Intracellular Oxygen-metabolizing Enzymes of *Phanerochaete chrysosporium*

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The levels of catalase and superoxide dismutase present in the white-rot fungus *Phanerochaete chrysosporium* were investigated. The specific activities of both enzymes increased and reached a maximum after 4-6 d growth before falling to a constant low activity. Catalase was purified and found to be the typical eukaryotic enzyme. Activity staining, in the presence and absence of cyanide, revealed that the cytosol of *P. chrysosporium* contained only the copper/zinc superoxide dismutase commonly found in eukaryotes. These results show that *P. chrysosporium* meets the challenge of raised levels of activated oxygen by producing enhanced levels of the usual detoxifying enzymes rather than any novel isoenzymes.

INTRODUCTION

Knowledge of the mechanisms by which white-rot fungi degrade lignin has increased dramatically in the last few years. The most significant advances have been the realization that lignin biodegradation is a secondary metabolic event strictly controlled by cultural parameters (Keyser et al., 1978; Jeffries et al., 1981) and the discovery of two separate 'ligninases' (Tien & Kirk, 1984; Kuwahara et al., 1984; Paszczynski et al., 1986). These enzymes attack a variety of lignin model compounds and lignin itself to a limited degree and require H$_2$O$_2$ for activity. Apart from H$_2$O$_2$, another activated reduced form of dioxygen, the superoxide anion, has also been implicated in lignin biodegradation (Crawford & Crawford, 1984). In generating potentially lethal reduced forms of oxygen, the white-rot fungi create for themselves a hostile environment. This paper examines certain mechanisms for the elimination of oxygen toxicity, including the production of catalase and superoxide dismutase.

METHODS

Organism. *Phanerochaete chrysosporium* was obtained from the Commonwealth Mycology Institute (CMK 174727) and maintained as described previously (Morpeth, 1985).

Culture conditions. *P. chrysosporium* was grown at 30 °C in 500 ml flasks either statically (50 ml culture volume) or as an orbitally shaken culture (100 ml culture volume, 200 r.p.m.). The medium was based on that described by Jeffries et al. (1981) and had the following composition (per 1000 ml distilled water): KH$_2$PO$_4$, 2 g; CaCl$_2$, 0.01 g; MgCl$_2$,6H$_2$O, 0.44 g; Na$_2$SO$_4$, 0.029 g; 1 ml of the mineral salt solution and 0.5 ml of the vitamin solution described in Jeffries et al. (1981). Nitrogen was added as (NH$_4$)$_2$HPO$_4$ to a final concentration of either 24 mM (low nitrogen) or 24 mM (high nitrogen). Carbohydrate growth substrates were added to the media at a concentration of 5 g l$^{-1}$. All cultures were buffered at pH 4-5 with 0.01 M-sodium 2,2-dimethylsuccinate.

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Abbreviation: PMSF, phenylmethylsulphonyl fluoride.
Electrophoresis. Non-denaturing PAGE was done at 5 °C in 7.5% (w/v) gels in 5 mM-Tris/50 mM-glycine buffer, pH 8.5, on a LKB Multiphor horizontal electrophoresis unit. Each well contained 5–100 μg protein and electrophoresis was done for 3 h at a constant voltage of 150 V. Gels were stained for protein using Brilliant blue R 250. Superoxide dismutase activity was revealed as described by Beauchamp & Fridovich (1971). Electrophoresis in the presence of SDS was done by the method of Weber & Osborn (1969). Each sample contained 20–50 μg protein and electrophoresis was done at 10 °C at a constant current of 150 mA for 5 h.

Enzyme assays. Superoxide dismutase activity was assayed by the xanthine oxidase/cytochrome c method of McCord & Fridovich (1969). Catalase activity was followed using an oxygen electrode under the conditions described by McCord et al. (1971). A unit of catalase activity is defined as the amount of enzyme that catalyses the destruction of 1 μmol H₂O₂ min⁻¹ under the assay conditions. To measure cellular enzyme activity, the mycelium was harvested by filtration through six layers of cheese cloth and washed three times with 0.1 M-potassium phosphate buffer, pH 7.4, containing 0.5 M-NaCl. This removed an extracellular peroxidase with catalase activity from the mycelium (Pasczynski et al., 1986). The washed mycelium was resuspended in 0.1 M-potassium phosphate buffer, pH 7.4, containing 0.5 mM-phenylmethylsulphonyl fluoride (PMSF), 5 mM-EDTA and 1 mM-DTT and sonicated for three 2 min periods with cooling in between. This suspension was centrifuged at 13000 g for 10 min and the supernatant was decanted and assayed for total protein and for catalase and superoxide dismutase activities.

