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THE PHENOLOXIDASES OF THE ASCOMYCETE *PODOSPORA ANSERINA*

X. ELECTRON MICROSCOPIC STUDIES ON THE STRUCTURE OF LACCASES I, II AND III

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SUMMARY

Electron microscopy of three intracellular laccases from the Ascomycete *Podospora anserina* showed that the high molecular weight laccase I ($M_r = 390\,000$) appears mainly as tetramers with the four subunits arranged at the corners of rectangles ($13.3\text{ nm} \times 8.5\text{ nm}$). In size and shape the subunits of laccase I equal the monomeric molecules of laccase II ($M_r = 70\,000$, $6.8\text{ nm} \times 5.5\text{ nm}$) and laccase III ($M_r = 80\,000$, $7.0\text{ nm} \times 5.8\text{ nm}$). The dimensions of the monomeric laccases estimated from the electron micrographs are in good agreement with the hydrodynamic measurements of their molecular weight.

INTRODUCTION

The phenoloxidase laccase (*p*-diphenol:O₂ oxidoreductase, EC 1.10.3.2) catalyzes the oxidation of various diphenols and other substances by molecular oxygen¹. This copper-containing glycoprotein has been investigated since the early beginning of biochemistry (see review by Levine²) because of its widespread occurrence in the plant and animal kingdom and its various biological functions. Beside investigation of its catalytic reaction, its composition, the amount, type and function of the copper, laccase recently has been examined in our laboratory also in connection with genetic problems in the Ascomycete *Podospora anserina*³⁻⁷.

In the wild strain and in the pleiotropic one-factor mutant "zonata" of this fungus, three different intracellular laccases were found, a high molecular weight laccase I ($M_r = 390\,000$), and two low molecular weight laccases, laccase II ($M_r = 70\,000$) and III ($M_r = 80\,000$). Whereas no qualitative differences between the respective laccases from the two strains were observed, large differences in the amount of the individual laccases were found⁷. When the purified laccases were characterized^{6,7}, several phenomena pointed to an oligomeric structure of laccase I and, furthermore,

led to the assumption that it consists of subunits identical or similar to the low molecular weight laccases.

Since laccase I is within the range of resolution of electron microscopy, we intended to verify the oligomeric structure of this enzyme by this method and to obtain more detailed information about its substructure in comparison to laccase II and III.

From the data reported in this communication it follows that laccase I indeed represents an oligomer, consisting of four subunits similar in shape and dimension to laccase II and III.

MATERIAL AND METHODS

Strains. Details concerning origin, ontogenesis and genetics of the wild type and mutant "zonata" of *P. anserina* have been published elsewhere⁷.

Purification and characterization of laccase I^{4,6,7}, laccase II^{5,7,8} and laccase III^{5,7} have already been described.

Electron microscopy. Negative staining of the preparations was performed at room temperature and at 4 °C using the droplet method and the spraying method, respectively, with 0.5–1.0% uranyl oxalate (pH 6.0–7.0) as contrast medium.

For the droplet method a drop of the cold enzyme (protein concentration 20 µg/ml) was placed on a carbon-coated grid for about 1 min. After blotting with filter paper until nearly dry one drop of stain solution was added and withdrawn after 1 min. For the spraying technique the enzyme solution was mixed with the contrast medium to the desired dilution (200 µg/ml protein; 0.5–1.0% uranyl oxalate) and sprayed on to carbon-coated grids at 4°.

For the height measurements enzyme solution mixed with polystyrene latex spheres was sprayed on carbon-coated grids. These grids were then shadowed with platinum at an angle of 10°, a distance of 12 cm and a calculated thickness of 2.0 nm.

Electron micrographs were taken at a magnification of 22 000 to 100 000 on 35-mm film or plates with a Philips EM 200 or Jeol 100B electron microscope operating at 80 kV.

Magnification calibration was performed with the aid of a carbon grating replica having 21 600 lines/cm.

