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Intracellular Substrates of a Heme-Containing Ascorbate Oxidase in *Pleurotus ostreatus*

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A novel heme-containing ascorbate oxidase isolated from oyster mushroom, *Pleurotus ostreatus*, catalyzes oxidation of ascorbic acid (Kim *et al.*, 1996). In this report, we describe the identification of intracellular substrates of the enzyme in the mushroom. Six compounds, which can serve as substrate of the heme-containing ascorbate oxidase, were identified as L-ascorbic acid, D-erythroascorbic acid, 5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, 5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid, 5-methyl-5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, and 5-methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid. All of the compounds were oxidized at a significant rate by the heme-containing ascorbate oxidase. Oxidation of the compounds produced equimolar amounts of hydrogen peroxide per mole of substrate.

***Keywords*:** ascorbate oxidase, substrates, *Pleurotus ostreatus*

Ascorbic acid is a γ-lactone with enediol as a functional group. The compound is oxidized by a successive reversible one-electron transfer process to produce ascorbate free radical and subsequently dehydro-L-ascorbic acid (Sapper *et al*., 1982). Dehydro-L-ascorbic acid is unstable and can be further degraded through the formation of intermediates including 2,3-diketo-L-gulonic acid, α-ketoaldehydes, and some enediol compounds (Kang *et al*., 1982; Kang, 1985). In the cell, the ascorbate redox system consisting of L-ascorbic acid, ascorbyl free radical and dehydro-L-ascorbic acid seems to be controlled by ascorbate oxidase (Lee and Dawson, 1973), NADH-dependent semidehydro-L-ascorbate oxidoreductase (De Leonardis *et al*., 1995), glutathione-dependent dehydro-L-ascorbate oxidoreductase (Arrigoni *et al.*, 1981), and NADPH-dependent thioredoxin reductase (May *et al*., 1997).

The oxidation of L-ascorbic acid in higher plants is catalyzed by ascorbate oxidase (Lee and Dawson, 1973; Marchesini and Kroneck, 1979). According to the crystal structure of green zucchini ascorbate oxidase, the enzyme contains copper in the active site (Messerschmidt *et al*., 1992). The copper catalyzes the transfer of electrons from L-ascorbic acid to oxygen, resulting in the formation of H_2O.

However, the oxidation of L-ascorbic acid in microorganism seems to be controlled by a novel type of ascorbate oxidase. An ascorbate oxidase purified from an oyster mushroom, *Pleurotus ostreatus* catalyzes the oxidation of L-ascorbic acid to dehydro-L-ascorbic acid (Kim *et al*., 1996). In contrast to the copper-containing ascorbate oxidase, the enzyme contains heme as a prosthetic group that catalyzes the transfer of electrons from L-ascorbic acid to oxygen, resulting in the formation of H_2O_2. The enzyme also catalyzes the oxidation of L-ascorbic acid analogs such as D-ascorbic acid, L-erythroascorbic acid and D-erythroascorbic acid that are chemically synthesized. From this substrate specificity of the enzyme it has been suggested that the C-4 configuration of the substrate is essential for its binding to the enzyme. On the other hand, there is no report on the presence of L-ascorbic acid and its analogs in *P. ostreatus*.

In the present study, we report the identification of intracellular substrates for the heme-containing ascorbate oxidase in *P. ostreatus*.

Materials and Methods

Organism and culture condition for the development of fruit body

A white-rot fungus, *Pleurotus ostreatus* NFFA2, was obtained from National Federation of Forestry Association in Korea. The cultures were maintained on an yeast malt extract (YM) agar slant at 4°C and subcultured every four weeks. Dikaryotic vegetative mycelia were inoculated into a Petri dish containing 20 ml of sucrose-asparagine (SA) medium. The mycelia were incubated in the dark at 25±2°C for 10 days and further incubated in alternate light and dark (8 h-light per day) at 10±2°C for 20 days. The white light intensity was 1200-1700 Lux. The relative humidity was 55±5%. The YM medium was composed of 3 g of malt extract, 3 g of yeast extract, 5 g of bacto-peptone, 10 g of glucose per liter of distilled water. The SA medium consisted of following con-

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stituents per liter of deionized water (Prillinger and Molitoris, 1979): 20 g of sucrose, 0.88 g of asparagine, 2 g of $NH_4H_2PO_4$, 1 g of L-valine, 0.224 g of $K_2HPO_4{\cdot}3H_2O$, 0.803 g of KH_2PO_4, 0.99 g of $MgSO_4{\cdot}7H_2O$, 0.02 g of $CaCl_2$, 5 ml of trace element solution, 5 ml of vitamin nucleotide solution. The trace element solution consisted of 0.89 g of $ZnSO_4{\cdot}7H_2O$, 0.765 g of $MnSO_4{\cdot}H_2O$, 0.73 g of Fe(III)-citrate, 0.20 g of $CuSO_4{\cdot}5H_2O$ and distilled water to 1,000 ml. The vitamin-nucleotide solution consisted of 1.60 g of adenosine, 0.02 g of thiamine-HCl and distilled water to 1,000 ml. To prepare solid agar medium 15 g of agar powder was added to 1 L of medium solution.

