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Purification and Characterization of Lipoxygenase from *Pleurotus ostreatus*

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Lipoxygenase was purified homogeneously from cups of *Pleurotus ostreatus* by Sephacryl S-400 HR gel filtration, Dyematrex Green A affinity, and DEAE-Toyopearl 650M ion-exchange chromatographies. The molecular weight of the enzyme was estimated to be 67 000 by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and 66 000 by gel filtration; the isoelectric point was pH 5.1. The optimum pH and temperature of the enzymatic activity were 8.0 and 25 °C, respectively. The enzyme contained non-heme iron, and a thiol group seemed to be involved in its activity. The K_m, V_{max}, and k_{cat} values of the enzyme for linoleic acid were 0.13 mM, 23.4 μmol·min^{-1}·mg^{-1}, and 25.7 s^{-1}, respectively. The enzyme showed high specificity toward linoleic acid. When linoleic acid was incubated with the enzyme, 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid was found to be the main oxidative product.

KEYWORDS: Lipoxygenase; mushroom; *Pleurotus ostreatus*; purification; flavor; 1-octen-3-ol

INTRODUCTION

Mushrooms have been consumed for a long time in many countries. The aroma contributes significantly to the characteristic flavor of mushrooms, and it is one of the attractive elements as a food material. Therefore, the aroma compounds have been analyzed in various species of mushrooms and many volatile compounds have been identified (*1*−*3*). These analyses have also revealed that the C_8 volatile compounds such as 3-octanol, 3-octanone, 2-octen-1-ol, and 1-octen-3-ol contribute to the aroma in most of species. Among them, 1-octen-3-ol, which is well-known as mushroom alcohol, is the most important aroma because of its abundance and low threshold value (*4*, *5*).

In general, 1-octen-3-ol is formed enzymatically by the oxidative cleavage of linoleic acid. Two pathways have been proposed in the biosynthesis. Tressl et al. (*6*) proposed the following pathway: 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-*Z*,*E*-HPOD) first results from the hydroperoxidation of linoleic acid by lipoxygenase (LOX) and is converted subsequently into 1-octen-3-one and 10-carbon compounds by a cleaving enzyme, and the former is finally reduced by alcohol oxidoreductase to 1-octen-3-ol. In contrast, Wurzenberger and Grosch (*7*−*10*) demonstrated in *Psalliota bispora* that linoleic acid is converted into 1-octen-3-ol via 10-hydroperoxyoctadecadienoic acid (10-HPOD) by LOX- and hydroperoxide lyase-like enzymes. However, these pathways are not completely clear, because little information has been obtained about the enzymes involved in the biosynthesis. For example, the LOX that produces 10-HPOD from linoleic acid has not yet been found in nature.

LOX is considered to be a key enzyme in the biosynthetic pathways of 1-octen-3-ol (*11*). This enzyme, which catalyzes the oxidation of polyunsaturated fatty acid containing a *Z*,*Z*-1,4-pentadiene moiety, is widely distributed in nature (*12*, *13*). The activity has been detected also in the various species of mushrooms (*14*−*16*). However, the homogeneous purification and characterization of LOX from mushroom has not been reported until now, as far as we know.

We examined LOX activity in eight species of industrial-cultivated mushrooms in Japan: *Lentinus edodes*, *Pleurotus ostreatus*, *Pholiota nameko*, *Flammulina velutipes*, *Hypsizigus marmoreus*, *Grifora frondosa*, and white and brown types of *Agaricus bisporus*. The result revealed that *P. ostreatus*, which emits a fairly pleasant aroma, shows the highest activity. In the present study, to solve the biosynthetic mechanism of 1-octen-3-ol, we purified LOX homogeneously from caps of the mushroom and examined the properties.

MATERIALS AND METHODS

Mushroom Material. Fresh mushroom, *P. ostreatus*, was obtained from Shirone garden (Niigata, Japan). Cut caps were frozen with liquid nitrogen and stored at −85 °C.

Chemicals. 4-Chloromercuribenzoic acid (*p*CMB), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and glycine ethyl ester hydrochloride (GEE) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and soybean LOX-1 were from Nacalai Tesque (Kyoto, Japan) and Serva

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Feinbiochemica GmbH.& Co. (Heidelberg, Germany), respectively. All other chemicals were of analytical grade.

Enzyme and Protein Assays. A substrate solution was prepared as described by Ben-Aziz et al. (*17*). LOX activity was determined either by a spectrophotometric procedure based on the formation of conjugated diene (*18*) or by a polarographic procedure based on the consumption of oxygen (*19*). The spectrophotometric procedure was performed in 3 mL of the standard reaction mixture containing 50 mM potassium phosphate buffer (pH 8.0), 125 μM linoleic acid, 0.0125% (v/v) Tween 20, and an appropriate amount of enzyme solution. Adding the substrate solution started the reaction, and the increase in absorbance at 234 nm ($\epsilon = 2.5 \times 10^4$ M^{-1} cm^{-1}) was measured at 25 °C with a spectrophotometer (U-2000, Hitachi, Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of hydroperoxide per min under the assay condition. The polarographic procedure was performed by using the same reaction mixture as described above except for the concentration of linoleic acid and Tween 20, 1.25 mM and 0.0075% (v/v), respectively. After the addition of the substrate solution, the oxygen consumption was measured at 25 °C with a Clark-type oxygen electrode (Rank Brothers, England). One unit of enzyme activity was defined as the amount of the enzyme consuming 1 μmol of O_2 per min under the assay condition.