RESULTS

Pure samples (disc electrophoresis and ultracentrifugation) of laccase I, II and III were used for preparation of the negatively stained specimens. Best results were obtained using uranyl oxalate at pH values between 6 and 7 with both, the droplet and the spraying method. Fixation experiments with glutaraldehyde according to Valentini *et al.*⁹ gave no significant differences.

The typical form of laccase I molecules is shown in Fig. 1. The molecules are observed as rectangles of 13.3 (± 0.6) times 8.5 (± 1.6) nm; one dimension being rather sharply confined, the other less clear. In most particles a substructure is visible.

In contrast to this, laccase II (Fig. 2) and laccase III (Fig. 3) appear as monomers measuring 6.8 nm \times 5.5 nm and 7.0 nm \times 5.8 nm, respectively (Fig. 4).

When laccase I was shadowed with platinum at an angle of 10°, the height of the molecules was measured as 5.4 (± 0.8) nm.

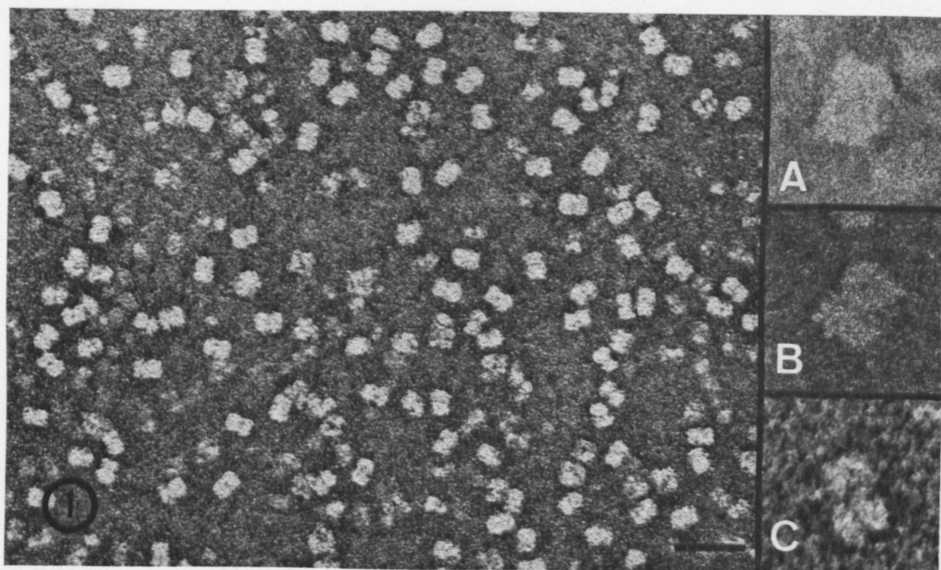


Fig. 1. A typical electron micrograph of laccase I. The molecules appear mainly as rectangles. Insets A, B and C show particles at higher magnification with different degrees of substructure visible. Negative staining with 1.0% uranyl oxalate, pH 6.6; spray method; scale line represents 50 nm.

