Galactose Oxidase of *Polyporus circinatus*: a Copper Enzyme*

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In 1959, Cooper et al. (1) described the isolation of an enzyme from *Polyporus circinatus* Fr., which catalyzed the oxidation of galactose by molecular oxygen. We have previously described a procedure for the purification of such an enzyme from culture filtrates and have shown the reaction product to be D-galacto-hexodialdose, oxidation occurring at the C-6 rather than the C-1 position (2). The present paper is concerned with further improvement in production of the enzyme, which has now been crystallized, and the determination of the molecular weight and identification as a copper-containing metalloenzyme.

**EXPERIMENTAL PROCEDURE**

Materials—For assay of galactose oxidase we used the Glucostat reagent (Worthington Biochemical Corporation) without glucose oxidase as previously described (3). Protein concentration was measured by the phenol method (3). All samples containing ammonium sulfate were dialyzed against buffer before protein assay because of interference of color development by this salt.

Radioactive iodinated serum albumin was obtained from Squibb in the form of Albumatope and was counted conveniently in a well-type scintillation counter. Purified alkaline phosphatase from *Escherichia coli* was kindly provided by Mr. M. Malamy.

Copper content was determined by the Scheinberg and Morell modification1 of the dicyclohexanoneoxalyldihydrazone (Eastman Organic Chemicals No. 7175) method for microdetermination of copper (4, 5) as developed for ceruloplasmin copper. All glassware used for enzyme purification was treated with a 10-5 M CuSO4 medium to eliminate these failures. The addition of 10-4 M CuSO4 medium tends to eliminate these failures.

Cultures were harvested 24 hours after full growth was obtained, approximately 48 hours after inoculation. The cultures were filtered through layers of cheesecloth to remove mycelia and each liter of culture filtrate were added 10 g of cellulose powder and 700 g of ammonium sulfate. The suspension was stirred for a minimum of 2 hours, and the cellulose and precipitated protein were collected on a 2-liter, coarse sintered funnel. The cellulose and precipitated protein were transferred to a column and washed with 100 ml of 60% saturated ammonium sulfate solution followed by 100 ml of 0.1 M phosphate buffer, pH 7, for each liter of media. Air was passed through the column between each wash.

The phosphate buffer eluate was allowed to run slowly down the side of a large cylinder. After about 30% of the calculated volume had been eluted, two darkly pigmented bands appeared in the eluate. These contained about 90 to 95% of the enzyme. This fraction was carefully siphoned off and brought to saturation with ammonium sulfate. The precipitate was collected by centrifugation for 1 hour at top speed in the Lourdes VRA centrifuge.

The precipitate was resuspended in 30 to 50 ml of 0.1 M phosphate buffer, pH 7, and 0.050 ml of purified beef liver catalase was added. The suspension was placed in dialysis tubing and dialyzed for 12 hours against 0.02 M phosphate buffer, pH 7. As dialysis starts some effervescence takes place. This has been shown to be due to oxygen release from peroxide formed in the solution and if catalase is not added, inactivation of the enzyme may occur. The source of hydrogen peroxide has not been identified but is not present in the dialysis tubing and is occasionally present even when the mold is grown on glucose.

After dialysis, the enzyme is purified as previously described except that the Mg++ concentration was 0.02% (2). However, an increased yield of enzyme has been obtained by reducing the rate of agitation of the growing cultures. Mycelia are now grown at 25° in 2-liter flasks containing 500 ml of medium with rotary shaking at about 200 r.p.m. and a 2-inch radius. Smaller culture flasks will tolerate higher speeds without loss of enzyme activity. Aeration of cultures by means of spargers also resulted in enzyme denaturation. Optimal conditions of growth yield up to 50 to 60 units of galactose oxidase per ml of media.

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RESULTS

**Purification and Growth**

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the sucrose gradient method. The molecular weight of galactose
known proteins, as described by Martin and Ames (6).

oxidase was calculated from its location relative to that of the
molecular weight 68,000 (lo), in 1% sucrose solution, and E. coli alka-
tivity over the next 48 hours.

III in mineral oil; the end point in the recovery of fractions
were formed. Linearity was checked with methylene blue. To mark the menis-
by means of the apparatus of Martin and Ames (6). Again
(9), 5 to 20% sucrose gradients in Tris buffer (0.1 M, pH 7.4)
precipitates under polarized light but no structural details are evident. At the end of
72 hours, large rhomboid plates appear and increase in quanti-
ty over the next 48 hours.

The specific activity of this material is about 8,000 units per
mg of protein. The over-all yield of activity is about 30%.

