A novel type of ascorbate oxidase was purified 420-fold from the cytosolic fraction of the mycelia of Pleurotus ostreatus with an overall yield of 13%. The molecular mass of the native enzyme determined by high performance gel permeation chromatography was 94 kDa. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the enzyme consists of two subunits with a molecular mass of 46 kDa. The N-terminal amino acid sequence of the enzyme was Asp-Val-Lys-Thr-Leu-Glu-Glu-His-Leu-Gln-Leu-Ala-Leu-Met-Val-. The enzyme was optimally active at pH 5.2, monitored at 37 °C. The enzyme had affinity toward L-ascorbic acid, D-ascorbic acid, and L-erythroseoarobic acid, and D-erythroseoarobic acid. Under optimal conditions, the \( K_m \) value of the enzyme toward L-ascorbic acid was 0.48 mM. The absorption spectra of the native enzyme exhibited a Soret maximum at 418 nm in its oxidized form and at 426 nm in its reduced form, and \( \alpha \) and \( \beta \) bands at 558 and 572 nm only in its reduced form, respectively. On the basis of spectral changes after treatment with cyanide and carbon monoxide, the enzyme is a hemoprotein, quite similar to b-type cytochrome, and contains 2 mol of heme per molecule. The reaction catalyzed by the enzyme was L-ascorbic acid + \( O_2 \) → dehydro-L-ascorbic acid + \( H_2O_2 \).

L-Ascorbic acid is oxidized by a successive reversible one-electron transfer process with a free radical intermediate, and thus the ascorbate redox system consists of L-ascorbic acid, L-ascorbyl free radical, and dehydro-L-ascorbic acid (1). In biological systems, the relative steady state level of L-ascorbic acid and dehydro-L-ascorbic acid seems to be maintained by ascorbate oxidase (2), glutathione-dependent dehydro-L-ascorbate oxidoreductase (3), and NADH-dependent semidehydro-L-ascorbate oxidoreductase (4).

The oxidative degradation of L-ascorbic acid in plants seems to be controlled by copper-containing ascorbate oxidase (2). After the oxidation by ascorbate oxidase, it has been suggested that dehydro-L-ascorbic acid can be transformed chemically or through the biological catalytic pathway composed of 2,3-diketogulonic acid, \( \alpha \)-ketoaldehyde, and some enediol compounds (5, 6).

Ascorbate oxidase is found mostly in the peripheral parts of the plant, closely associated with the cell-wall material during plant growth, such as in the germination of pumpkin seeds (7, 8). Although various biochemical and biological functions of ascorbate oxidase have been discussed, the in vivo role of ascorbate oxidase in plants is still under debate.

Ascorbate oxidases, which have been purified from higher plants, such as green zucchini (2, 9) and cucumber (10), catalyze the oxidation of L-ascorbic acid to dehydro-L-ascorbic acid and belong to blue copper oxidases including laccase and ceruloplasmin. The refined crystal structure of the fully oxidized form of ascorbate oxidase from green zucchini was recently reported (11).

On the other hand, there are almost no reports on the oxidation of ascorbic acid in microorganisms. Even though the occurrence of ascorbate oxidase was reported in Myrothecium verrucaria (12) and there was a report on the purification and characterization of the enzyme from the culture filtrate of Acremonium sp. H1-25, which contains copper in its active site (13, 14), the properties of intracellular ascorbate oxidase in microorganisms are not yet clarified. In the present study, we report the purification and characterization of a novel type of an intracellular heme-containing ascorbate oxidase from an oyster mushroom, Pleurotus ostreatus.

**EXPERIMENTAL PROCEDURES**

**Materials—** L-Ascorbic acid and D-ascorbic acid were purchased from Merck, Sepharose CL-6B, reactive red 120-Sepharose CL-4B, Sephadex G-200, S Sepharose CL-6B, and molecular mass markers for high performance gel filtration chromatography from Sigma, Protein PAK SW300 and Pico-Tag column from Waters, poly(vinylidene difluoride) membrane from Millipore, and molecular mass standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis from Boehringer Mannheim. L-Erythroseoarobic acid and D-erythroseoarobic acid were prepared according to the methods of Gan and Seib (15). All other reagents used were of the highest quality generally available.