Chemicals

D-Erythroascorbic acid was synthesized by the method described (Liang *et al*., 1990). Deuterium oxide, sulfuric acid, L-ascorbic acid, and methanol were purchased from Merck. Sephadex G-10, Sephadex G-15, dithiothreitol, 2,6-dichlorophenol indophenol and trifluoroacetic acid were purchased from Sigma Chemical Company. Other reagents used were of analytical grade of commercial sources.

Detection of L-ascorbate-like compounds

The fruit body was immersed in 1.5 volumes of 95% methanol containing 0.1% dithiothreitol. After 1 h-extraction, the sample was centrifuged at 10,000×g for 30 min. To detect L-ascorbic acid-like compounds in the extracts, the supernatant was analyzed by the method with modification (Tsao and Young, 1985). The supernatant was loaded onto two tandem-linked ODS hypersil columns (Hewlett Packard, 100 mm × 4.6 mm), and chromatographed on a Waters 510 HPLC equipped with 460 electrochemical detector. The eluent was 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The potential of the detector was set at 0.80 V vs. Ag/AgCl reference electrode. In order to determine which EC-reactive compounds can serve as a substrate of ascorbate oxidase, methanol was partially removed by evaporation using speed vacuum concentrator (Savant Instrument), and then the mixture was incubated with 2 units of heme-containing ascorbate oxidase in 50 mM citrate-phosphate buffer (pH 5.6) at 25°C for 40 min. The reaction mixture was analyzed by HPLC described above. In order to compare retention times of the ascorbate oxidase-reactive compounds with those of L-ascorbic acid and D-erythroascorbic acid, the extracts were supplemented with L-ascorbic acid or D-erythroascorbic acid and then analyzed by HPLC as described.

Isolation of the L-ascorbate-like compounds

The fruit bodies were frozen and then immersed in 1.5 volumes of 95% methanol containing 0.1% dithiothreitol at 20°C. The sample was thawed and blended at 4°C with Omni Mixer for 2 min. The suspension was stored on ice for 1 h, then was centrifuged at 10,000×g for 30 min. The supernatant was passed through a column containing Dowex 50-X8 (200-400 mesh) cation-exchange resin in the hydrogen form equilibrated with 95% methanol. Methanol was removed from the solution by evaporation under reduced pressure at 40°C using Rotavapor EL 131 (Büchi, Switzerland), and then the mixture was freeze dried. The powder was resuspended with 0.1% trifluoroacetic acid and then centrifuged at 10,000×g for 30 min. The supernatant was filtrated through a Sephadex G-15 gel column with 0.05% trifluoroacetic acid as a eluent. The fractions containing compounds, as assayed by titration with 2,6-dichlorophenol indophenol, were pooled. The pooled solution was freeze-dried. The powder was resuspended with 0.1% trifluoroacetic acid solution. The sample was applied to a DeltaPak C_{18} column and chromatographed isocratically with 0.1% trifluoroacetic acid at a flow rate of 20 ml/min using a Waters Delta Prep 4000 Preparative Chromatography System (Waters, USA). The compounds were monitored at 254 nm. Four well-separated peaks (P3, P4, P5, and P6) were detected. Each peak was pooled and freeze-dried. The powder was dissolved in 5 mM sulfuric acid and then applied to an Aminex HPX-87H column in the hydrogen form (Bio-Rad, 30 cm×7.8 mm). The column was eluted with 5 mM sulfuric acid at a flow rate of 0.7 ml/min, and fractions were collected and their UV-spectra were recorded. In the final step, the sample was filtrated through Sephadex G-10 gel column with 0.025% trifluoroacetic acid as an eluent. The eluent containing the compounds was freeze-dried and then used to identify its structure.

Spectrophotometric analysis

The isolated compounds were dissolved in 0.1% trifluoroacetic acid (pH 1.8) and then the absorbance between 200 nm and 400 nm was measured. The pH of the solution was adjusted to 7.5 by addition of 10 μl of 1M of K_2HPO_4 and then absorbance between 200 nm and 400 nm was measured again with Shimadzu UV-265 spectrophotometer.

Elemental analysis

In order to find out what kinds of atoms are present and to determine the relative numbers of the different kinds of atoms in the molecule, the amounts of carbon, hydrogen, oxygen, nitrogen and sulfur were determined by elemental analyzer (Carlo Erba Co. EA 1108) three times. The combustion temperature was 1,000°C.

Negative ion thermospray liquid chromatography mass spectrometry

Thermospray mass spectra were recorded using a VG Quattro quadruple mass spectrometer equipped with a thermospray interface. Sample was introduced via a Waters HPLC system (Waters Associates, USA) equipped with an ODS hypersil column (Hewlett Packard, 100 mm×4.6 mm). The mobile phases were methanol/0.01 M ammonium acetate (70:30) at a flow rate of 0.6 ml when P3 and P4 components were analyzed, and methanol/0.1 M ammonium acetate (70:30) at a flow rate of 0.3 ml when P5 and P6 components were analyzed. The temperatures of ion source and probe were 250°C and 200°C, respectively.