The protein concentration was estimated from the absorbance at 280 nm or by the method of Bradford (*20*) with bovine serum albumin as the standard protein.

Purification. All steps were conducted at less than 4 °C. The frozen caps were homogenized with 20 mM potassium phosphate buffer (pH 7.0) containing 10 mM EDTA by using an Osterizer blender (Oster, Mexico) at 16 800 rpm for 30 s, and next an ultrasonic disruptor (UR-200P, Tomy Seiko, Tokyo, Japan) four times for 5 min. After the homogenate was centrifuged at 30000*g* for 20 min, the supernatant was recentrifuged at 90000*g* for 60 min. The supernatant was concentrated with poly(ethylene glycol) (PEG) 20 000 as described by Pohl (*21*), and the concentrate was used as a crude enzyme solution. The enzyme solution was passed through a Sephacryl S-400 HR column (2.6 × 70 cm, Amersham Pharmacia, Uppsala, Sweden) which was equilibrated with 20 mM potassium phosphate buffer (pH 7.0) at a flow rate of 1.0 mL/min. The eluate was collected in 4-mL fractions. The active fractions were combined and concentrated with PEG 20 000. The concentrate was loaded on a Dyematrex Green A column (1.4 × 8.5 cm, Amicon, Beverly, MA) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) at a flow rate of 0.4 mL/min. The column was thoroughly washed with the same buffer, and the adsorbed protein was eluted from the column with 500 mM potassium phosphate buffer (pH 7.0). The eluate was collected in 2-mL fractions. The active fractions were pooled and applied to a DEAE-Toyopearl 650M column (1.4 × 6.5 cm, Tosoh, Tokyo, Japan) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) at a flow rate 0.5 mL/min. The column was thoroughly washed with 70 mM potassium phosphate buffer (pH 7.0), and 2-mL fractions were collected. The adsorbed protein was eluted from the column with a linear gradient of 70−370 mM potassium phosphate buffer (pH 7.0) and collected in 1-mL fractions. The active fractions were combined, concentrated with PEG 20 000, dialyzed against 20 mM potassium phosphate buffer (pH 7.0), and used for subsequent studies.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed by the method of Laemmli (*22*). Proteins were visualized by silver stain (*23*).

Analysis of Iron. To remove extraneous metals, the enzyme solution was dialyzed against 250 volumes of metal-free water for 24 h at 4 °C. The iron concentration of the purified enzyme was determined by atomic absorption spectrophotoscopy by using an atomic absorption spectrophotometer (Z-8200, Hitachi, Tokyo, Japan).

Isoelectric Focusing. The isoelectric point of the enzyme was determined by two-dimensional electrophoresis with IPGphor IEF system (Amersham Pharmacia, Uppsala, Sweden) and Immobiline DryStrip Gel (pH 4−7, 11 cm) according to the method of Tasaki et al. (*24*).

Analysis of Reaction Products. The enzyme preparation of 1 unit was incubated at 25 °C in 10 mL of the reaction mixture containing 2.5 mM linoleic acid, 0.015% (v/v) Tween 20, and 50 mM potassium phosphate buffer (pH 8.0) with gentle aeration for 30 min. After extraction by the Bligh−Dyer extraction method (*25*), the reaction products were dried by the stream of nitrogen, dissolved in 2 mL of *n*-hexane/2-propanol/acetic acid (984:15:1, v/v/v), and then injected onto a LiChrosorb Si-60 column (0.46 × 25 cm, 5 μm, GL Sciences, Tokyo, Japan), which was equipped with a L-6300 pump (Hitachi, Tokyo, Japan) and a L-4250 UV−vis detector (Hitachi, Tokyo, Japan). The reaction products were eluted with *n*-hexane/2-propanol/acetic acid (984:15:1, v/v/v) at a flow rate of 1 mL/min and detected at 234 nm. The HPOD standards were prepared with partially purified potato LOX (*26*) and commercial soybean LOX-1.

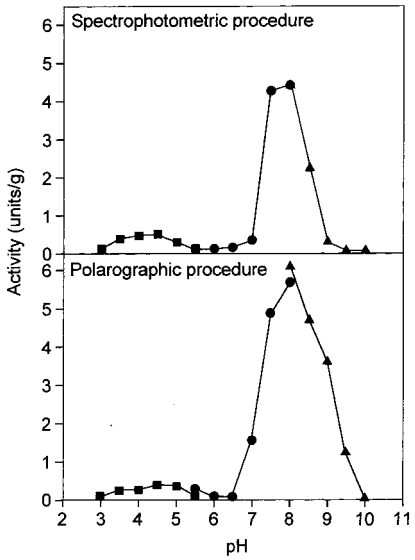

Figure 1. pH-LOX activity profile in the crude enzyme from *P. ostreatus*. The activity was assayed by spectrophotometric and polarographic procedures with the following buffers: ■, 50 mM acetate buffer (pH 3.0–5.5); ●, 50 mM potassium phosphate buffer (pH 5.5–8.0); ▲, 50 mM borate buffer (pH 8.0–10.0).

RESULTS

LOX Activity in the Crude Enzyme. The activity in the crude enzyme was assayed by spectrophotometric and polarographic procedures in the pH range from 3.0 to 10.0. As shown in **Figure 1**, two peaks were detected by both procedures: a major peak at pH 8.0 and a minor peak at pH 4.5.