**Microorganism and Growth Conditions—** The mycelium of white-rot fungus Pleurotus ostreatus, a kind gift from the Korean Forest Research Laboratory, was grown in complete medium containing 2% malt extract (w/v), 0.5% peptone (w/v), 0.5% yeast extract (w/v), and 1% glucose (w/v). It was cultivated for 4 days at 28°C in a 500-mL Erlenmeyer flask containing 200 mL of medium in a reciprocally shaking water bath operating at 100 rpm.

**Enzyme Assay—** The activity of ascorbate oxidase was determined using 0.1 mM sodium phosphate, dithiothreitol buffer (pH 5.4) containing 0.5 mM EDTA, according to the method of Oberbacher and Vines (16). The oxygen consumption rate was also measured using Clark-type oxygen electrodes (YSI Instrument Co.). One unit of enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 \( \mu \)mol of L-ascorbic acid/min. For the determination of \( K_m \) values, the enzyme activities were assayed at 290 nm, using a molar absorption coefficient of 2,800 \( \text{M}^{-1}\text{cm}^{-1} \) (17), in the concentration range of 0.025–0.75 mM ascorbic acid by means of Shimadzu model UV-265 spectrophotometer. \( K_m \) values were determined from Lineweaver-Burk plots.

**Purification of the Enzyme—** The mycelia from the exponential phase were used as a starting material for purification. All of the purification steps were performed at 4°C. The grown mycelia of P. ostreatus were collected by filtration on Whatman No. 1 filter paper and washed three times with 500 mL of 50 mM potassium phosphate buffer (pH 6.6) containing 0.5 mM EDTA (buffer A). The washed mycelia were homogenized in buffer A containing aluminum oxide. The aluminum oxide and unbroken mycelia were removed by centrifugation. The supernatant was used as a crude extract during the enzyme purification. Cold acetone was added to 65% saturation to the crude extract and the
**TABLE I**

Purification step of ascorbate oxidase from *P. ostreatus*

| Step                                      | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) | Purification |
|-------------------------------------------|--------------------|------------------------|-------------------------------|-----------|--------------|
| Crude extract                             | 672.5              | 8.74                   | 0.013                         | 100       | 1            |
| Acetone precipitation                     | 239.8              | 6.00                   | 0.025                         | 68.6      | 1.9          |
| Sepharose CL-6B chromatography            | 18.4               | 4.10                   | 0.223                         | 46.9      | 17.2         |
| Red-reactive chromatography               | 8.1                | 3.00                   | 0.370                         | 34.3      | 28.5         |
| Sephacryl G-200 chromatography            | 2.9                | 2.30                   | 0.793                         | 26.3      | 61.0         |
| S-Sepharose chromatography                | 0.2                | 1.10                   | 5.500                         | 12.6      | 423.1        |

**FIG. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ascorbate oxidase. Lane 1, purified enzyme; lane 2, molecular mass markers; β-galactosidase (a, 116.4 kDa), fructose-6-phosphate kinase (b, 85.2 kDa), glutamate dehydrogenase (c, 55.4 kDa), lactate dehydrogenase (d, 36.5 kDa), and soybean trypsin inhibitor (e, 20.1 kDa). Relative mobilities of the standard markers versus common logarithms of their molecular weights were plotted. With the linear regression output, the molecular weight of ascorbate oxidase was estimated.