Nuclear magnetic resonance spectroscopy

All NMR experiments were performed on a Bruker AMX 500 spectrometer equipped with 5 mm probes at 303 K. One dimensional ^{1}H-NMR spectra in D_2O were acquired with a spectral width of 3.0 kHz. Two dimensional ^{1}H-^{1}H correlation spectra in D_2O were recorded. The spectral width was 2767 Hz in both dimensions. This spectrum was

acquired with 2048 complex data points in t_1 and 2048 data points in t_2 in the TPPI mode. One dimensional ^{13}C-NMR spectra in D_2O were acquired with a spectral width of 26.3 kHz. Two-dimensional ^{1}H-^{13}C correlation experiment in D_2O at natural abundance was recorded in the inverse mode with 256 complex points in t_1 and 2048 complex points in t_2. The spectral widths were 8064 Hz in t_1 and 2525 Hz in t_2.

Gas chromatography

The identity of sugar residues in the compounds was determined by preparing alditol acetates from its respective sugar residues and comparing retention times of the alditol acetates in gas chromatograph with those of the alditol acetates of standard monosaccharides. The alditol acetates were prepared by the method described (Albersheim *et al*., 1967).

Circular dichroism spectroscopy

The CD spectra were recorded with a CD-spectropolarimeter JASCO J720 in 0.02 M phosphate buffer (pH 7.2). P4 compound was hydrolyzed with 2 N trifluoroacetic acid in a sealed tube at 121°C for 30 min. The erythroascorbic acid residue were isolated from the hydrolyzate by HPLC on an Aminex HPX-87H column with 0.005 M sulfuric acid as an eluent.

Kinetic studies of oxidation of L-ascorbate-like compounds

The heme-containing ascorbate oxidase was purified from homogenate prepared from the vegetative mycelia as described (Kim *et al*., 1996). The reactivity of compounds present in *P. ostreatus* to the purified ascorbate oxidase was studied by incubation of 0.578 μM of enzyme in 1 ml of 0.1 M sodium phosphate citrate buffer, pH 5.4 containing 0.5 mM EDTA and ascorbate or its analogs at various concentrations. The reaction mixture incubated at 25°C for 5 min, and then the amount of substrates was measured by the method described (Oberbacher and Vines, 1963). All experiments were conducted two times. K_m and k_{cat} values were calculated from Lineweaver-Burk plots of the data.