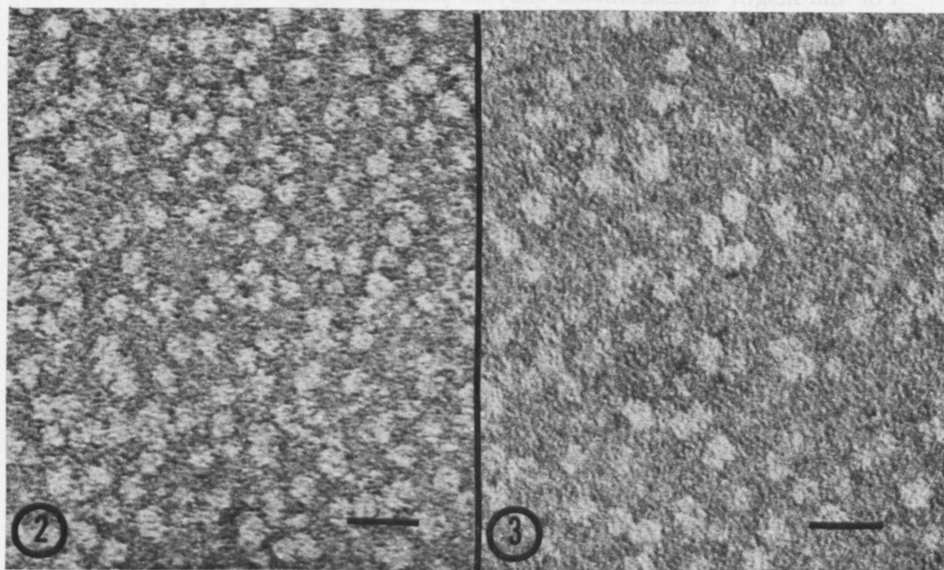


Fig. 2. A typical electron micrograph of laccase II. Negative staining with 0.5% uranyl oxalate, pH 6.0; spray method; scale line represents 25 nm.

Fig. 3. A typical electron micrograph of laccase III. Negative staining with 0.5% uranyl oxalate, pH 6.0; droplet method; scale line represents 25 nm.

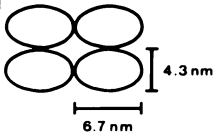


Laccase I	Laccase II	Laccase III
M_r 390 000	M_r 70 000	M_r 80 000
		

Fig. 4. Models and molecular data for laccase I, laccase II and laccase III. The molecular weight given is derived from hydrodynamic studies (see Table I), the dimensions in nm are estimated from the electron micrographs.

DISCUSSION

Previous work^{6,7} on the three laccases suggested an oligomeric structure of laccase I. Properties which led to this assumption are given in Table I.

With a molecular weight of 70 000 and 80 000, respectively, the low molecular

TABLE I

PROPERTIES OF THE PURIFIED LACCASES I, II AND III

Property	Laccase I	Laccase II	Laccase III	Ref.
Molecular weight				
Gel chromatography (Sephadex G-150)		70 000	80 000	7
$s_{20,w}/D_{20,w}$	390 000		82 000	6, 7
Sedimentation equilibrium	383 000	71 000	78 000	6, 7
Electron microscopy*				
ellipsoid ($c = 4.3$ nm $\approx 1/2$ width of laccase I molecule)		74 000	76 000	This work
rotational ellipsoid		95 000	102 000	This work
Partial specific volume (\bar{v})	0.701	0.680	0.726	6, 7
Sedimentation constant ($s_{20,w}$)	14.5	5.4	5.3	6, 7
Ratio of mol. wts** (laccase I/laccase II and laccase I/laccase III, respectively)		4.3	4.6	This work
Copper content (% dry matter)	0.30	0.30	0.31	6, 7
Approximate number of copper atoms/enzyme molecule	16	4	4	6, 7
Dissociation into defined subunits***	1:5	—†	—†	

* Calculation from the dimensions using the formula $M_r = (1/\bar{v}) \cdot N \cdot V$, where M_r is the molecular weight, \bar{v} is the partial specific volume, N is Avogadro's number and V is the volume of the molecule. The volume for an ellipsoid (V_e) with the half axes a , b and c was calculated according to $V_e = 4/3 \cdot \pi \cdot a \cdot b \cdot c$; the volume for a rotational ellipsoid (V_r) with the rotational half axis a according to $V_r = 4/3 \cdot \pi \cdot a \cdot b^2$; the volume for a sphere (V_s) with radius r according to $V_s = 4/3 \cdot \pi \cdot r^3$.

** Calculations according to Scheraga and Mandelkern¹⁰ from the sedimentation constants.

*** Dialysis against sodium dodecylsulfate, urea or guanidine HCl.

§ H. P. Molitoris (unpublished observation).