**TABLE II**

Amino acid compositions of ascorbate oxidases purified from *P. ostreatus* and various plants

| Amino acid | *P. ostreatus* | Green zucchini* | Cucumis sativus* |
|------------|---------------|-----------------|------------------|
| Lys        | 29.9          | 51.1            | 26.6             |
| His        | 53.0          | 42.6            | 20.5             |
| Arg        | 13.1          | 33.2            | 19.1             |
| Asp + Asn  | 62.2          | 122.6           | 59.9             |
| Thr        | 6.8           | 59.2            | 28.6             |
| Ser        | 18.4          | 54.7            | 26.6             |
| Glu + Gln  | 52.4          | 92.4            | 44.3             |
| Pro        | 77.4          | 99.0            | 50.1             |
| Gly        | 35.3          | 86.3            | 43.2             |
| Ala        | 52.9          | 64.8            | 32.8             |
| Val        | 53.4          | 68.3            | 35.5             |
| Met        | 22.8          | 18.6            | 14.9             |
| Ile        | 19.0          | 66.1            | 32.7             |
| Leu        | 34.2          | 80.8            | 44.6             |
| Tyr        | 34.9          | 42.6            | 21.7             |
| Phe        | 13.4          | 46.5            | 25.8             |
| Trp        | ND            | 40.45           | 13.9             |
| Cys        | ND            | 12.3            | 5.5              |

* Data from Marchesini and Kroneck (9).
* Data from Ohkawa et al. (29).
* ND, not determined.

**Sources**

- *P. ostreatus*: D V K L Q E H L Q L A L M V
- *Cucumis sativus*: G F K P K I K H Y K W D V E Y M
- *Cucurbita pepo medullaris*: S Q I R H Y K W E V E Y M F W
- *Cucurbita sp. Ebitu Nakini*: S Q I R H Y K W E V E Y M F W

**FIG. 2.** The comparison of N-terminal amino acid sequences between several ascorbate oxidases, a, data from Ohkawa et al. (29); b, data from Messerschmidt et al. (11); and c, data from Esaka et al. (30).

**Spectroscopic Studies**—UV absorption spectra were obtained in 0.1 M phosphate buffer (pH 6.0) at 25 °C with a Shimadzu model UV-265 spectrophotometer. Reduced-minus-oxidized difference spectra were obtained by recording the spectrum of the enzyme reduced with 5 mM ascorbic acid and a few crystals of solid Na2S2O4, and the spectrum of the sample was oxidized by bubbling oxygen for 20 min. For ligand-binding study, the samples were reduced with a few granules of Na2S2O4, making an anaerobic condition by exchanging the sample headspace with argon and then gently mixing. After several cycles of argon exchange, CO was passed through the sample solution and (reduced-CO)-minus-reduced difference spectra were obtained. The spectra of potassium cyanide complex were recorded through the same procedure. The spectra of pyridine hemochrome were obtained according to the method proposed by Berry and Trumpower (24), and the
content of protoporphyrin was determined at 562 nm using a molar absorption coefficient of 30,000 M$^{-1}$ cm$^{-1}$ (25).

***Stoichiometric Determination—***All experiments for stoichiometric analysis were performed at 25°C using 0.1 M phosphate buffer (pH 5.4) containing 0.5 mM EDTA. The reaction was monitored using an UV-visible spectrophotometer and oxygen monitor. The concentration of hydrogen peroxide was determined according to the method of Nishikimi et al. (26).

Analysis of Reaction Product—To 3 ml of the enzyme solution (0.25 mg/ml), 3 mg of L-ascorbic acid were added, and this mixture was flushed with oxygen at 37°C for 1 h. The reaction process was traced by measuring the absorbance at 265 nm every 10 min during the reaction. After the oxidation, the solution was freeze-dried immediately. The freeze-dried preparation was redissolved in 0.5 ml of D$_2$O. $^1$H NMR spectra of enzymatic reaction products dissolved in D$_2$O were obtained at room temperature by means of VXR-200S FT-NMR spectrometer from Varian, using 3-(trimethylsilyl)-1-propane sulfonic acid as an internal reference.