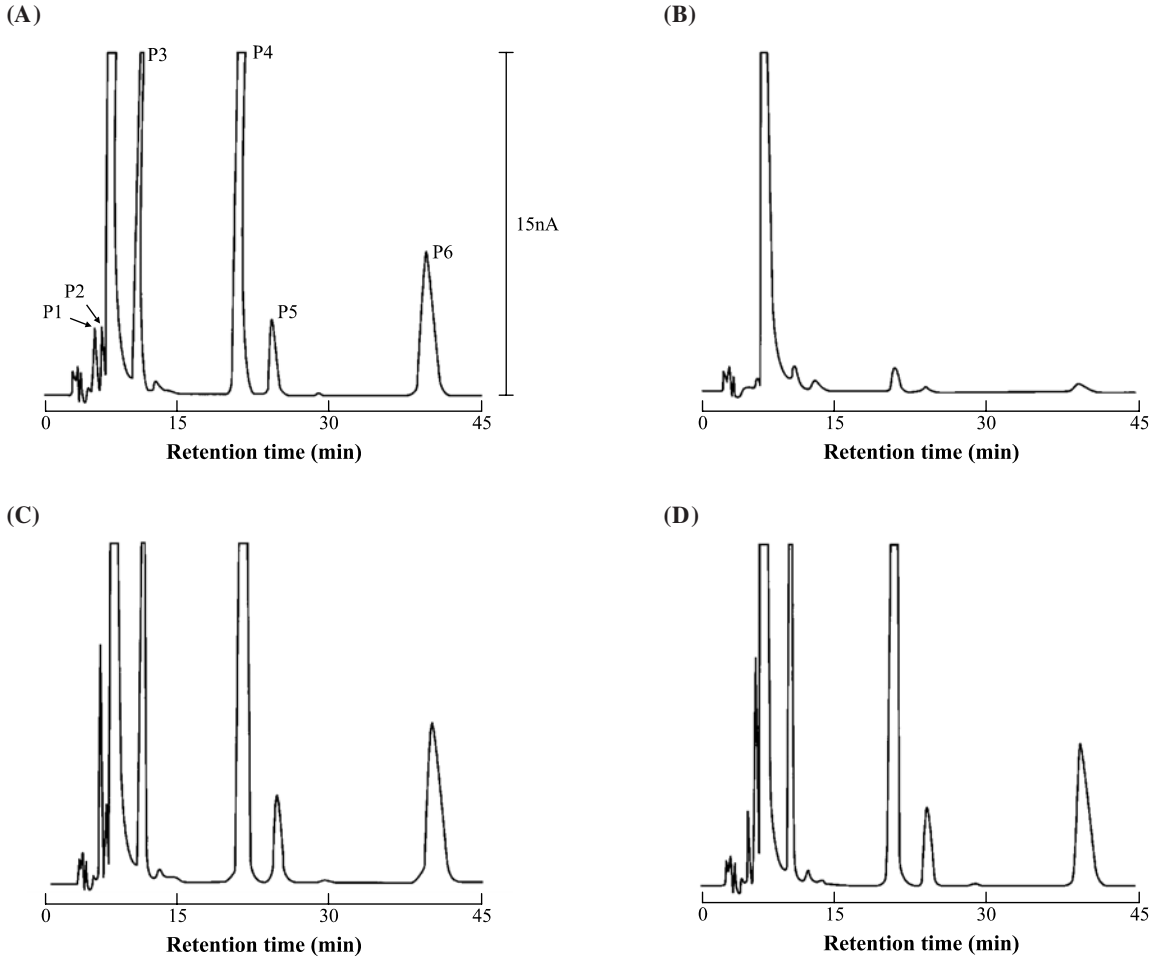

Fig. 1. HPLC chromatograms of the L-ascorbate-like compounds that can serve as a substrate for the ascorbate oxidase found in oyster mushroom, *Pleurotus ostreatus*. (A) Chromatogram of extract prepared from fruit body of oyster mushroom. (B) Resulting chromatogram of extract after incubation with ascorbate oxidase (2 units) from *P. ostreatus*. (C) Chromatogram of extract supplemented with L-ascorbic acid (4 ng). (D) Chromatogram of extract supplemented with D-erythroascorbic acid (4 ng).

Measurement of hydrogen peroxide

Hydrogen peroxide generated during the oxidation of the compounds by the heme-containing ascorbate oxidase. The content of the hydrogen peroxide was measured by a HPLC-fluorometry method (Guibualt *et al*., 1969) with some modifications: the ascorbate oxidase reaction was stopped by addition of 10% *m*-phosphoric acid and then hydrogen peroxide was separated from the remaining compounds by HPLC equipped with a ODS hypersil column (Hewlett Packard, 200 mm×4.6 mm) and then reacted with in the incubation mixture. Mobile Phase was 80% methanol containing 0.5 mM KH_2PO_4 at a flow rate of 1.0 ml/min. The post-column incubation mixture was 0.2 M potassium phosphate (pH 8.0) containing 10 mM *p*-hydroxyphenylacetic acid, 2 mM EDTA, 2 mM sodium azide, and 31.2 units/ml horseradish peroxidase at a flow rate of 0.5 ml/min. the fluorescence was measured in a spectrofluorometer (SLM Aminco 48000S) equipped with a flow cell. The excitation and emission wavelength used is 318 nm and 405 nm, respectively. A standard curve was prepared using 1~100 μM of hydrogen peroxide

Results

An oyster mushroom, *Pleurotus ostreatus* produced primordia and subsequently fruit bodies from dikaryotic vegetative mycelia on a synthetic medium, when incubated in the alternate light and dark for additional 18 days at 10±2°C after the incubation in the dark for 10 days at 25±2°C. However, the fungus remained mycelia if it was continuously incubated in the dark at 10±2°C. These results are consistent with the previous observations (Eger *et al*., 1974; Zadražil, 1974) that light stimulated development of fruit body from vegetative mycelia.

The fruit body was harvested and then ground with Omni Mixer using 1.5 volumes of 95% methanol containing 0.1% dithiothreitol. The cell-free extracts were centrifuged at 4°C for 30 min at 10,000×g. The resulting supernatant was evaporated partially to remove methanol using speed vacuum concentrator. The residue was then incubated with heme-containing ascorbate oxidase of *P. ostreatus* in 50 mM sodium phosphate citrate buffer, pH 5.6 at 25°C for 40 min, since the enzyme was found to catalyze the oxidation of the compounds that contain a lactone ring carrying a vicinal enediol adjacent to a carbonyl group (Kim *et al*., 1996). The reaction mixture was analyzed by high performance liquid chromatography (HPLC) coupled with electrochemical detector set at 0.80 V *vs* Ag/AgCl reference electrode. We found six compounds that can serve as substrate of the ascorbate oxidase in the extract (Fig. 1A and B). When the extract was cochromatographed with L-ascorbic acid or D-erythroascorbic acid, two (P1 and P2 peaks) of the compounds were co-eluted with L-ascorbic acid and D-erythroascorbic acid, respectively, and four others were eluted later than D-erythroascorbic acid (Fig. 1C and D). The results indicated that in addition to L-ascorbic acid and D-erythroascorbic acid (hereafter called as P1 and P2 compounds), four other compounds (hereafter called as P3, P4, P5, and P6) present in the extracts of fruit body can serve as substrate of ascorbate oxidase.

