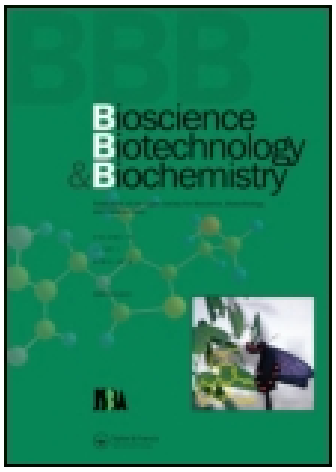


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# Linoleic Acid 10-Hydroperoxide as an Intermediate during Formation of 1-Octen-3-ol from Linoleic Acid in Lentinus decadetes

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*Note*

# Linoleic Acid 10-Hydroperoxide as an Intermediate during Formation of 1-Octen-3-ol from Linoleic Acid in *Lentinus decadetes*

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**In order to confirm the biosynthetic pathway to 1-octen-3-ol from linoleic acid, a crude enzyme solution was prepared from the edible mushroom, *Lentinus decadetes*. When the reaction was performed in the presence of glutathione peroxidase, which can reduce organic hydroperoxide to the corresponding hydroxide, the amount of 1-octen-3-ol formed from linoleic acid was decreased. At the same time, an accumulation of linoleic acid 10-hydroxide could be detected. The 10-hydroperoxide therefore seems to be an intermediate on the biosynthetic pathway.**

**Key words:** *Lentinus decadetes*; flavor compound; 1-octen-3-ol, linoleic acid 10-hydroperoxide

The flavor compounds formed by mushrooms are important factors to determine their quality as food materials. The volatile compositions of many mushrooms have therefore been analyzed and reported.[1] Among the volatiles, 1-octen-3-ol can be found in almost all mushrooms and in some fungi, and it is known as one of the key compounds for the earthy flavor.[2] It has been reported that the C8 vinyl alcohol is derived from linoleic acid through oxygenation of the fatty acid and subsequent cleavage of the fatty acid hydroperoxide.[3,4] However, the precise pathway to 1-octen-3-ol in mushrooms has not been fully elucidated, and until now there has been no direct evidence for the formation of 10-HPOD as an intermediate to form the C8 alcohol, although a possible reaction to form the alcohol from 10-HPOD has been proposed.[3] Screening of several kinds of mushrooms showed that the fruiting bodies of *Lentinus decadetes* had relatively high activity to form 1-octen-3-ol from linoleic acid. In this present study, we could identify 10-HPOD as an intermediate of the enzyme system in this mushroom.

Fruiting bodies (at the fully opened stage) of *L. decadetes* were kindly provided by Ringyou Center (Yamaguchi, Japan). After harvesting (during September to October), the fruiting bodies were immediately frozen in liquid nitrogen, and kept frozen at −80°C until needed. 13-HPOD and 15-hydroperoxy-(*Z*, *E*)-11,13-eicosadienoic acid (15-HPED) were prepared by using soybean lipoxygenase from linoleic acid and (*Z*, *Z*)-11,14-eicosadienoic acid (98% pure; Sigma, St. Louis, MO, USA), respectively.

The fruiting bodies (30 g fresh weight) were homogenized with 90 ml of a 0.1 M sodium phosphate buffer (pH 6.5) with a Polytron mixer for 20 sec. The homogenate was filtered through four layers of cheesecloth, and transferred to a 22-ml screw-cap vial. The solution was incubated at 26°C for 30 min with or without 180 $\mu$l of 50 mM linoleic acid (in 0.2% Tween 20), before 3 ml of a saturated $CaCl_2$ solution was added to kill the enzyme activity. As an internal standard, 20 $\mu$l of 1 mM *n*-octanal was added to the mixture. Sampling of the headspace was carried out by inserting an SPME fiber (65 $\mu$m; Carbowax-divinylbenzene, Spelco) for 30 min while the vial was incubated at 40°C. The adsorbed compounds were analyzed by GC-MS apparatus (Shimadzu GC/MS-QP 5050A) equipped with a DB-WAX column (0.25 mm i.d. × 60 m) and using helium as the carrier gas. The column temperature was held at 40°C for 2 min and programmed to increase at 10°C/min to 220°C. The SPME fiber was kept inserted into the injection port until the end of each analysis. The ionization energy was 70 eV.

Fruiting bodies (5 g fresh weight) of *L. decadetes* were cut into small pieces and ground with sea sand in 5 ml of a 0.1 M Tris-HCl buffer (pH 8.0) containing 0.2% polyclar VT (w/v), 10% sucrose (w/v) and 1 mM PMSF. The slurry was filtered through four layers of cheesecloth, and the resulting filtrate was centrifuged at 15,000 × *g* for 20 min. The supernatant was used as a crude enzyme solution. This crude enzyme solution (0.5 ml) was added to 2.5 ml of a 0.1 M sodium phosphate buffer (pH 6.5) containing 20 $\mu$l of 50 mM linoleic acid (dispersed in 0.2% Tween 20) or in 6 $\mu$l of 0.1 M 13-HPOD (in ethanol).