Molecular Weight of Galactose Oxidase

By means of the gradient apparatus of Britten and Roberts
(9), 5 to 20% sucrose gradients in Tris buffer (0.1 M, pH 7.4)
were formed. Linearity was checked with methylene blue added to
the dense solution. The tubes were emptied drop by drop
by means of the apparatus of Martin and Ames (6). Again
linearity was checked with methylene blue. To mark the meniscus
we overlaid the protein layer at the meniscus with Sudan
III in mineral oil; the end point in the recovery of fractions
after centrifugation was thus marked by the appearance of a
bright red drop at the outflow needle.

Standards consisted of radioactive iodinated serum albumin,
molecular weight 68,000 (10), in 1% solution, and E. coli alka-
line phosphatase, molecular weight 80,000 (11), containing 140
units per ml. Galactose oxidase was at a concentration of
1,200 units per ml. The protein layer contained 0.05 ml of
galactose oxidase, 0.05 ml of alkaline phosphatase, and 0.005
ml of radioactive iodinated serum albumin containing 115,000
C.p.m. All tubes were spun for 12 hours at 38,000 r.p.m. and
emptied by needle puncture into 5-ml serum tubes, 5 drops
(approximately 0.08 ml) per tube. The entire tube was counted
for 113I in a well counter, and aliquots were taken for galactose
oxidase and alkaline phosphatase assays. This method measures
relative sedimentation constants and involves the assumptions
that the molecules are roughly spherical and have the same
partial specific volume. With these assumptions the relative
molecular weight can be calculated. The approximate molecular
weight of the enzyme, assuming a partial specific volume of
0.725 cm³ per g, was calculated to be about 75,000 g per mole
(Fig. 1).

Enzyme Inhibition and Reactivation

Diethyldithiocarbamate—Diethyldithiocarbamate at a concen-
tration of 10⁻⁴ M stopped the enzymatic action of galactose
oxidase completely and instantly. In an attempt to define the
action of this inhibitor, the purified enzyme was diluted to 0.5
mg per ml in 0.1 m phosphate buffer, pH 7, containing 10⁻³ M
diethyldithiocarbamate and the light yellow metal-diethyl-
dithiocarbamate complex which formed was completely extracted
from the enzyme solution with isoamyl alcohol. The remaining
diethyldithiocarbamate was removed by three subsequent
dialyses against buffer. The inactive apoenzyme was reacti-
ated by incubation with copper salts for 24 hours at 25°C.
Activity was measured by the Glucostat or Somogyi-Nelson
methods (Table I). With increasing incubation time, further
reactivation could be achieved, approaching 100% at 36 hours
(see Fig. 2). Results with enzyme inactivated by dialysis against
H₂S solution were similar. Other metals tested, including
Co++, Ni++, Zn++, Mn++, Mg++, Fe++, Mo++, were completely inactive.

Cyanide—In an attempt to produce a metal-free apoenzyme by
a procedure similar to that used by Kubowitz with tyrosinase
(12), cyanide was employed as an inhibitor. Complete inacti-
vation was produced at 1 X 10⁻⁴ CN⁻ but addition of CN⁻
to reaction mixtures showed inhibition to be slow and progressive.
After dialysis to remove excess cyanide, reactivation was achieved

![FIG. 1. Molecular weight determination of galactose oxidase by
the sucrose gradient method. The molecular weight of galactose
oxidase was calculated from its location relative to that of the
known proteins, as described by Martin and Ames (6).](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Specific activity*</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, no treatment</td>
<td>3700</td>
<td>100</td>
</tr>
<tr>
<td>Treated with diethyldithiocarbamate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Incubated with Cu⁺, 1.1 X 10⁻⁴ M</td>
<td>1660</td>
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* Specific activity is defined as units per mg of protein (see (2)).
† The enzyme concentration was 0.5 mg per ml in 0.1 m phos-
phate buffer, pH 7.
not only with copper but also with nickel, silver, or cobalt ions. It was postulated that cyanide complexes with, but does not remove, the essential metal group(s) of the enzyme molecule.

Other Inhibitors—The following inhibitors were also tested: ethylenediaminetetraacetic acid, benzoin oxime, o-phenanthroline, and α,α-dipyridyl. None of these affected the enzyme activity when present in the assay mixture at $10^{-3}$ M. Azide and hydroxylamine inhibited completely at $10^{-3}$ and 25% and 35%, respectively, at $10^{-4}$ M. Dialysis for several days against $10^{-3}$ M EDTA did not affect the activity of the enzyme.