The compound co-eluted with L-ascorbic acid was iso-

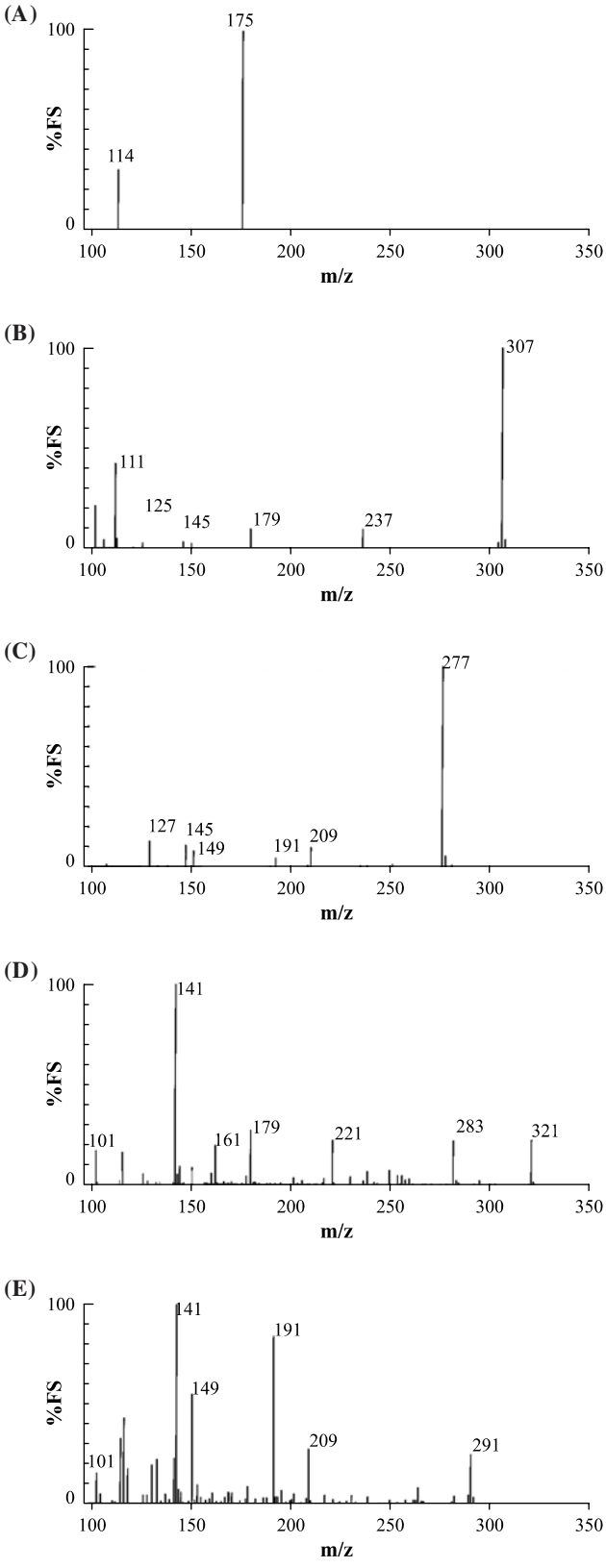

Fig. 2. Negative ion thermospray LC-MS spectra of the P1, P3, P4, P5, and P6 compounds isolated from oyster mushroom. (A) P1 compound, (B) P3 compound, (C) P4 compound, (D) P5 compound, (E) P6 compound.

lated by chromatography and its identity was confirmed by comparing their mass spectra obtained by thermospray LC-MS (Fig. 2A). The four compounds designated as P3, P4, P5, and P6 were isolated from the extract of fruit body by sequential chromatographies. Each purified compound had the ability to reduce 2,6-dichlorophenol-indophenol (data not shown). It was also oxidized by copper-containing ascorbate oxidase from *Cucurbita* species (data not shown). Their U.V.-absorption spectra exhibited bathochromic shift accompanied by hyperchromic effect upon increasing the pH of the solvent. These properties were quite similar to those of L-ascorbic acid and D-erythroascorbic acid, indi-

Table 1. Selected physical data for the substrates of ascorbate oxidase

Method	P1	P2	P3	P4	P5	P6
HPLC, t_R[a]	5.12	5.87	9.38	19.34	22.78	37.92
LC-MS, m/z[b]	175	145	307	277	321	291
GC			Glucose	Xylose	Glucose	Xylose
^{1}H-NMR[c]						
H-4	4.97 (1.82)	4.94	5.20	5.06	4.76 (1.73)	4.91 (1.66)
H-5	4.08	4.05 (2.54, 11.83)	4.16 (2.54, 11.83)	4.03 (2.52, 12.08)	4.07	4.18
H-5		3.86 (3.59)	4.10 (3.79)	3.95 (3.79)		
5-CH_3					1.23 (6.53)	1.39 (6.60)
H-6	3.77 (6.17, 11.54)					
H-6	3.75 (6.75)					
H-1'[d]			5.06 (3.68)	4.89 (3.58)	4.85 (3.53)	4.97 (3.21)
H-2'			3.65 (9.90)	3.50 (9.60)	3.34 (9.40)	3.50 (8.74)
H-3'			3.75 (9.17)	3.55 (9.04)	3.37 (8.33)	3.47 (8.67)
H-4'			3.54 (9.94)	3.58	3.29 (10.06)	3.57
H-5'			3.74	3.67 (5.10, 10.40)	3.36	3.65 (5.62, 10.76)
H-5'				3.45 (10.54)		3.40 (10.80)
H-6'			3.96 (2.39, 12.39)		3.67 (2.29, 12.57)	
H-6'			3.90 (4.59)		3.61 (3.50)	
^{13}C-NMR[c]						
C-1	173.614	173.616	173.571	173.582	173.743	173.790
C-2	118.095	118.159	117.898	118.213	117.711	117.759
C-3	156.072	155.597	156.079	155.572	156.501	156.563
C-4	76.550	77.572	76.194	76.279	79.235	79.245
C-5	69.251	59.703	65.047	65.357	69.478	70.000
5-CH_3					15.316	15.504
C-6	62.407					
C-1'			98.575	98.964	96.136	96.499
C-2'			71.558	71.703	71.344	71.389
C-3'			73.189	73.449	73.059	73.237
C-4'			69.667	69.707	69.168	69.498
C-5'			72.290	61.765	72.299	61.811
C-6'			60.678		60.215	