Purification. LOX, which showed high activity in the alkaline region, was purified from *P. ostreatus* by Sephacryl S-400 HR gel-filtration chromatography (**Figure 2A**), Dyematrex Green A affinity chromatography (**Figure 2B**), and DEAE-Toyopearl 650M ion-exchange chromatography (**Figure 2C**). A summary of the purification is given in **Table 1**. The homogeneity of the purified enzyme was analyzed by SDS−PAGE. The result showed that it gives a single band (**Figure 3**). With the final step, the recovery of the purified enzyme activity was 5.4% with 126.3-fold purification from the crude enzyme solution (**Table 1**). Although the recovery was low, the purified LOX was used in the following experiments.

Molecular Weight and Isoelectric Point. The molecular weight of the enzyme was estimated to be approximately 67 000 and 66 000 by SDS−PAGE and gel filtration, respectively (**Figures 2A and 3**). The results show that the enzyme is a monomeric protein. The isoelectric point of the purified enzyme was found to be 5.1 by two-dimensional PAGE.

Effects of pH and Temperature. The effects of pH and temperature on the enzyme activity were examined with linoleic acid as substrate (**Figure 4**). The optimum pH of the enzyme was 8.0. A relatively small peak was also found at pH 4.5. After

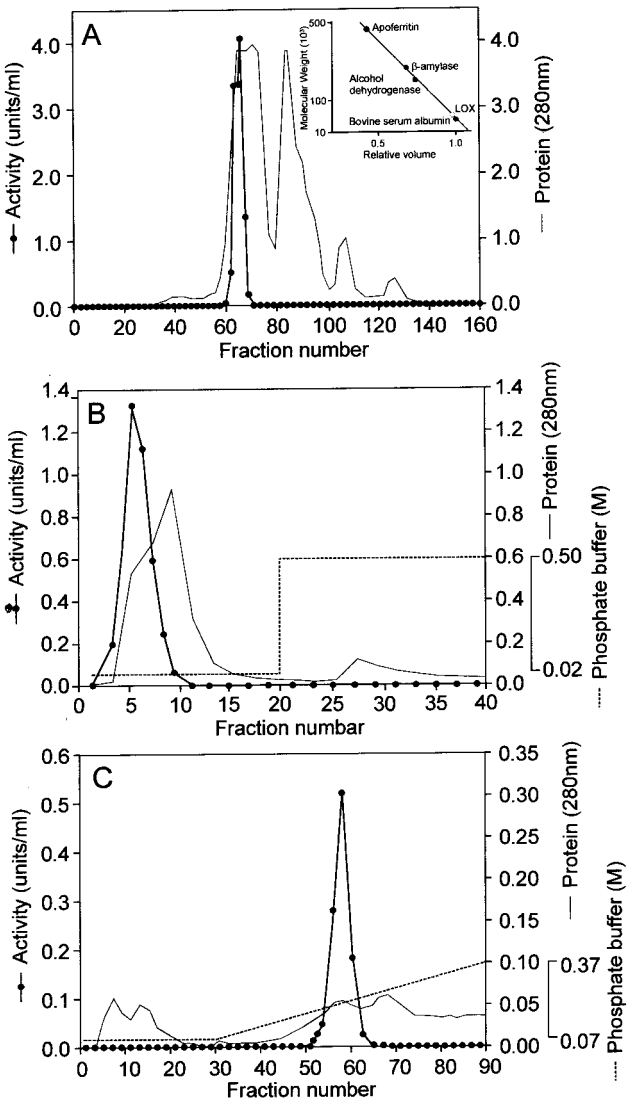

Figure 2. Purification of LOX from *P. ostreatus*. (A) Sephacryl S-400 HR gel filtration chromatography; (B) Dyematrex Green A chromatography; (C) DEAE-Toyopearl 650M chromatography. The inset in **Figure 2A** shows the estimation of molecular weight of LOX by Sephacryl S-400 HR.

Table 1. Summary of Purification of LOX from *P. ostreatus*

	protein (mg)	LOX activity (units)	specific activity (units/mg)	recovery (%)	purification (fold)
crude enzyme	451.9	117.8	0.3	100.0	1.0
Sephacryl S-400 HR	43.3	49.1	1.1	41.9	4.3
Dyematrex Green A	6.6	18.1	2.7	15.4	10.5
DEAE-Toyopearl 650M	0.2	6.4	33.0	5.4	126.3

the incubation at 4 °C for 24 h at various pH values, the activity was measured. The enzyme was stable at pH values between 5.0 and 8.5. The optimum temperature was estimated to be 25 °C. Pretreatment of the enzyme at various temperatures for 5 min revealed that the enzyme was relatively stable below 40 °C, but rapidly lost the activity above 40 °C.

Analysis of Iron. Atomic absorption spectrometry was used to investigate whether the purified enzyme contains Fe atom. The results revealed that only every fourth molecule of the enzyme still contains iron. Further, the absorbance spectrum of the enzyme was recorded with a spectrophotometer (U-2000, Hitachi, Tokyo, Japan). The absorption spectrum of the enzyme

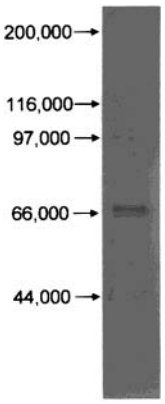

Figure 3. SDS–PAGE analysis of the purified LOX from *P. ostreatus*. About 2 μg of protein from the adsorbed active fractions in DEAE-Toyopearl chromatography, nos. 51–63 in **Figure 2C**, was submitted to electrophoresis and stained by a silver stain method. Molecular weights of the maker proteins are given to the left of the figure.

