Efficient Incorporation of Free Oxygen into Volicitin in *Spodoptera litura* Common Cutworm Larvae

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Note

Volicitin [N-(17-hydroxylinolenyl)-l-glutamine] has previously been identified from the lepidopteran larval regurgitant as an elicitor of plant volatile emission. The efficient incorporation of free oxygen into volicitin by *Spodoptera litura* larvae is demonstrated here by rearing them under $^{18}$O$_2$ for three days. $^{18}$O-labeling of the hydroxyl group of volicitin was confirmed by liquid chromatography/mass spectrometry-ion trap-time-of-flight (LC/MS-IT-TOF) and suggests the activity of a monooxygenase in volicitin biosynthesis.

Key words: volicitin; plant defense signal; *Spodoptera litura*

Plants damaged by caterpillar feeding release a mixture of volatiles which help parasitoids, natural enemies of caterpillars, to locate their hosts. Volicitin [N-(17-hydroxylinolenyl)-l-glutamine] was first identified from the oral secretion of *Spodoptera exigua* (Noctuidae) as an elicitor of plant volatile emission (Fig. 1). Since then, analogs such as N-linolenoyl- and N-linoleoyl-l-glutamine have also been found in several other lepidopteran species. Our recent report has shown that N-linolenoyl-l-glutamine was only about 30% as active as volicitin when assayed on Zea mays seedlings, suggesting that hydroxylation at the 17-position (cis-1 hydroxylation) of the linolenic acid moiety of volicitin is important for elicitor activity. The absolute configuration at the 17-position in some Noctuid species has been determined to be 17S in all cases, although the two synthesized enantiomeric isomers showed no significant difference in an elicitor activity assay. It has been assumed that the cis-1 hydroxylation of volicitin might be catalyzed by such enzymes as cytochrome P450 (subsequently referred to as P450). For example, CYP6A8 in *Drosophila melanogaster* has been reported to catalyze the cis-1 hydroxylation of lauric acid, although long-chain unsaturated fatty acids such as oleic acid were not metabolized by this enzyme. Kandel et al. have suggested that CYP709C1, the first plant P450 found to catalyze cis-1 hydroxylation, might be involved in volicitin biosynthesis. This hypothesis was based on Pare’s report that the fatty acid moiety of volicitin originated from plant tissues. However, we can rule out this possibility by the fact that the hydroxylation of volicitin occurred after conjugation of fatty acids with glutamine in larval midgut tissues. At present, there is no experimental evidence to indicate any involvement of P450 in volicitin biosynthesis. In this study, we determined the oxygen source for this oxidative reaction of N-linolenoyl-l-glutamine to develop a better understanding of the cis-1 hydroxylation of volicitin.

The volicitin fraction was extracted from the gut contents of *S. litura* larvae reared for three days under atmospheric conditions with $^{18}$N$_2$/$^{18}$O$_2$ = 4/1, and was analyzed with an LC/MS 2010 A system. Volicitin and volicitin-related compounds were identified by comparing the retention time and molecular ions with authentic samples, and the amount of each of those compounds was within the range broadly consistent with those reported previously. As shown in Fig. 2, the ions of normal volicitin and v$^{18}$O-labeled volicitin were detected at $m/z$ 421 and 423 [(M – H)$^-$] ($r_k = 7.5$ min), respectively, using a negative ion mode analysis. The labeled ratio of volicitin was approximately 74.5% as an average of three replicates.

Both v$^{18}$O-labeled and unlabeled volicitin were then analyzed by an LC/MS-IT-TOF system for detailed structural analysis. Unlabeled volicitin ($m/z$ 421.26) produced fragment ions at $m/z$ 403.26 (dehydrated ion, calculated as 403.2597), 385.23 (doubly dehydrated ion, calculated as 385.2491), 231.20 (dehydrated and decarboxylated hydroxylinolenate ion, calculated as 231.2113), and 145.05 (glutamine-derived ion, calculated as 145.0614). In contrast, v$^{18}$O-labeled volicitin ($m/z$ 423.26) produced fragment ions at $m/z$ 405.25 (dehydrated ion, calculated as 405.2515), 385.24 (doubly dehydrated ion, calculated as 385.2491), 231.20 (dehydrated and decarboxylated hydroxylinolenate ion, calculated as 231.2113), and 145.05 (glutamine-derived ion, calculated as 145.0614). These results clearly show that v$^{18}$O-labeled oxygen was introduced to the hydroxyl group of volicitin by a monooxygenase-type enzyme such as P450.