[a] t_R, retention time (min).
[b] m/z, mass to charge ratio of (M-H) ion.
[c] Chemical shifts (ppm) in δ-scale and coupling constants (Hz) in parenthesis.
[d] The prime numbering system is used to refer to positions of carbon atoms on the sugars because the structure of erythroascorbic acid is numbered as in Fig. 3.

cating that all the compounds have a enediol group as a functional group.

The structure of the compounds were identified by HPLC, UV absorption spectroscopy, molecular ion analysis by thermospray liquid chromatography mass spectrometry, ^{1}H- and ^{13}C-NMR spectrometry, carbohydrate analysis of the sugar residues using gas chromatography, and circular dichroism spectroscopy of the erythroascorbic acid residue.

First, the structure of the P4 compound was determined. Molecular ion analysis of the P4 compound by thermospray LC-MS revealed its mass to be 278. A peak at 145 m/z was attributable to an erythroascorbic acid residue (Fig. 2C).

The ^{1}H-NMR spectrum and ^{13}C-NMR spectrum of the P4 compound indicated that it is a glycoside of erythroascorbic acid. The ^{13}C-NMR spectrum showed 10 carbon signals at 173.582, 155.572, 118.213, 98.964, 76.279, 73.449, 71.703, 69.707, 65.357, and 61.765 ppm. The resonance at 173.582 ppm was characteristic of a lactone carbonyl carbon of the erythroascorbic acid residue and resonances at 155.572 and 118.213 ppm were characteristic of carbons of the enediol functional group of the erythroascorbic acid residue. The resonances with chemical shifts in the range of 61.765 to 98.964 ppm also indicated the presence of a sugar residue. The ^{1}H-NMR spectrum contained five quartets at 5.06, 4.03, 3.95, 3.67, 3.50 ppm, a doublet at 4.89 ppm, two triplets at 3.55 and 3.45 ppm and an octet at 3.58 ppm.

The carbon resonance at 98.964 ppm showed correlation to its directly bonded proton resonance at 4.89 ppm which was characteristic of the anomeric proton of the sugar residue in the ^{13}C-^{1}H single-bond correlation spectrum. The ^{13}C-^{1}H correlation spectrum also showed the presence of two methylene groups with geminal protons and five methenyl groups in the P4.

Starting from the downfield proton resonances, connectivity to the rest of the protons for the individual residues can be assigned from cross-peaks in the ^{1}H-^{1}H correlation spectrum. The anomeric resonance of the sugar residue, at 4.89 ppm, gave a cross-peak to its H-2' signal at 3.89 ppm, and connectivity to the H-5' (methylene) protons at 3.67 and 3.45 ppm could be traced out. Similarly, the H-4 signal of the erythroascorbic acid residue at 5.06 ppm gave cross-peaks to H-5 (methylene) signals at 4.03 and 3.95 ppm.

The proton coupling constant, $^{3}J_{H\text{-}H}$, can be used to distinguish axial from equatorial protons and to determine the anomeric configuration and the stereochemical configuration of the sugar residue. The coupling constants between transdiaxial protons are in the range of 7 to 10 Hz and the constants involving equatorial protons are in the range of 0 to 3 Hz (Abeygunawardana *et al*., 1991). For the sugar residue, the ^{1}H-^{1}H correlation spectrum showed that $^{3}J_{H2'\text{-}H3'}$ and $^{3}J_{H3'\text{-}H4'}$ values were large. These results imply that H-2', H-3', and H-4' are axial in the residue and that it has gluco-configuration. The small value of $^{3}J_{H1'\text{-}H2'}$ implies that the residue has the α configuration. Therefore, the sugar residue are identified as a pentopyranose with the α-gluco configuration.

Once the proton assignment is made, the carbon assignment can be obtained by direct correlation to their respective protons in the ^{13}C-^{1}H correlation spectrum. The proton and carbon assignments of the P4 compound were given in Table 1.

In order to identify the α-pentopyranose residue, carbohydrate analysis was performed by gas chromatography. The gas chromatograms of the alditol acetates prepared from the sugar residue and the standard monosaccharides. Gas chromatography of the sugar residue gave a peak at the same retention time as that of xylose. Therefore, the sugar residue was assigned to be α-xylose.

The CD spectrum of the erythroascorbic acid residue was quite similar to D-erythroascorbic acid. This implies that the erythroascorbic acid has D-configuration.