Table 2. Effects of Various Chemicals on the Activity of LOX from *P. ostreatus*

	relative activity (%)	
chemicals	1 mM	10 mM
control	100.0	100.0
sodium thiocyanate	90.6	89.9
o-phenanthroline	90.2	53.2
KCN	94.2	0.1
NaN_3	89.7	85.5
*p*CMB	1.1	–[a]
iodoacetate	99.5	0.1
TNBS	62.3	31.8
N-ethylmaleimide	97.4	83.5
EDC and GEE	89.3	83.6
Ca^{2+}	49.8	13.1
Mn^{2+}	21.6	8.8
Hg^{2+}	0.0	–[a]
Co^{2+}	12.9	4.6
Cu^{2+}	110.3	0.1

[a] LOX activity from *P. ostreatus* was not detected by the polarographic procedure.

showed a peak at 280 nm, and no characteristic absorbance of a heme protein in 300−800 nm was found, suggesting that the enzyme is a non-heme iron protein.

Effect of Various Chemicals. The effect of chemicals on the enzyme activity was examined. Chemicals were added to the reaction mixture without the substrate and incubated at 25 °C for 1 h. After the addition of substrate, the activity was monitored by the polarographic procedure. Because *N*-ethylmaleimide and *p*CMB were solubilized in ethanol, the control reaction was assayed in the mixture containing ethanol without the chemicals. Treatment with EDC and GEE was performed as described by Sanz et al. (*27*). For the investigation of metal ions, because they form insoluble salts with phosphate in the buffer used in the purification process, the enzyme was dialyzed at 4 °C for 5 h against 250 volumes of 20 mM Tris-HCl buffer (pH 7.0), and 50 mM Tris-HCl buffer (pH 8.0) was used as the reaction buffer. In addition, the substrate solution containing 50 mM borate buffer (pH 9.0) was used (*28*). The results are shown in **Table 2**. A chelating reagent for Fe^{3+}, sodium thiocyanate, had no stimulatory effect on the activity, whereas a chelating reagent for Fe^{2+}, *o*-phenanthroline, inhibited 50% of the activity at 10 mM. Although an inhibitor of heme protein, KCN inhibited the enzyme activity at 10 mM; NaN_3 also did not affect it. Among thiol-blocking agents tested, *p*CMB,

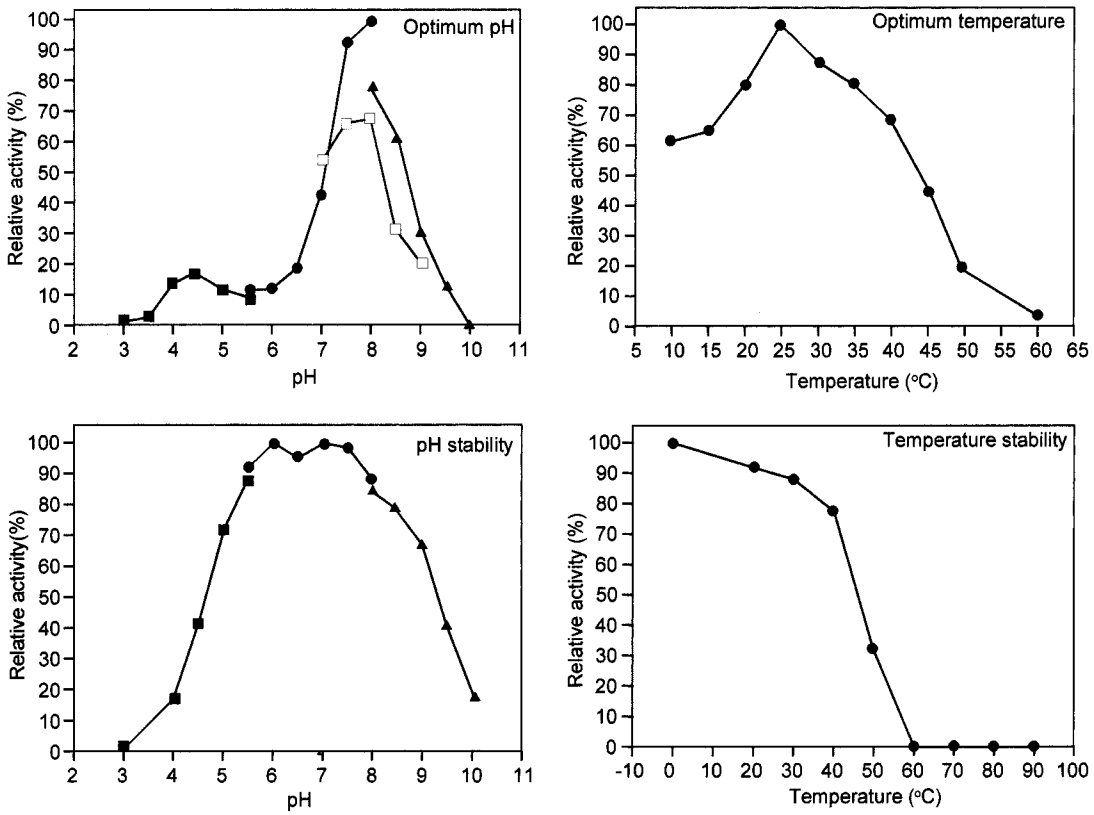

Figure 4. Effects of pH and temperature on the purified LOX activity. Optimum pH and pH stability of the enzyme were determined by using the following buffers: ■, 50 mM acetate buffer (pH 3.0–5.5); ●, 50 mM potassium phosphate buffer (pH 5.5–8.0); □, 50 mM Tris-HCl buffer (pH 7.0–9.0); ▲, 50 mM borate buffer (pH 8.0–10.0).