Kinetics of Reactivation

The apoenzyme, prepared by treatment with $10^{-5}$ M diethyl-dithiocarbamate for 20 minutes, followed by extraction with an equal volume of isoamyl alcohol, was dialysed for 6 hours against 0.1 M Tris buffer, pH 7.4, with four changes of buffer during this time. On addition of copper and incubation at room temperature there was a slow but progressive reactivation of the enzyme, which was strongly dependent on the concentration of copper (Fig. 2). At $10^{-2}$ M Cu++, nearly full activity was restored in 24 hours, and little further change was observed when the enzyme-Cu mixture was incubated for several days longer at the same temperature. With $10^{-4}$ M Cu++ reactivation was slower, and reached a maximal value of only 35% of the expected value. At $10^{-5}$ M Cu++ no significant reactivation was observed, although in other experiments the apoenzyme was capable of extensive reactivation by Cu++ at this concentration.

The failure to reactivate at low levels of Cu++ may be related to the instability of the apoenzyme in the absence of Cu++ (Fig. 3). If the apoenzyme is treated with $10^{-3}$ M Cu++ immediately after preparation and dialysis, it can be reactivated fully. After storage at 0°, however, the apoenzyme can no longer be fully reactivated. After storage for 48 hours, very little reactivation is observed.

Metal Content of Galactose Oxidase

The specificity of copper in the reactivation of galactose oxidase after treatment with H₂S or diethyl-dithiocarbamate suggested that this was the true metal cofactor. This conclusion was supported by the results of copper analysis on the native enzyme. These assays were performed at each step in purification, on the apoenzyme after diethyldithiocarbamate and after cyanide inactivation. The results are given in Table II. The ratio of copper to protein does not change markedly as the enzyme is purified, suggesting that galactose oxidase is not the only copper protein present in the medium.

On the basis of the molecular weight of 75,000, the purified enzyme contains 1 atom of copper per mole of enzyme.
The $\beta$-galactose oxidase of Polyporus circinatus exhibits a number of unusual properties. Among these is its ability to catalyze oxidation of $\beta$-galactose at position 6 to form $\beta$-galacto-hepaldialdose, a compound hitherto unknown in biological systems. Although it is specific for the $\beta$-galactopyranose structure, it is more active with polysaccharides containing this structure than with the monosaccharides. The enzyme has proved highly useful for the quantitative determination of galactose, for the identification of galactose in polymers (2), and for converting galactosides to the corresponding galactosiduronic acid derivatives. Since glycosiduronic linkages are more stable to acid hydrolysis than are glycosidic linkages, this conversion facilitates characterization of the galactoside linkage in polysaccharides.

The enzyme does not contain a flavin prosthetic group (2), unlike glucose oxidase which contains FAD as the prosthetic group (13). Our early experiments with metal-binding agents suggested that galactose oxidase is a metalloprotein although the results with such agents as EDTA or cyanide were confusing; the former because it was not at all inhibitory and the latter because the inhibited enzyme after dialysis was reactivated by a number of metal ions. Vallee (14) has discussed this problem in detail.

The evidence presented here supports the conclusion that galactose oxidase is a copper enzyme, containing about 0.85% Cu, which represents 1 g atom per mole of enzyme of molecular weight 75,000. The copper can most conveniently be removed by dialysis against H$_2$S or by treating the enzyme solution with diethylidithiocarbamate and extracting the diethylidithiocarbamate-Cu complex with isoamyl alcohol. The apoenzyme is fully reactivated by incubation with Cu$^+$ or Cu$^{++}$ salts. The apoenzyme loses its ability to be reactivated when it is stored at O°, although the native enzyme is completely stable under these conditions. Studies are now underway to identify the site of binding of copper.

It is noteworthy that the ratio of copper to protein does not change significantly during purification of the enzyme from the culture filtrates. A possible implication of this result is that galactose oxidase is the only extracellular protein produced by the organism and that it has become partially inactivated. This inactivated enzyme would be removed during purification. This explanation is consistent with the lability of the enzyme in the culture medium. For example, enzyme activity is lost if the shaking speed is increased. An alternative explanation is that other extracellular copper proteins are elaborated by the organism.

The nature of linkage of copper in the protein is not understood, but the evidence suggests that it is very tightly bound. It is not removed by cyanide or by passage over Chelex 100. The slow combination of copper with apoenzyme suggests that the protein may undergo changes in conformation when copper is removed. These changes in conformation may become irreversible since the apoenzyme loses its ability to be reactivated when it is stored at O°, although the native enzyme is completely stable under these conditions. Studies are now underway to identify the site of binding of copper.

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REFERENCES


Note added in proof—The organism employed in these studies has now been identified as Dactylium dendroides, rather than Polyporus circinatus (M. K. Nobles and C. Madhosingh, Biochem. and Biophys. Research Commun., in press).
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