The position of the linkage between the erythroascorbic acid residue and the α-xylose residue was inferred from ^{13}C-NMR spectral data. Substitution of the hydroxyl hydrogen atom with a carbon atom causes shift of chemical shifts of the contiguous and adjacent carbon atoms. In general, *O*-alkylation causes an approximately 5 to 10 ppm downfield shift of the contiguous carbon atom and a small upfield shift of the neighboring carbon atoms (Eugan, 1980). The more downfield chemical shift of C-5 carbon and more upfield chemical shift of C-4 carbon of the erythroascorbic acid residue in comparison with those of their respective carbons of erythroascorbic acid imply that the linkage position is C-5-O on the erythroascorbic acid residue. Therefore, The P4 compound was identified as 5-*O*-(*α*-D-xylopyranosyl)-D-erythroascorbic acid (XyloEASC).

When the P4 compound was submitted to elemental analysis, it was found that the compound was composed of 41.2% of carbon, 5.2% of hydrogen, and 51.0% of oxygen. This result was quite similar to the composition calculated for $C_{10}H_{14}O_9$: C, 43.2%; H, 5.1%; O, 51.8 %.

The structure of the purified P3, P5, and P6 compounds was similarly determined. The results were summarized in Table 1. Molecular ion analysis of P3 compound by thermospray LC-MS revealed its mass to be 308. Peaks occurring at 145 and 179 m/z were attributable to an erythroascorbic acid residue and a sugar residue, respectively (Fig. 2B). The ^{1}H- and ^{13}C-NMR spectra and the GC analysis of the sugar residue of the P3 compound indicated that the compound was 5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid (GlcEASC).

Molecular ion analysis of P5 compound by thermospray LC-MS revealed its mass to be 322. Peaks occurring at 141 and 179 m/z were attributable to the fragments produced by the cleavage of *O*-glycosidic bond (Fig. 2D). The ^{1}H- and ^{13}C-NMR spectra and the GC analysis of the sugar residue of the P5 compound indicated that the compound was 5-methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid (MxyloEASC).

Molecular ion analysis of P6 compound by thermospray LC-MS revealed its mass to be 292. Peaks occurring at 141 and 145 m/z were attributable to the fragments produced by the cleavage of *O*-glycosidic bond (Fig. 2E). The ^{1}H- and ^{13}C-NMR spectra and the GC analysis of the sugar residue of the P6 compound indicated that the component was 5-methyl-5-*O*-(*α*-D-glucopyranosyl)-D-erythroascorbic acid (MGlcEASC). In addition, ^{1}H-^{13}C and ^{1}H-^{1}H correlation spectra (data not shown) of the P6 compound verified the ^{1}H/^{13}C and ^{1}H/^{1}H interactions.

The data in Table 1 reveal that P1, P2, P3, P4, P5, and P6

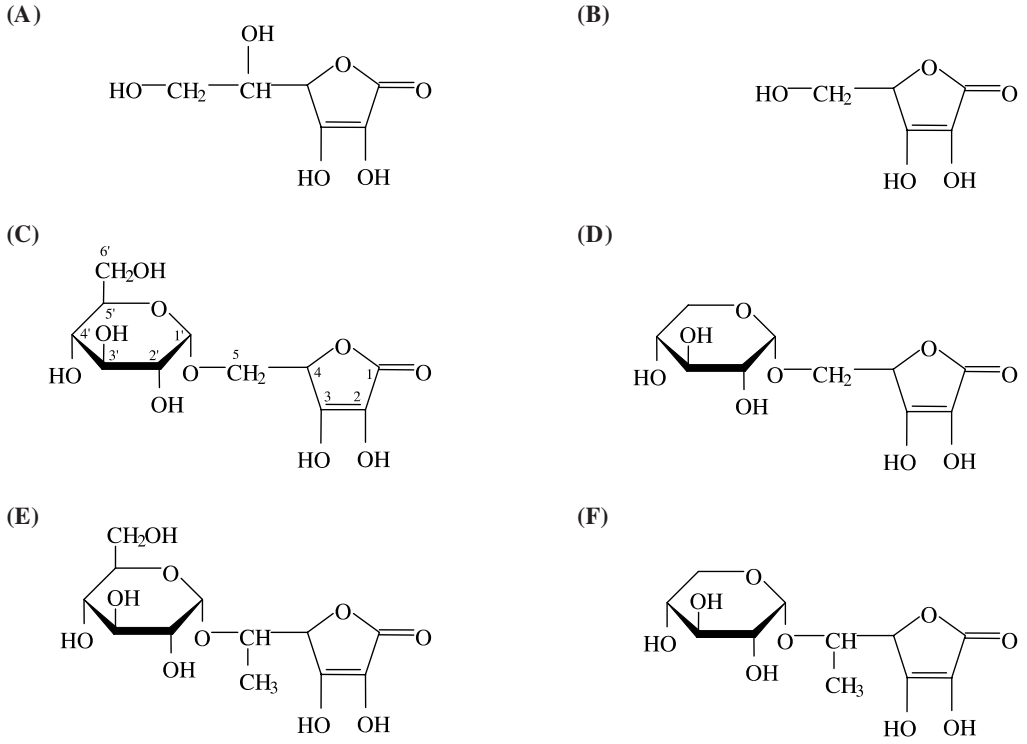

Fig. 3. Structure of substrates of the heme-containing oxidase in *P. ostreatus*. (A) P1 compound; L-ascorbic acid. (B) P2 compound; D-erythroascorbic acid. (C) P3 compound; 5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid. (D) P4 compound; 5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid. (E) P5 compound; 5-methyl-5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid. (F) P6 compound; 5-methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid.

are L-ascorbic acid, D-erythroascorbic acid, 5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, 5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid, 5-methyl-5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, and 5-methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid, respectively (Fig. 3).