Table 3. Substrate Specificity of LOX from *P. ostreatus*

substrate	relative activity (%)
linoleic acid	100.0
linolenic acid	76.3
γ-linolenic acid	25.5
1-monolinolein	13.7
1,3-dilinolein	0.4
trilinolein	0.0

iodoacetate, and TNBS inhibited the activity, but *N*-ethylmaleimide did not change it. EDC and GEE, a modifying agent for carboxyl group, had no significant effect on the activity. Ca^{2+}, Mn^{2+}, Hg^{2+}, and Co^{2+} greatly inhibited the enzyme activity. Cu^{2+} had no effect on the activity at 1 mM, but inhibited it at 10 mM.

Substrate Specificity. Relative activities of the purified enzyme toward various substrates at 125 μM are shown in **Table 3**. The enzyme exhibited the highest activity toward linoleic acid among the free polyunsaturated fatty acids tested, and a high level of activity toward linolenic acid. However, the activity toward γ-linolenic acid was low. Acylglycerols such as 1-monolinolein, 1,3-dilinolein, and trilinolein were poor substrates. The K_m, V_{max}, and k_{cat} values for linoleic acid were determined from Lineweaver−Burk plots under the conditions of a spectrophotometric procedure. They were found to be approximately 0.13 mM, 23.4 μmol·min^{-1}·mg^{-1}, and 25.7 s^{-1}, respectively.

Analysis of Reaction Products. For characterization of the specificity of purified enzyme, the products obtained from linoleic acid were analyzed by straight-phase high-performance liquid chromatography (**Figure 5**). The elution profile showed one major peak at 24.05 min and one minor peak at 32.43 min. The standards of 13-*Z*,*E*-HPOD and 13-hydroperoxy-9*E*,11*E*-

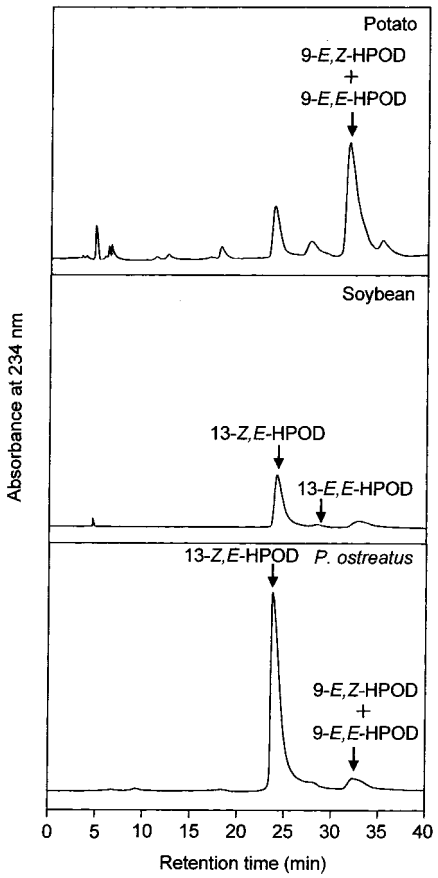

Figure 5. High-performance liquid chromatography analysis of linoleic acid oxygenized by the purified LOX.

octadecadienoic acid (13-*E*,*E*-HPOD) prepared with LOX-1 from soybean had retention times of 24.42 and 28.38 min, respectively. The standards of 9-hydroperoxy-10*E*,12*Z*-octadecadienoic acid (9-*E*,*Z*-HPOD) and 9-hydroperoxy-10*E*,12*E*-octadecadienoic acid (9-*E*,*E*-HPOD) obtained from partially purified potato LOX were found as one peak with a retention time of 32.09 min. The comparison of retention times with the standards revealed that the products of purified enzyme are 13-*Z*,*E*-HPOD and 9-HPODs, which are produced with a ratio of 9:1. In addition, the correspondence of retention time between the reaction products and the standards was confirmed by cochromatography analysis.

DISCUSSION

In this study, we purified LOX homogeneously from *P. ostreatus* by using gel-filtration, affinity, and ion-exchange chromatographies. LOX is regarded as a key enzyme in the biosynthesis of 1-octen-3-ol in mushrooms. However, to the best of our knowledge, the isolation of this enzyme from mushrooms has not been reported. Therefore, the catalytic properties of the purified enzyme would provide the biosynthesis of 1-octen-3-ol with the important information.

The apparent LOX activity in the crude enzyme was measured at various pH values. The pH-activity profile showed two active peaks in both spectrophotometric and polarographic procedures. Their activities at the optimum pH 8.0 were 4.3 and 6.0 units/g, respectively, and only a small difference was found between the procedures. Therefore, we considered that it was possible to monitor the major LOX activity by the simple spectrophotometric procedure at pH 8.0 during the purification. The result also shows that the LOX in *P. ostreatus* mainly converts linoleic acid to the products containing conjugated diene, that is, not 10-HPOD but 13-*Z*,*E*-HPOD may be the intermediate of 1-octen-3-ol biosynthesis in the cap from *P. ostreatus*.