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*Abbreviations*: H(P)OD, hydro(pero)xy octadecadienoic acid; HPED, hydroperoxy eicosadienoic acid

The mixture was incubated at 26°C for 30 min. 1-Octanol (10 $\mu$g) was added as an internal standard to the mixture, and then 0.1 ml of 1 N NaOH was added to stop the reaction. The products were extracted with pentane (1 ml), and the extract was concentrated. The residue was analyzed by GC-MS. To quantify the aldehydes, *n*-heptanal (10 $\mu$mol) was added as an internal standard just before stopping the reaction. After this, 2 ml of a 0.1% 2,4-dinitrophenylhydrazine solution in ethanol containing 2% acetic acid was added, and the mixture was incubated at 25°C for 30 min. The hydrazone derivatives were analyzed as previously described.[5]

The crude enzyme solution (2.5 ml) was added to 12.5 ml of a 0.1 M sodium phosphate buffer (pH 6.5) containing 100 $\mu$l of 50 mM linoleic acid (in 0.2% Tween 20), 150 $\mu$l of 0.3 M glutathione and 450 units of glutathione peroxidase (Sigma). The mixture was incubated at 26°C for 30 min. As internal standards, 50 $\mu$l each of 1 mg/ml 1-octanol and 1 mM *n*-heptanal were added. 1-Octen-3-ol and *n*-hexanal were quantified as already shown. To quantify the oxygenated fatty acids, 100 $\mu$l of 2 mM 15-HPED was added as an internal standard to the reaction mixture (9 ml), and then 9 ml of tetrahydrofuran, 12 g of solid $NH_4SO_4$ and enough solid NaCl for saturation. The organic layer was collected. The oxygenated fatty acids were reduced by adding 50 $\mu$l of 0.1 M triphenylphosphine. The HODs were purified with preparative TLC (Merck, silica gel 60, 10 × 20 cm, *n*-hexane/ethyl acetate (2/1, v/v)). After being reacted with ethereal diazomethane, the resulting methylated HODs were quantitatively analyzed by HPLC with a Zorbax SIL column (0.25 m × 4.6 mm, 5 $\mu$m; Agilent Technologies). Elution was performed with hexane/ether (9/1, v/v) at a flow rate of 1.5 ml/min and the absorption at 205 nm was monitored. As authentic specimens, a mixture of 9-, 10-, 12- and 13-HPODs was prepared according to the reported procedure[6] by using methylene blue (Wako Pure Chemicals) as a photosensitizer. The structure of each HPOD was confirmed by a GC-MS analysis after reduction of the hydroperoxy group, catalytic hydrogenation of the double bonds with platinum oxide as a catalyst, and silylation of the hydroxy group with BSTFA (Aldrich).

An SPME analysis of the volatiles emitted from the homogenized fruiting bodies of *L. decadetes* indicated that the most abundant volatile was 1-octen-3-ol (Table 1). Other C8 compounds such as *n*-octan-1-ol, 3-octanone, 1-octen-3-one, and (*E*)-2-octen-1-ol could also be detected, although the composition of the volatiles varied considerably depending on the growth, freshness, and season of cultivation of the mushrooms (data not shown). This instability in volatile composition has also been observed with the shiitake mushroom.[7] After incubating with linoleic acid, the amount of 1-octen-3-ol had significantly increased, suggesting that it was formed enzymatically from the fatty acid. On the contrary, that of *n*-octan-1-ol showed little change. The oxidized form of 1-octen-3-ol, namely 1-octen-3-one, could be detected with the control, but it seemed to have been converted into other molecules during the 30-min incubation. C6 compounds such as *n*-hexanal and *n*-hexan-1-ol, or C9 compounds such as (*E*)-2-nonenal, which is usually formed through a lipoxygenase/fatty acid hydroperoxide lyase system in higher plants,[8] could also be detected, their amounts increasing after incubating with linoleic acid.

**Table 1.** Composition of the Volatile Compounds Identified by SPME in Fruiting Bodies of *L. decadetes*

| Compound | Content ($\mu$g/100 g fresh weight)* | |
|---|---|---|
| | Control | Incubation with linoleic acid |
| toluene | 15.7 ± 1.5 | 20.8 ± 5.8 |
| *n*-hexanal | 42.6 ± 30.0 | 175 ± 30.6 |
| 3-octanone | 31.0 ± 4.6 | 117 ± 38.9 |
| 1-octen-3-one | 40.1 ± 7.9 | nd |
| *n*-hexan-1-ol | 11.9 ± 2.8 | 40.7 ± 13.5 |
| acetic acid | 18.7 ± 2.9 | 36.1 ± 8.9 |
| 1-octen-3-ol | 699 ± 136 | 1088 ± 36.1 |
| *n*-heptan-1-ol | 12.6 ± 4.3 | 13.8 ± 6.0 |
| 2-ethyl-1-hexenol | 10.6 ± 2.7 | 12.9 ± 5.3 |
| (*E*)-2-nonenal | nd | 31.1 ± 5.7 |
| *n*-octan-1-ol | 403 ± 59.5 | 362 ± 117 |
| 2,2-dimethyl-propanoic acid | 13.5 ± 4.4 | 17.2 ± 3.9 |
| (*E*)-2-octen-1-ol | 43.7 ± 10.2 | 63.3 ± 19.7 |
| hexanoic acid | 12.8 ± 2.6 | 32.8 ± 8.2 |

* Each value is the average (n = 3) ± S.D.