Protein estimation. Protein was determined by the Lowry method with BSA fraction V as a standard. The concentration of BSA was determined by using a value of 6.67 for A₂₅⁴₆ (Janatova et al., 1968).

Purification of catalase. P. chrysosporium grown in shaking culture at 30 °C in medium with high nitrogen and glucose as sole carbon source was harvested after 30 h growth. The mycelium was separated by filtration through cheese cloth and washed with 0.5 M-NaCl before being ground to a fine powder under liquid nitrogen in a mortar. This powder was either stored at −20 °C or immediately resuspended in 0.1 M-potassium phosphate buffer, pH 7.4, containing 0.5 mM-EDTA and 1 mM-DTT. The suspension was passed twice through a precooled Mauton-Gaulon milk homogenizer and bulk material was removed by centrifugation. A 40–65% saturation ammonium sulphate fractionation was done and the final precipitate was dissolved and dialysed exhaustively against 5 mM-potassium phosphate buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-DTT. The resultant solution was clarified by centrifugation and applied to a column of DEAE-cellulose (80 × 5 cm) equilibrated with the dialysis buffer. Enzyme activity was eluted just after the main protein peak by washing with the dialysis buffer. After a second 40–65% ammonium sulphate fractionation the precipitate obtained was dissolved in and dialysed against 40 mM-potassium phosphate buffer, pH 7, containing 1 mM-EDTA and 1 mM-DTT. After 4 h dialysis to dissolve the precipitate and reduce viscosity, the solution was loaded onto a Sephacryl S-200 gel filtration column (110 × 5 cm). This column was eluted with the dialysis buffer and peak fractions were pooled and concentrated by ultrafiltration. The protein solution was reapplied to the Sephacryl S-200 column as before and the catalase was concentrated and stored in 0.5 ml lots at −20 °C.

RESULTS

Effects of growth conditions on catalase and superoxide dismutase activity

A time course for the appearance of catalase and superoxide dismutase activity in P. chrysosporium grown statically under atmospheric oxygen levels in a low-nitrogen medium is shown in Fig. 1. The specific activities of both enzymes reached a maximum after 4–6 d of 150 ± 39 units (mg protein)⁻¹ for catalase and 27 ± 8 units (mg protein)⁻¹ for superoxide dismutase. They then decreased to a constant low level of 8 ± 3 units (mg protein)⁻¹ for catalase and 13 ± 4 units (mg protein)⁻¹ for superoxide dismutase.

When P. chrysosporium was grown in shaking culture in a low-nitrogen medium, a similar result was obtained. After 2–3 d the specific activities of catalase and superoxide dismutase were 210 ± 30 and 31 ± 8 units (mg protein)⁻¹, respectively. These decreased after 5–7 d to constant specific activities of 8 ± 3 units (mg protein)⁻¹ for catalase and 12 ± 5 units (mg protein)⁻¹ for superoxide dismutase. Within the limits of error, identical results were obtained in the high-nitrogen medium.

Purification and properties of catalase

The purified catalase showed one main protein band on electrophoresis. In gels which had been loaded with 50 μg protein samples several additional faint bands were visible; though these probably accounted for less than 10% of the total protein. The purification procedure for catalase from P. chrysosporium is summarized in Table 1. The purified enzyme had a maximum
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![Graph](image)