The purified enzyme is considered to consist of a single subunit with a molecular weight of about 66 000. This molecular weight is smaller than that of the typical LOXs from plant, which are generally about 90 000 (*29*−*31*). Because a part of native LOXs in plants has been reported to be proteolytically cleaved to produce a fragment of about 65 000, it is possible that the purified enzyme in this study is a proteolytic product. However, in some plant sources such as avocado (*32*) and horse bean (*33*), LOXs with the molecular weights of 66 000−74 000, which are almost the same as that of *P. ostreatus*, have also been found. On the other hand, the LOXs from fungal sources seem to have a broad range of molecular weights from 13 000 in *Fusarium oxysporum* (*34*) to 100 000 in *Thermomyces lanuginosus* (*35*), although the reports are few.

The pH-activity profile of the purified enzyme was similar to that of crude enzyme. Moreover, there was no active fraction that was detectable by the spectrophotometric procedure in the purification process besides the purified enzyme. These indicate that this enzyme is the main LOX in cap of *P. ostreatus*. In general, enzymes have one optimum pH. However, the purified LOX shows two active peaks at pH 4.5 and 8.0. A similar property has been reported in a few enzymes; for example, a homogeneous glutamyl endopeptidase from *Bacillus intermedius* has two pH optima at 7.5 and 9.0 for casein (*36*). The property of the purified LOX is probably natural.

The purified LOX showed the maximum activity at 25 °C and had high activity even at relatively low temperatures; for example, at 10 °C, the enzyme retained about 60% of its maximum activity. The activity of purified LOX at low temperatures is higher than that of other LOXs such as soybean (*37*) and sweet corn germ (*38*). The initiation of fruiting is usually caused by the stimulation of low temperatures (*39*). Mau et al. (*40*) reported that 10-oxo-*trans*-8-decenic acid produced concurrently with 1-octen-3-ol might be involved in the development of fruit bodies. Because it has been assumed that the formation of 1-octen-3-ol requires actions of LOX and hydroperoxide lyase-like enzyme, LOX in the mushroom may play some role in the fruiting.

Many LOXs from plant and animal sources have one non-heme iron atom per enzyme molecule (*13*, *41*). The purified enzyme from *P. ostreatus* also seems to contain non-heme iron. This is supported by the lack of absorption in the wavelength range of 300−800 nm, the inhibitory effect of *o*-phenanthroline, a chelating reagent for Fe^{2+}, and the lack of inhibitory effect of KCN at 1 mM and NaN_3, inhibitors of heme protein. KCN at 10 mM strongly inhibited the enzyme activity. Although cyanide is used frequently to discriminate between heme protein and non-heme LOX, the use at a high concentration causes a cleavage of a disulfide bond. Actually, KCN at a high concentration reduces the activities of LOXs (*42*−*44*). The atomic absorption spectrometry of the enzyme also demonstrated the presence of iron atom, but only every fourth molecule of it still contained iron. Although the purified enzyme was stored at −20 °C until the amount required for the analysis of iron was accumulated, the stored enzyme had lost about 90% of its original activity. Percival (*45*) described that the catalytic iron atom could not be detected from inactivated LOX, because the enzyme was destroyed and the iron atom was lost by the exposure to oxygen. Therefore, the result means that the iron atom, which is probably contained in the ratio of one atom per molecule, might come off the enzyme during the storage. Typical thiolytic inhibitors inhibited the activity significantly when the enzyme was preincubated with them, indicating that the thiol groups are involved in the enzymatic activity, similarly to other LOXs (*46*, *47*). This also suggests that the oxidation of thiol groups may cause the inactivation of enzyme during the storage. As shown in **Table 1**, the recovery in the purification process was low. One of the reasons is also considered to be the oxidation of thiol groups; the addition of an appropriate reducing regent such as dithiothreitol and 2-mercaptoethanol to the buffers for purification and storage may be effective to increase the recovery of enzyme and iron atom.

The increase of LOX activity by stimulation of metal ions has been reported in some sources such as *F. oxysporum* (*34*), soybean (*48*), and sea algae (*49*). However, the purified enzyme was strongly inhibited by all metal ions tested. Only two concentrations of metal ions used are not enough to explain the inhibition mechanism. In addition to their direct effects on the enzyme, they could be considered to affect the substrate, for example, the oxidation by oxidizing metal ions such as Cu^{2+}.

The analysis of substrate specificity of the purified LOX showed that free linoleic acid, which is the most abundant fatty acid (72.4% of total fatty acid) in the pileus of *P. ostreatus* (*50*), was the best substrate. The analysis of reaction products revealed that the purified LOX mainly converts linoleic acid to 13-*Z*,*E*-HPOD. As described in the introduction, 13-*Z*,*E*-HPOD and 10-HPOD have been proposed as precursors of 1-octen-3-ol, suggesting that the purified LOX occupying most of the LOX activity in *P. ostreatus* may be involved in the synthesis of 1-octen-3-ol via 13-*Z*,*E*-HPOD. However, Assaf et al. (*51*, *52*) have indicated that 13-*Z*,*E*-HPOD was not converted into 1-octen-3-ol in mycelial homogenate of *Pleurotus pulmonarius*.

More detailed studies are required about whether the purified LOX is actually involved in the formation of 1-octen-3-ol in *P. ostreatus*.

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LITERATURE CITED

(1) Lizárraga-Guerra, R.; Guth, H.; López, M. G. Identification of the most potent odorants in huitlacoche (*Ustilago maydis*) and austern pilzen (*Pleurotus* sp.) by aroma extract dilution analysis and static head-space samples. *J. Agric. Food Chem.* **1997**, *45*, 1329−1332.