### RESULTS AND DISCUSSION

**Purification of the Enzyme—**The ascorbate-oxidizing activity in aerobically grown P. ostreatus was detectable in the mycelium from the lag phase to the exponential phase. The activity of ascorbate oxidase was overlapped with that of laccase, because laccase occurs in the mycelial crude extract of P. ostreatus and can oxidize ascorbic acid. However, after the first gel filtration was performed, the enzyme activity was reproducible.

Ascorbate oxidase was purified from P. ostreatus, as summarized in Table I. The enzyme purified 420-fold relative to the crude cell extract with a recovery of 13%. The purity of the enzyme was confirmed by an electrophoretic method. The preparation of the purified enzyme gave a single band with enzyme activity after non-denaturing polyacrylamide gel electrophoresis.

**Molecular Properties—**The apparent molecular mass of the purified enzyme was determined to be 94 kDa by high performance gel permeation chromatography. On the other hand, when the enzyme was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a single band of 46 kDa was found (Fig. 1), indicating that the enzyme is composed of two identical subunits. The ascorbate oxidases from cucumber and squash are dimeric enzymes with a molecular mass in the range of 66–70 kDa for the monomer, while the enzyme from the culture filtrate of Acremonium was a monomer with a molecular mass of 80 kDa (13).

The pl value of this enzyme is 4.5. The ascorbate oxidase contains approximately 12.5% carbohydrate by weight, which is estimated according to the method proposed by Dubois et al. (21). All of the ascorbate oxidases previously reported were also glycoproteins: ascorbate oxidase from zucchini contains 10% carbohydrates (27) and has two N-linked oligosaccharide chains per subunit (28).

**Amino Acid Composition and N-terminal Amino Acid Sequence of the Enzyme—**As shown in Table II, the enzyme contained very different compositions of amino acids from those of ascorbate oxidases from green zucchini (9) and Cucumis sativus (29). And the N-terminal amino acid sequence of the enzyme is Asp-Val-Lys-Thr-Leu-Gln-Glu-His-Leu-Gln-Leu-Ala-Leu-Met-Val-. As shown in Fig. 2, this sequence is also very different from those of ascorbate oxidases from Cucurbita pepo medulloidea (11), Cucumis sativus (29), and Cucurbita sp. Ebisu Nankin (30).

**Effects of pH and Temperature—**The enzyme exhibited maximum activity at pH 4.8–5.5 (Fig. 3A). L-Ascorbic acid can be easily autoxidized at alkaline pH, thus the enzymatic oxidation can be distinctly observed only at acidic pH. The enzyme was relatively stable at acidic pH. The highest rates of the enzymatic reaction were observed at 40°C, and above 60°C the enzyme lost its activity. When the enzyme was incubated at various temperatures for 1 h at pH 5.4, it was found to be stable below 50°C, and 95% of the initial activity was lost at 55 and 60°C, respectively (Fig. 3, B and C).

**Kinetic Calculations—**The relationship between enzyme activity and substrate concentration was Michaelis-Menten type. The $K_m$ and $V_{max}$ values for L-ascorbic acid determined from Lineweaver-Burk plot were estimated to be 0.48 mM and 1.4 $\mu$M min$^{-1}$, respectively. The oxidation rate of L-ascorbic acid by the purified enzyme was compared with various cytochromes to confirm whether the enzyme was another kind of cytochrome. The purified enzyme exhibited much higher affinity toward ascorbate than any other cytochromes (Table III). Ascorbate oxidase from P. ostreatus has similar kinetic parameters relative to those observed with purified enzyme from Acremonium

![Fig. 3](image-url)

**Fig. 3. Effect of pH and temperature on the activity and stability of ascorbate oxidase.** A, changes of the enzyme activity measured in each pH buffer solution at 37°C (solid line) and those measured in 0.1 M phosphate buffer (pH 5.4) at the same temperature. After the incubation in each pH buffer solution for 12 h (dashed line). B, changes of the enzyme activity measured at each temperature using 0.1 M phosphate citrate buffer (pH 5.4) and C, those assayed with the same buffer at 37°C, after the incubation at each temperature for 1 h.