**Table 2.** Formation of 1-Octen-3-ol and *n*-Hexanal from Linoleic Acid or Linoleic Acid 13-Hydroperoxide in a Crude Enzyme Solution Prepared from *L. decadetes*

| | Content ($\mu$mol/100 g fresh weight) | | |
|---|---|---|---|
| | Control | Linoleic acid | 13-HPOD |
| 1-octen-3-ol | 0.7 ± 0.4 | 11.0 ± 2.4 | 0.6 ± 0.2 |
| *n*-hexanal | 8.6 ± 0.2 | 170 ± 3.6 | 20.5 ± 0.3 |

In order to confirm the substrate-product relationship to form 1-octen-3-ol and *n*-hexanal, a crude enzyme extract was prepared from the fruiting bodies and incubated with linoleic acid or 13-HPOD as a substrate. As shown in Table 2, the amounts of both the volatiles increased after incubating the extract with linoleic acid. The reaction with 13-HPOD resulted in the formation of *n*-hexanal, while the amount of 1-octen-3-ol showed no change. The pH-activity profile to form 1-octen-3-ol from linoleic acid showed the highest activity at pH 6.5, and no activity at pH 8.0. On the contrary, the pH-activity profile to form *n*-hexanal from linoleic acid showed a broad pH optimum at around pH 6 to 7, and even at pH 8.0, half of the maximum activity could be observed. These results suggest that the fatty acid

**Table 3.** Effect of Glutathione Peroxidase on the Amounts of Volatiles and Linoleic Acid Hydroxides

| | Content (nmol/g fresh weight) | | |
|---|---|---|---|
| | Control | Linoleic acid | Linoleic acid + glutathione peroxidase |
| 1-octen-3-ol | 27.7 ± 3.9 | 147 ± 11.0 | 76.3 ± 6.8 |
| *n*-hexanal | 350 ± 188 | 1641 ± 79.2 | 1007 ± 62.1 |
| 10-HOD | 87.2 ± 29.7 | 61.6 ± 16.6 | 250 ± 38.9 |
| 13-HOD | 90.2 ± 14.7 | 226 ± 21.2 | 1987 ± 328 |

hydroperoxide lyases involved in the formation of the two volatile compounds can be discriminative.

It has been postulated that 10-HPOD is the intermediate for 1-octen-3-ol formation;[4,9] however, there is no direct evidence for the formation of 10-HPOD as the intermediate to form the C8 compound from linoleic acid. Glutathione peroxidase is an enzyme that reduces organic hydroperoxides to the corresponding hydroxides in the presence of glutathione and it has been widely used to trap an intermediate having a hydroperoxy group.[10] As shown in Table 3, the addition of linoleic acid enhanced the formation of 1-octen-3-ol, but when the reaction was performed in the presence of glutathione peroxidase and glutathione, this formation was effectively inhibited. This suggests that a fatty acid hydroperoxide(s) was the intermediate for the formation of 1-octen-3-ol. When the crude enzyme solution was incubated without any additive, 10- and 13-H(P)OD could be detected. In the presence of linoleic acid, no increase in the amount of 10-H(P)OD was apparent; however, a four-fold greater amount of it could be detected after the further addition of glutathione peroxidase, concomitant with a decrease of the amount of 1-octen-3-ol. The decrease in the amount of 1-octen-3-ol in the presence of the peroxidase (*ca.* 70 nmol/g fresh weight) was smaller than that of 10-HOD trapped (*ca.* 190 nmol/g fresh weight). Although the difference between these two values cannot be fully explained, it can be assumed that some of 10-HPOD formed might have been converted to compounds other than 1-octen-3-ol such as the 10-oxo form of linoleic acid. The amount of 13-H(P)OD increased more than two fold after additing linoleic acid, and further addition of glutathione peroxidase resulted in an almost 20-fold higher amount of 13-H(P)OD. Again, the amount of *n*-hexanal inhibited was smaller than that of 13-HPOD trapped. Other than these two oxygenated fatty acids, a compound that was thought to be 11-HOD from its MS spectrum could be detected, although its amount showed no change (data not shown). Neither 9- nor 12-H(P)OD could be detected. As we have shown, 13-H(P)OD cannot be converted into 1-octen-3-ol; thus, of the HPODs detected here, only 10-HPOD could have been the intermediate for the formation of 1-octen-3-ol.

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