(2) Rapior, S.; Gavalié, S.; Andary, C.; Pélissier, Y.; Marion, C.; Bessière, J.-M. Investigation of some volatile components of seven fresh wild mushrooms (Basidiomycetes). *J. Essent. Oil Res.* **1996**, *8*, 199−201.

(3) Tressl, R.; Bahri, D.; Engel, K.-H. Formation of eight-carbon and ten-carbon components in mushrooms (*Agaricus campestris*). *J. Agric. Food Chem.* **1982**, *30*, 89−93.

(4) Maga, J. A. Mushroom flavor. *J. Agric. Food Chem.* **1981**, *29*, 1−4.

(5) Mau, J.; Beelman, R. B.; Ziegler, G. R. Aroma and flavor components of cultivated mushrooms. *Dev. Food Sci.* **1994**, *34*, 657−684.

(6) Tressl, R.; Bahri, D.; Engel, K.-H. Lipid oxidation in fruits and vegetables. *ACS Symp. Ser.* **1981**, *170*, 213−232.

(7) Wurzenberger, M.; Grosch, W. The enzymic oxidative breakdown of linoleic acid in mushrooms (*Psalliota bispora*). *Z. Lebensm.-Unters.-Forsch.* **1982**, *175*, 186−190.

(8) Wurzenberger, M.; Grosch, W. Origin of the oxygen in the products of the enzymatic cleavage reaction of linoleic acid to 1-octen-3-ol and 10-oxo-*trans*-8-decenoic acid in mushrooms (*Psalliota bispora*). *Biochim. Biophys. Acta* **1984**, *794*, 18−24.

(9) Wurzenberger, M.; Grosch, W. The formation of 1-octen-3-ol from the 10-hydroperoxide isomer of linoleic acid by a hydroperoxide lyase in mushrooms (*Psalliota bispora*). *Biochim. Biophys. Acta* **1984**, *794*, 25−30.

(10) Wurzenberger, M.; Grosch, W. Stereochemistry of the cleavage of the 10-hydroperoxide isomer of linoleic acid to 1-octen-3-ol by a hydroperoxide lyase from mushrooms (*Psalliota bispora*). *Biochim. Biophys. Acta* **1984**, *795*, 163−165.

(11) Hadar, Y.; Dosoretz, C. G. Mushroom mycelium as a potential source of food flavour. *Trends Food Sci. Technol.* **1991**, *2*, 214−218.

(12) Robinson, D. S.; Wu, Z.; Domoney, C.; Casey, R. Lipoxygenases and the quality of foods. *Food Chem.* **1995**, *54*, 33−43.

(13) Prigge, S. T.; Boyington, J. C.; Faig, M.; Doctor, K. S.; Gaffney, B. J.; Amzel, L. M. Structure and mechanism of lipoxygenases. *Biochimie* **1997**, *79*, 629−636.

(14) Belinky, P. A.; Masaphy, S.; Levanon, D.; Hadar, Y.; Dosoretz, C. G. Effect of medium composition on 1-octen-3-ol formation in submerged cultures of *Pleurotus pulmonarius*. *Appl. Microbiol. Biotechnol.* **1994**, *40*, 629−633.

(15) Mau, J.-L.; Beelman, R. B.; Ziegler, G. R. 1-Octen-3-ol in the cultivated mushroom, *Agaricus bisporus*. *J. Food Sci.* **1992**, *57*, 704−706.

(16) Bisakowski, B.; Atwal, A. S.; Kermasha, S. Characterization of lipoxygenase activity from a partially purified enzymic extract from *Morchella esculenta*. *Process Biochem.* **2000**, *36*, 1−7.

(17) Ben-Aziz, A.; Grossman, S.; Ascarelli, I.; Budowski, P. Linoleate oxidation induced by lipoxygenase and heme proteins. *Anal. Biochem.* **1970**, *34*, 88−100.

(18) Sanz, L. C.; Perez, A. G.; Olias, J. M. Purification and catalytic properties of chickpea lipoxygenases. *Phytochemistry* **1992**, *31*, 2967−2972.

(19) Ohta, H.; Ida, S.; Mikami, B.; Morita, Y. Purification and characterization of rice lipoxygenase component 3 from embryos. *Agric. Biol. Chem.* **1986**, *50*, 3165−3171.

(20) Bradford, M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(21) Pohl, T. Concentration of proteins and removal of solutes. *Methods Enzymol.* **1990**, *182*, 68−83.

(22) Laemmli, U. K. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (London)* **1970**, *227*, 680−685.

(23) Oakley, B. R.; Kirsch, D. R.; Morris, N. R. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **1980**, *105*, 361−365.

(24) Tasaki, S.; Awang, A. B.; Hara, T.; Joh, T. Effect of phosphate deficiency on growth and protein profile in three strains of *Pholiota nameko*. *Mycoscience* **2001**, *42*, 489−498.

(25) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.

(26) Sekiya, J.; Aoshima, H.; kajiwara, T.; Togo, T.; Hatanaka, A. Purification and some properties of potato tuber lipoxygenase and detection of linoleic acid radical in the enzyme reaction. *Agric. Biol. Chem.* **1977**, *41*, 827−832.

(27) Sanz, L. C.; Perez, A. G.; Rios, J. J.; Olias, J. M. Physicochemical properties of chickpea lipoxygenases. *Phytochemistry* **1992**, *31*, 3381−3384.

(28) Surrey, K. Spectrophotometric method for determination of lipoxygenase activity. *Plant Physiol.* **1964**, *39*, 65−70.

(29) Chen, A. O.; Whitaker, J. R. Purification and characterization of a lipoxygenase from immature English peas. *J. Agric. Food Chem.* **1986**, *34*, 203-211.