† W. Minuth, personal communication.

weight laccases II and III are well within the molecular weight range (60 000–110 000) commonly found for laccases, whereas laccase I shows with a value of 390 000 an unusually high molecular weight. Laccase contains in its active center divalent copper which is necessary for its catalytic activity^{11–13}. Generally, a minimum of 4 copper atoms per enzyme molecule is found in active laccase. As shown in Table I, laccase II and III also contain 4 copper atoms. It, therefore, seems probable that laccase I with a molecular weight of 390 000 and approximately 16 copper atoms, has an oligomeric structure consisting of 4–5 subunits. The fact, that only laccase I dissociates under certain conditions into defined subunits (H. P. Molitoris, unpublished observation) substantiates this hypothesis.

The feasibility of using electron microscopy to prove this hypothesis comes from the fact that the diameter calculated for a spherical subunit (see footnote to Table I) of the postulated tetrameric laccase I is in the range of 4.0–6.0 nm. Since the resolution is about 2 nm for negatively stained protein molecules, this method was used to verify the oligomeric structure of laccase I and to show details of its substructure.

Indeed, an oligomeric structure was found for laccase I and the dimensions of the low molecular weight laccases II and III are in the expected range, laccase III being slightly, but consistently the larger, which is in agreement with its higher molecular weight as determined by gel chromatography and ultracentrifugation.

The tetrameric laccase I molecules have about double the diameter of the laccase II and III monomers, respectively (Fig. 4). Therefore, they could very well consist of subunits like the laccase II and III monomers. The fact that the dimensions are somewhat less than double that of the low molecular weight laccases might be explained by a difference in the flattening of the monomers and tetramers during drying down on the grid. Another possible explanation would be an interlocking of the outer regions of the monomers which are closely packed to the tetrameric structure. This is in agreement with the incomplete separation observed in the electron micrographs (see Fig. 1, inset A and B).

Sometimes, however, a complete tetrameric substructure is observable in the molecules (see Fig. 1, inset C). The resolved structure clearly depends on the orientation of the particle on the carbon film during specimen preparation for electron microscopy.

From the dimensions determined for the laccases I, II and III, respectively, we conclude a tetrameric structure for the laccase I. In addition it is known from the ultracentrifuge analysis of the laccase I sample investigated that the molecules had a molecular weight of 400 000 corresponding with a tetramer.

The shape of the monomers of laccase I, II and III appears to be elliptical (Fig. 4) in agreement¹⁴ with the ratio of their frictional coefficients f/f_0 (1.25–1.46; Molitoris and Esser⁶; H. P. Molitoris, unpublished results).

The molecular weight of ellipsoids was calculated from the densities (Table I) and dimensions (Fig. 4) for the low molecular weight laccases, being 74 000 for laccase II and 76 000 for laccase III, respectively (Table I). These values are in good agreement with the hydrodynamic measurement of their size (Table I). From these calculations we arrive then at a molecular weight for the tetrameric laccase I of 298 000 when composed of laccase II and of 303 000 when composed of laccase III monomers.

If, however, the molecular weight for laccase II and III is calculated according to the formula for rotational ellipsoids (Table I) this leads to a value for the tetrameric

laccase I of 380 000 when composed of laccase II and of 408 000 when composed of laccase III monomers. These values are in good agreement with the hydrodynamic measurements of the size of laccase I ($M_r = 390\,000$).

The three laccases are similar or identical also in many other respects such as carbohydrate content⁷, carbohydrate composition (ref. 8 and H. P. Molitoris, unpublished results), copper content⁷ and ESR spectra (H. P. Molitoris, unpublished results). Furthermore, they are serologically related^{6,7}. Therefore, the data from electron microscopy are in accordance with the hypothesis that the subunits of laccase I are similar or identical with laccase II and/or laccase III.

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