with the five cloned VLX isozymes, running reactions under N_2 did not enhance pentenol production significantly. Low rates of pentenol formation could also be explained by the finding that pentenol is only one of numerous products arising from a putative pentene radical, produced upon C5,13-cleavage of 13(*S*)-HPOT (Gardner et al., 1996).

The reason for the large variability in the ratio of pentenol production to oxidative LOX activity by various VLX isozymes (Fig. 2) is uncertain. It is worth noting that all of the VLX isozymes tested here differ significantly with respect to regulation, substrate preference, pH profile, and reaction products (Fuller et al., 2001). Future experiments to address the relative rates of pentenol production by VLX isozymes will have to address these differences.

The results presented here suggest a model for formation of C5 VOCs after leaf wounding. The wound leads to release of α-linolenic acid, which can be rapidly peroxidized to 13(*S*)-HPOT by the high levels of LOX present in leaves. If the activity of hydroperoxide lyase, which catalyzes the C6,12-cleaveage of 13(*S*)-HPOT, becomes limiting due to substrate inhibition (Fauconnier et al., 1997), accumulation of 13(*S*)-HPOT could favor the C5,13-cleavage, increasing the appearance of C5 products. At present, we do not have enough data to ascertain which VLX isozyme(s) is responsible for pentenol production in vivo. And, the possibility that a different (i.e., non-soluble) leaf LOX isozyme is responsible for the majority of pentenol (and pentenone) production in freeze-damaged leaves cannot be ruled out. Grimes and co-workers have observed a novel LOX isozyme in chloroplasts of soybean leaves by fluorescent antibody staining (unpublished results). Given the abundance of α-linolenic acid in galactolipids of plant chloroplasts (Hitchcock and Nichols, 1971), it is possible that this LOX isozyme is responsible for a significant fraction of pentenol and pentenone produced upon freeze/thaw damage.

Notably, pentenone was not detected as a product of the in vitro VLX reactions described here. These results indicate that an additional factor, present in soybean leaf extracts, is required for the production of pentenone. Gardner et al. (1996) proposed that pentenone and pentenals arise from the oxidation of pentenol by alcohol dehydrogenase (ADH) in soybean seeds. However, ADH activity in soybean leaves is very low (Kimmerer, 1987), and ADH in leaves of higher plants appears to be inhibited by freeze/thaw damage (Fall et al., 2001). Further work is needed to establish the mechanism of pentenone formation in freeze-damaged plants.

Future work should also address whether LOX-dependent reactions are the sole source of abundant C5 VOCs released from leaves upon freeze–thaw damage.

It is known that Fe^{2+} and plant iron proteins can catalyze the homolytic cleavage of the 10-hydroperoxide of α-linolenic acid, 10-hydroperoxy-8,12-octadecadienoic acid; the resulting alkoxyl radical undergoes non-enzymatic β-scission to form an alkene radical and an aldehyde (Labeque and Marnett, 1987). It is likely that 13(*S*)-HPOT, produced in leaves upon freeze–thaw damage, undergoes similar reactions. Experiments could be directed at investigating the accumulation of 13(*S*)-HPOT in freeze–thawed leaves and testing whether leaf iron proteins catalyze its homolytic cleavage.

The release of pentenyl VOCs is not limited to plant damage caused by freezing. For example, emissions of pentenol and related C5 VOCs have been detected from plants after mowing (Konig et al., 1995), upon drying (Fall et al., 2001), and after exposure to ozone fumigation or pathogen attack (Scutareanu et al., 1997; Heiden et al., 1999). These results indicate that pentenol and similar C5 VOCs may be released from leaves in response to many forms of wounding, or from related processes. It is likely, but not proven, that the high levels of pentenol detected at a high mountain observatory site in the Austrian Alps in autumn, 1999 (Fall et al., 2001) were derived from leaf processes of this type in the nearby forests. Future work will clarify the mechanisms and magnitude of leaf C5 volatile formation and determine if this is a common process in the earth's abundant temperate forests and grasslands.

3. Experimental

3.1. Plant material and reagents

Soybean (*Glycine max* cv Williams 82) plants were grown from seed in a temperature-controlled greenhouse with supplemental lighting (500 μmol m^{-2} s^{-1}) for a 16 h photoperiod. Standard fertilization practices were used. Temperatures in the greenhouse ranged from 21 °C at night to 34 °C during the day.