Li et al. have already reported an intriguing link between herbivore P450 and plant-insect interaction. Jasmonate and salicylate, previously known as plant-

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**Abbreviations:** LC/MS, liquid chromatography/mass spectrometry; ESI, electron spray ionization; LC/MS-IT-TOF, liquid chromatography/mass spectrometry-ion trap-time-of-flight
produced signals that activate genes defending against herbivores, have also induced gene expression of P450 in corn earworm *Helicoverpa zea*. Insect P450s are best studied in terms of the mechanism by which they detoxify toxic compounds in plants or pesticides. Li *et al.* have postulated that P450s were induced in caterpillars to counter an array of defense substances in plants. It is also widely held that the many variants of P450 genes have been developed in herbivorous insects through co-evolution with host plants. Our latest study is significant, because although N-linolenoyl-L-glutamine has been found in a wide range of lepidopteran species including primitive Gerachiidae, volicitin, the ω-1 hydroxylated version, has previously only been reported in developed and polyphagous macrolepidopteran species (unpublished data). It also remains unclear why some of these caterpillars make the effort to oxygenate N-linolenoyl-ω-glutamine when linolenic acid is itself a vital nutrient for most lepidopteran larvae, and, what is worse, why this oxygenation increases the risk of parasitoid attack. Our hypothesis is that ω-1 hydroxylation plays an important role in caterpillar physiology, or that it is a by-product of certain multisubstrate enzymes such as P450.

Considering the impact of insect P450 on plant defenses, it is no wonder that corn plants recognize volicitin as an indicator of an attack by these developed herbivores. The data presented here suggest that a monoxygenase could be a key player in plant-insect interaction.

**Experimental**

*Chemicals and insect materials.* 18O-labeled oxygen was obtained from Isotec and N2 was obtained from Taiyo Nissan. Colonies of *Spodoptera litura* were reared in the laboratory on an artificial diet (Insecta-LFS, Nihon Nosan Kogyo, Yokohama, Japan) at 24°C under 16L/8D lighting.

18O2 application with *S. litura* larvae. 18O2 was mixed with non-labeled N2 at a ratio of 1/4 (v/v) in a glass desiccator (2.25 liters) with a rubber stopper in the top. The stopper was quickly removed, and then 26 g of an artificial diet and ten 3rd instar larvae of *S. litura* were

![Structure of Volicitin](image1)

**Fig. 1.** Structure of Volicitin.

![Extracted Ion Chromatograms](image2)

**Fig. 2.** Extracted Ion Chromatograms for Volicitin (tR = 7.5 min) in *S. litura* Larvae Reared under Atmospheric Conditions with N2/18O2 = 4/1 for Three Days. 

[18O]-labeled ions of normal volicitin and 18O-labeled volicitin were detected at m/z 421 and 423, respectively. Samples were analyzed by the LC/MS 2010 system.

![MS and MS2 Spectra](image3)

**Fig. 3.** MS and MS2 Spectra for Unlabeled Volicitin (A) and 18O-Labeled Volicitin (B) Analyzed by the LC/MS-IT-TOF System.
slowly moved into the desiccator. After rearing them for three days at 24 °C under 16L/8D lighting, the larvae were frozen at −20 °C to dissect and remove the gut contents.

Analysis of 18O-labeled volicitin by LC/MS and LC/MS-IT-TOF. The gut content of a larva was immediately boiled for 10 min, homogenized in 500 μl of 50% acetonitrile in water, and centrifuged at 14,000 g for 5 min. The supernatant was filtered through a 0.45-μm hydrophilic PTFE membrane (Millipore, Bedford, USA), diluted with distilled water, and then an internal standard (1 μg of N-palmitoleoyl-l-glutamine in 150 μl of acetonitrile) was added to the sample. To determine the ratio between labeled and unlabeled volicitin, each sample was analyzed by a 2010 A LC/MS instrument (Shimadzu, Kyoto, Japan) equipped with a Prominence HPLC system coupled to an LC/MS-IT-TOF instrument (Shimadzu, Kyoto, Japan). The same Cosmosil column was eluted (0.2 ml/min) with water containing 0.05% acetic acid over 15 min with a solvent of 30–90% acetonitrile containing 0.08% acetic acid, with the column temperature maintained at 40 °C in an oven. To obtain structural information on labeled and unlabeled volicitin by an MS analysis, each sample solution was analyzed by using a Prominence HPLC system coupled to an LC/MS-IT-TOF instrument (Shimadzu, Kyoto, Japan). The CDL temperature was 250 °C, a nebulizer gas flow of 1.5 l/min, an ion accumulation time of 18 min with a gradient of 20–90% acetonitrile containing 0.08% acetic acid, with the column temperature maintained at 40 °C. The MS system was operated with a probe voltage of 4.50 kV, a CDL (curved desolvation line) temperature of 200 °C, a block heater temperature of 200 °C, a nebulizer gas flow of 1.5 l/min, an ion accumulation time of 10 msec, an MS range of m/z 200 to 500, an MS2 range of m/z 100 to 500, and CID parameters of 80% energy and 100% collision gas. The precursor ions of unlabeled and labeled volicitin were m/z 421.26 (calculated for C21H24N2O10, 421.2702) and m/z 423.26 (calculated for C21H24N2O11O2, 423.2745). MS data were processed with LC/MS solution ver. 3.4 software (Shimadzu, Kyoto, Japan).

Labeled ratio of volicitin. The 18O-labeled ratio of volicitin was calculated by the LC/MS 2010 A instrument, using selected ion chromatograms, as follows: \( \frac{m/z \text{ of } 423}{m/z \text{ of } 421} \) [unlabeled volicitin (m/z 421)] + [labeled volicitin (m/z 423)].

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