(30) Fornaroli, S.; Petrussa, E.; Braidot, E.; Vianello, A.; Macrì, F. Purification of a plasma membrane-bound lipoxygenase from soybean cotyledons. *Plant Sci.* **1999**, *145*, 1−10.

(31) Clemente, A.; Olías, R.; Olías, J. M. Purification and characterization of broad bean lipoxygenase isoenzymes. *J. Agric. Food Chem.* **2000**, *48*, 1070−1075.

(32) Marcus, L.; Prusky, D.; Jacoby, B. Purification and characterization of avocado lipoxygenase. *Phytochemistry* **1988**, *27*, 323−327.

(33) Nicolas, J.; Drapron, R. Some physicochemical characteristics of horse bean lipoxygenase. (Vicia faba L.). *Ann. Technol. Agric.* **1977**, *26*, 119−132.

(34) Matsuda, Y.; Satoh, T.; Beppu, T.; Arima, K. Purification and properties of Co^{2+} requiring heme protein having lipoxygenase activity from *Fusarium oxysporum*. *Agric. Biol. Chem.* **1976**, *40*, 963−976.

(35) Li, D.-C.; Lui, Z.-W.; Lu, J. Purification and characterization of lipoxygenase from the thermophilic fungus *Thermomyces lanuginosus*. *Mycol. Res.* **2001**, *105*, 190−194.

(36) Leshchinskaya, I. B.; Shakirov, E. V.; Itskovitch, E. L.; Balaban, N. P.; Mardanova, A. M.; Sharipova, M. R.; Viryasov, M. B.; Rudenskaya, G. N.; Stepanov, V. M. Glutamyl endopeptidase of *Bacillus intermedius*, strain 3−19. *FEBS Lett.* **1997**, *404*, 241−244.

(37) Fauconnier, M.-L.; Marlier, M. Purification of soybean lipoxygenase isoenzyme-1 and characterization of its inhibition by 13-hydroperoxides. *Grasas Aceites* **1996**, *47*, 242−246.

(38) Theerakulkait, C.; Barrett, D. M. Lipoxygenase in sweet corn germ: isolation and physicochemical properties. *J. Food Sci.* **1995**, *60*, 1029−1033.

(39) Kües, U.; Liu, Y. Fruiting body production in basidiomycetes. *Appl. Microbiol. Biotechnol.* **2000**, 54, 141−152.

(40) Mau, J.-L.; Beelman, R. B.; Ziegler, G. R. Effect of 10-oxo-*trans*-8-decenoic acid on growth of *Agaricus bisporus*. *Phytochemistry* **1992**, *31*, 4059−4064.

(41) Shidata, D.; Axelrod, B. Plant lipoxygenases. *J. Lipid Mediators Cell Signaling* **1995**, *12*, 213−228.

(42) Sanders, T. H.; Patee, H. E.; Singleton, J. A. Lipoxygenase isozymes of peanut lipids. *Lipids* **1975**, *10*, 681−685.

(43) Kermasha, S.; Metche, M. Characterization of seed lipoxygenase of *Phaseolus vulgaris* cv, Haricot. *J. Food Sci.* **1986**, *51*, 1224−1227.
(44) de Lumen, B. O.; Stone, E. J.; Kazeniac, S. J.; Forsythe, R. H. Formation of volatile flavor compounds in green beans from linoleic and linolenic acids. *J. Food Sci.* **1978**, *43*, 698−702.
(45) Percival, M. D. Human 5-lipoxygenase contains an essential iron. *J. Biol. Chem.* **1991**, *266*, 10058−10061.
(46) Spaapen, L. J.; Verhagen, J.; Veldeink, G. A.; Vliegenthart, J. F. The effect of modification of sulfhydryl groups in soybean lipoxygenase-1. *Biochim. Biophys. Acta* **1980**, *618*, 153−162.
(47) Herman, R. P.; Hamberg, M. Properties of the soluble arachidonic acid 15-lipoxygenase and 15-hydroperoxide isomerase from the oomycete *Saprolegnia parasitica*. *Prostaglandins* **1987**, *34*, 129−139.
(48) Zimmerman, G. L.; Snyder, H. E. Role of calcium in activating soybean lipoxygenase 2. *J. Agric. Food Chem.* **1974**, *22*, 802−805.
(49) Kuo, J.-M.; Hwang, A.; Yeh, D.-B.; Purification, substrate specificity, and products of a Ca^{2+}-stimulating lipoxygenase from sea algae (*Ulva lactuca*). *J. Agric. Food Chem.* **1997**, *45*, 2055−2060.
(50) Hiroi, M. Fatty acid composition of mushroom lipids and chemotaxonomy. *Trans. Mycol. Soc. Jpn.* **1988**, *29*, 449−470.
(51) Assaf, S.; Hadar, Y.; Dosoretz, C. G. Biosynthesis of 13-hydroperoxylinoleate, 10-oxo-8-decenoic acid, and 1-octen-3-ol from linoleic acid by a mycelial-pellet homogenate of *Pleurotus pulmonarius*. *J. Agric. Food Chem.* **1995**, *43*, 2173-2178.
(52) Assaf, S.; Hadar, Y.; Dosoretz, C. G. 1-Octen-3-ol and 13-hydroperoxylinoleate are products of distinct pathways in the oxidative breakdown of linoleic acid by *Pleurotus pulmonarius*. *Enzyme Microb. Technol.* **1997**, *21*, 484−490.

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