Fig. 1. Time course of the induction of catalase (a) and superoxide dismutase (b) in *P. chrysosporium* grown statically in a low-nitrogen medium at 30 °C. Each value quoted is the mean of a minimum of four independent determinations. Vertical bars indicate SD.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity [units (mg protein)]⁻¹</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1380</td>
<td>18400</td>
<td>2.4 × 10⁶</td>
<td>138</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>150</td>
<td>2076</td>
<td>9.8 × 10⁵</td>
<td>472</td>
<td>41</td>
<td>3.4</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>350</td>
<td>425</td>
<td>6.8 × 10⁵</td>
<td>1600</td>
<td>28-3</td>
<td>12.1</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>7</td>
<td>360</td>
<td>6.7 × 10⁴</td>
<td>1860</td>
<td>27-9</td>
<td>14.3</td>
</tr>
<tr>
<td>1st Sephacryl</td>
<td>3</td>
<td>21</td>
<td>5.8 × 10⁴</td>
<td>28000</td>
<td>24-2</td>
<td>215</td>
</tr>
<tr>
<td>2nd Sephacryl</td>
<td>1</td>
<td>14</td>
<td>5.2 × 10⁴</td>
<td>37400</td>
<td>21-6</td>
<td>288</td>
</tr>
</tbody>
</table>

Specific activity of 30000-42000 units (mg protein)⁻¹ and, in the best preparations, the ratio of the absorbance at 408 nm to that at 280 nm was 0.9. Omission of the protease inhibitor PMSF from the buffer in the early stages of purification reduced the final yield of catalase from around 20% to less than 5%. Electrophoresis of catalase in the presence of SDS indicated a subunit *Mₙ* of 57000. The catalase eluted from a calibrated Sephacryl 5-200 column with an apparent *Mₚ* of 220000. Visible absorbance spectra of catalase and its complex with cyanide in 0-1 M-potassium phosphate buffer, pH 7, at 25 °C are shown in Fig. 2. Absorbance maxima for the native enzyme were at 408, 500, 537 and 627 nm; for the azide complex at 413, 500, 540 and 623 nm; and for the cyanide complex at 427, 558 and 590 nm. Under these conditions the dissociation constants for azide and cyanide were 2 mM and 24 μM, respectively. The anaerobic addition of sodium dithionite to the enzyme, from the side arm of a Thunberg cuvette, did not bring about reduction of the enzyme.

**Nature of the superoxide dismutase**

Cell-free extracts of *P. chrysosporium* grown on glucose or cellulose in high- and low-nitrogen medium were submitted to native gel electrophoresis. Samples from each culture were examined at 2, 4, 6, 12 and 20 d. In every case, activity staining of the gel revealed one band of superoxide dismutase activity of identical mobility. In the presence of 2 mM-cyanide, this activity band did not develop. This indicates that *P. chrysosporium* only produces a typical eukaryotic copper/zinc enzyme which is characteristically inhibited by cyanide.
DISCUSSION

The catalase and superoxide dismutase produced by *P. chrysosporium* are of the type most commonly found in eukaryotic micro-organisms. However, the levels of these enzymes found in *P. chrysosporium* during primary growth are comparable to the highest activities detected in any micro-organism (McCord *et al.*, 1971).

Lignin biodegradation is a secondary metabolic event controlled, to a large degree, by the level of nitrogen in the medium (Keyser *et al.*, 1978; Jeffries *et al.*, 1981). Interestingly, the specific activities of catalase and superoxide dismutase were independent of the concentration of the nitrogen source.

H$_2$O$_2$ is believed to be an essential component in lignin biodegradation. It is not known how *P. chrysosporium* generates H$_2$O$_2$, although it has been suggested that the flavocytochrome-b-containing cellobiose oxidase (Morpeth, 1985) could be involved (Paterson & Lundquist, 1985). This enzyme first appears during primary growth (unpublished observations), which is consistent with the time course of catalase and superoxide dismutase production.

Whatever the mechanism of H$_2$O$_2$ production, the dramatic fall in intracellular catalase and superoxide dismutase activity during secondary growth is at first sight surprising. However, it can be explained by two factors. Firstly, *P. chrysosporium* will be more susceptible to oxidative damage when it is actively growing. Secondly, when the catalase levels inside the cell decrease at the end of the primary-growth phase, the concentration of peroxidases outside the cell increases. Thus, when one H$_2$O$_2$-detoxifying enzyme disappears another is being generated. Since H$_2$O$_2$ is able to cross biological membranes (Chance *et al.*, 1979), the fact that one of the peroxidases is bound to the mycelium (Paszczynski *et al.*, 1986) may be significant.

REFERENCES


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