The heme-containing ascorbate oxidase was purified from mycelia, and its activity for the compounds was determined. All of the compounds tested were oxidized at a significant rate by the enzyme. The kinetic data were summarized in Table 2. The K_m values of L-ascorbic acid, D-erythroascorbic acid, 5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, 5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid, 5-methyl-5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid and 5-methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid were 338, 304, 402, 642, 433, and 162 μM, respectively. And their k_{cat}/K_m values were 0.12, 0.22, 0.09, 0.12, 0.07, and 0.08 $\mu M^{-1}min^{-1}$, respectively. Oxidation of glycosides of erythroascorbic acid produced equimolar amounts of hydrogen peroxide per mole of substrate, like that of L-ascorbic acid (Table 2).

Discussion

L-Ascorbic acid is a ubiquitous and essential substance in higher organisms such as plants and animals (Szent-Györgyi, 1928). Its derivatives, ascorbate-3-sulfate and 2-methylascorbate are present in human and rat, respectively (Blaschke and Hertting, 1970; Baker *et al*., 1971). Its five-carbon analog, erythroascorbic acid is present in the ascomycetes (Dumbrava and Pall, 1987) and imperfect fungus, *Candida albicans* (Huh *et al*., 1994). Erythroascorbic acid is produced *in vitro* during the oxidative degradation of L-ascorbic acid (Kang *et al*., 1982; Kang, 1985). In this study, we showed that in

Table 2. Kinetic parameters for the reaction of the heme-containing ascorbate oxidase with the substrates present in oyster mushroom

Substrate	K_m	k_{cat}	k_{cat}/K_m	Ratio of H_2O_2 produced to substrate
	μM	min^{-1}	μM^{-1} min^{-1}	
L-Ascorbic acid	338	39.7	0.12	0.8
D-Erythroascorbic acid	304	67.1	0.22	0.9
5-*O*-(α-D-Glucopyranosyl)-D-erythroascorbic acid	402	36.0	0.09	1.0
5-*O*-(α-D-Xylopyranosyl)-D-erythroascorbic acid	642	77.2	0.12	0.8
5-Methyl-5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid	433	33.2	0.07	1.0
5-Methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid	162	14.6	0.08	0.9

addition to L-ascorbic acid and D-erythroascorbic acid, glycosides of D-erythroascorbic acid and their methylated derivatives; 5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid, 5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid, 5-methyl-5-*O*-(α-D-glucopyranosyl)-D-erythroascorbic acid and 5-methyl-5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid were present in oyster mushroom, *Pleurotus ostreatus*. The glycosides of D-erythroascorbic acid and their methylated compounds were also found in the fruit bodies of *Lentinus edodes* and *Agaricus bisporus* (data not shown) and other mushrooms (Okamura, 1994). This implies that they are fairly ubiquitous metabolites in mushrooms.

The oxidation of L-ascorbic acid in plants is catalyzed by copper-containing ascorbate oxidase to produce dehydroascorbic acid and water as reaction products (White and Smith, 1961; Lee and Dawson, 1973; Marchesini and Kroneck, 1979). In contrast to plants, in fungi, all of L-ascorbic acid, D-erythroascorbic acid and glycosides of D-erythroascorbic acid were oxidized by a heme-containing ascorbate oxidase to produce their oxidized compounds and hydrogen peroxide as reaction products. Glycosylation of erythroascorbic acid resulted in a slight decrease of the affinity for the enzyme, while methylation of the glycosylated compounds resulted in a slight increase of the affinity for the enzyme. These results indicated that there is almost no steric restriction in the side-chain binding domain of the ascorbate oxidase and glycosylation and/or methylation at C-5 of erythroascorbic acid slightly affect binding of the substrates to the active site of enzyme.

Many species of higher fungi develop fruit body from vegetative mycelia upon stimulation of light (Carlile, 1974; Manachère, 1980). However, the detailed mechanism for the differentiation is not elucidated yet. On the other hand, the changes of oxidation-reduction state can induce or accompany physiological changes that culminate in differentiation (Allen *et al*., 1988; Hansberg *et al*., 1993). In *P. ostreatus*, upon stimulation by light at low temperature, a burst increase in production of L-ascorbic acid, glycosides of erythroascorbic acid and their methylated compounds occurred in the vegetative mycelia. The contents of glycosides of erythroascorbic acid and their methylated compounds increased again in the primordia and the fruit bodies (Lee and Kang, unpublished data). From the results, the heme-containing ascorbate oxidase and its substrates; L-ascorbic acid, glycosides of erythroascorbic acid and their methylated compounds might play an important role in the development of fruit body in *P. ostreatus.*

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