**Table III**

| Ascorbate oxidase | 100 |
|-------------------|-----|
| Cytochrome b from yeast | 1.33 |
| Cytochrome c from yeast | 2.47 |
| Cytochrome c from bovine heart | 1.35 |

**Comparison of activities of ascorbate oxidase purified from P. ostreatus and various cytochromes toward ascorbic acid**
Substrate Specificity—As shown in Table IV, the ascorbate oxidase from P. ostreatus is more specific for L-ascorbic acid and its analogs. D-Ascorbic acid and D-erythroascorbic acid which share similar conformation to L-ascorbic acid in the C-4 position have Km values in the 0.4–0.5 mM range, whereas the somewhat different L-erythroascorbic acid has a Km of 2.45 mM. These data indicate that the C-4 configuration of the substrate is essential for its binding to the enzyme.

Effects of Various Compounds and Metal Ions—The effects of various compounds listed in Table V on the enzyme activity were examined using L-ascorbic acid as a substrate. 1,10-Phenanthroline caused complete inhibition at the concentrations shown in Table V. The straight lines obtained in the double reciprocal plot, 1/V against 1/[ascorbate] at different 1,10-phenanthroline concentrations, crossed at a point (Fig. 4A), whereas linear lines were obtained for a plot of 1/V against [1,10-phenanthroline] (Fig. 4B). This reaction mode accounts for the competitive inhibition, and from these results, Kᵢ was calculated to be 8.3 μM. Sulphydryl reagents, such as iodoacetate and p-chloromercuribenzoate, showed no inhibition. This result supports the conclusion that ascorbate oxidase from plants is not sulphydryl-dependent. Also, the enzyme was inhibited by azide and cyanide.

Spectroscopic Studies—The final homogeneous preparation of the enzyme was brownish red in solution, suggesting the presence of the heme group in its active site. The absorption spectra of the purified enzyme revealed a Soret maximum at 418 nm in its oxidized form, at 426 nm in its reduced form, and α and β bands at 558 and 527 nm only in its reduced form, respectively, as shown in Fig. 5. Judged from the position of α band in the difference spectrum of the reduced-minus-oxidized enzyme, this enzyme seems to belong to the group of b-type cytochromes, and the absorption maximum at 562 nm observed in the pyridine hemochrome spectrum is indicative of a protoporphyrin (data not shown). This spectrum was obtained through the acid/acetone extract from an ether-soluble heme fraction. The solubility in ether confirmed that the enzyme belongs to the group of b-type cytochromes, not covalently bound to the enzyme. On the basis of the molar absorption coefficient for the α band of the reduced-minus-oxidized pyridine hemochrome-

![Fig. 4. Inhibition of ascorbate oxidase by 1,10-phenanthroline. A, Lineweaver-Burk plot for ascorbic acid as a function of the concentration of 1,10-phenanthroline, and B, slopes from A are replotted against concentrations of 1,10-phenanthroline.](image-url)
gen, a quantity of 22.5 nmol of protoheme per mg of protein was calculated. Given the molecular mass of 94 kDa, 2.12 mol of protoheme per enzyme were present. Therefore, assuming that a protoheme is not removed during purification procedures, the ascorbate oxidase from P. ostreatus may contain one protoheme per monomeric subunit. Calculation of the molar absorption coefficient of the ascorbate oxidase at 562 nm, by using the heme concentration derived from the pyridine hemochromogen spectra, gave a value of 30,000 M$^{-1}$ cm$^{-1}$. Absorption spectra of CN-enzyme were recorded after the addition of 30 mM sodium azide to the 1.48 μM native enzyme solution (solid line) in 0.1 M sodium phosphate buffer, pH 6.0. The right inset shows the 5-fold enlarged spectra in the 500–600 nm region.

**Fig. 5.** Absorption spectra of ascorbate oxidase. A, spectra of oxidized (solid line) and reduced form (dashed line) of the enzyme. B, difference spectrum of reduced minus oxidized enzyme. Purified enzyme (2 μM) was dissolved in 0.1 M sodium phosphate buffer (pH 6.0) and reduced by 5 mM L-ascorbic acid.