13(*S*)-HPOT was prepared by oxidation of α-linolenic acid with LOX and purified on silicic acid as described previously (Gardner, 1991). The 13-hydroperoxide isomer was determined to be over 99% pure by HPLC analysis with UV detection at 235 nm. Stock solutions of 13(*S*)-HPOT (at ~30 mg/ml in MeOH) were stored at −20 °C. Aqueous solutions (2 mM) of the hydroperoxide were prepared fresh daily as potassium salts of the fatty acid. The pH of the solution was adjusted to 8.5 with KOH, and 0.032% (v/v) Tween 20 was added to ensure even dispersal of the hydroperoxide.

Linolenic acid (99%), LOX (type 1S from soybean seed), silicic acid (chromatography grade), salicylhydroxamic acid (SHAM), *n*-propyl gallate (PG), IGEPAL CA-630 and other reagents were purchased from Sigma (St. Louis, MO).

3.2. Enzyme preparation

Soybean leaves (20 g) from mature plants were harvested fresh daily, washed in a solution containing 2% (v/v) bleach and 0.05% (v/v) IGEPAL CA-630 [(octylpenoxy)-polyethoxyethanol] nonionic detergent (Sigma; St. Louis, MO). The leaves were then frozen in liquid N_2 and ground to a fine powder in a mortar and pestle. The resulting powder was added to ice-cold extraction buffer containing 5 mM sodium acetate (pH 4.5), 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 5% (w/v) insoluble polyvinyl-polypyrrolidone (PVPP). After stirring on ice for 20 min, the solution was filtered through a layer of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 13,000 *g* for 20 min at 4 °C to remove solid debris. Protein was precipitated from the resulting solution between 30 and 60% (w/v) saturation with ammonium sulfate. The precipitate was collected by centrifugation at 24,000 *g* for 20 min at 4 °C and resuspended in a minimal volume of extraction buffer containing no PVPP. The resuspended precipitate was dialyzed overnight against 2 l of 50 mM Hepes–KOH (pH 7.5). This preparation was used as the enzyme source.

3.3. LOX expression in E. coli

Full-length LOX isozymes (VLXB, VLXC, VLXD, and VLXE) from soybean leaves cloned into the pSBET vector were used for the studies presented here. The construct containing full-length soybean leaf VLXA in pSBET was not expressed consistently (Fuller et al., 2001). Therefore, a Maltose Binding Protein fusion protein of VLXA expressed in the pMAL vector was used (Fischer et al., 1999). *E. coli* BL21(DE3)pLysS strains containing the LOX constructs were grown, induced with IPTG, harvested, and lysed as described previously (Fuller et al., 2001). Cell debris was removed by centrifugation, and the resulting supernatants, containing soluble LOX proteins, were assayed for LOX activity and C5 volatile production as described below.

3.4. Enzyme assays

Pentenol and pentenone production were assayed in the headspace of reaction vials using an isothermal capillary gas chromatography (GC) system employing a reduction gas detector (RGD). The GC–RGD system was constructed in our laboratory and has been described previously (Fisher et al., 2000). Pentenol and pentenone were separated on a DB-624 capillary column (60 m×0.32 mm, 18 μm film thickness; J&W scientific, Folsom, CA). For enzyme assays, 250-μL aliquots of enzyme extract were incubated with 250 μL α-linolenic acid in 25 mM Hepes–KOH (pH 7.5) containing 0.032% (v/v) Tween 20, or 250 μl aqueous 13(*S*)-HPOT,

in 4.8-ml glass vials sealed with Teflon-lined septa for 10 min at 30 °C. After incubation, 2 mL of vial headspace was withdrawn with a gas-tight syringe and analyzed by GC–RGD. Reported concentrations of pentenol and pentenone are estimates based on calibration curves in which standard aqueous solutions of each were incubated for 10 min at 30 °C, and reaction headspace was analyzed by GC–RGD. As pentenol and pentenone produced in the vial are not at equilibrium, true rates of enzymatic pentenol and pentenone production cannot be determined.

LOX activity was assayed photometrically, following hydroperoxide formation (Axelrod et al. 1981). For plant extracts, assays contained 0.25 mM linoleic acid in 25 mM Hepes–KOH (pH 7.5) and 10–100 μl of enzyme. For *E. coli* BL21(DE3)pLysS lysates expressing LOX isozymes, assays contained 0.5 mM α-linolenic acid in 50 mM BisTris propane containing 50 mM acetic acid and 0.00025% (v/v) Tween 20, adjusted to pH 6.0 with acetic acid, and 50-250 μL of lysate (Fuller et al., 2001). Lysates of *E. coli* BL21(DE3) control cells without LOX expression plasmids (no IPTG was added) were analyzed identically.

Production of the C13 cleavage product, 13-oxo-9(*Z*), 11(*E*)-tridecadienoic acid (13-OTA), was assayed photometrically as described previously (Salch et al., 1995). Protein was determined according to Bradford (1976).

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