**Fig. 6.** Absorption spectra of CN-treated ascorbate oxidase. CN-enzyme (dashed line) was prepared by the addition of 30 mM sodium azide to the 1.48 μM native enzyme solution (solid line) in 0.1 M sodium phosphate buffer, pH 6.0. The right inset shows the 5-fold enlarged spectra in the 500–600 nm region.

Stoichiometry—The ascorbate oxidase from P. ostreatus was estimated to be capable of using only molecular oxygen, not cytochrome and ferricyanide as an electron acceptor. The reaction catalyzed by the ascorbate oxidase was L-ascorbic acid $+$ O$_2$ $\rightarrow$ dehydro-L-ascorbic acid $+$ H$_2$O$_2$, which is confirmed by the result shown in Table VI. The purified enzyme was rather inhibited by hydrogen peroxide. In this study, it has been shown conclusively that the enzyme really does use molecular oxygen as an electron acceptor, and oxygen is converted to...
hydrogen peroxide (Fig. 8).

Analysis of the Reaction Product—In the $^1$H NMR spectrum of the reaction mixture of L-ascorbic acid with ascorbate oxidase in D$_2$O, the H-4 resonance peak was obscured by a strong solvent peak. The strong geminal coupling between H-6 protons indicates that the hydroxymethyl group at C-6 does not freely rotate. From these results, it is concluded that dehydro-L-ascorbic acid exists in the bicyclic hydrated monomer through hemiketal linkage between 6-OH and C-3, as reported previously (1).

Conclusion—Considering that ascorbate oxidase is detected only in the mycelial stage of P. ostreatus and produces hydrogen peroxide as a reaction product, this enzyme might play an important role in catalyzing oxidation reactions during development of this mushroom.

Ascorbic acid is well known as a scavenger for active oxygen species, such as superoxide, hydroxyl radical, and hydrogen peroxide, especially in the chloroplast and in the nitrogen-fixing root nodules. That function, however, is mediated by another enzyme, ascorbate peroxidase (33). Ascorbate oxidase is unlikely to be involved in scavenging activity because the reaction consumes molecular oxygen, rather than hydrogen peroxide. Considering that water is one of the reaction products of copper-containing ascorbate oxidase, it is noteworthy that the heme-containing ascorbate oxidase from P. ostreatus produces hydrogen peroxide. The electron transfer mechanism in

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**TABLE VI**

Stoichiometric determination of the oxidation of L-ascorbic acid with ascorbate oxidase

In cases of L-ascorbic acid and O$_2$, their consumption rates were measured and in case of H$_2$O$_2$, its production rate was determined according to the method described under "Experimental Procedures." 1 µg of protein was used.

| Compound    | Rate       | Ratio to L-ascorbic acid |
|-------------|------------|--------------------------|
| L-Ascobic acid | 1.45 ± 0.25 | 1                        |
| O$_2$       | 1.37 ± 0.23 | 0.94                     |
| H$_2$O$_2$  | 1.19 ± 0.20 | 0.82                     |

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**Fig. 7. CO difference spectra of ascorbate oxidase.** A, CO-binding spectra were obtained by sodium dithionite in an anaerobic condition. Time interval was 2 min in the spectra above. B, difference spectrum of (reduced plus CO)-minus reduced enzyme.

**Fig. 8. Monitoring of oxygen consumption during the enzymatic oxidation of L-ascorbic acid.** At the left-hand arrow, 2.5 µg of ascorbate oxidase were added to 0.6 ml of reaction solution containing 5 mM ascorbic acid in 0.1 M phosphate citrate buffer (pH 5.4), and 2.5 units of catalase were added at the right-hand arrow. Oxygen consumption was measured at 25 °C using a Clark-type oxygen electrode.
the reaction of the ascorbate oxidase from P. ostreatus might be different from that of the copper-containing one.

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