Purification and Properties of the Catalase of Bakers' Yeast

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SUMMARY
Catalase from bakers' yeast has been purified to homogeneity in the analytical ultracentrifuge and in gel electrophoresis; sedimentation measurements permit an estimation of its molecular weight as 248,000. Under denaturing conditions, polyacrylamide gel electrophoresis revealed dissociation of a major component of molecular weight 61,000, which constituted 90% of the total protein of the stained gel, suggesting that the native enzyme is tetrameric. The iron content was 0.096%, corresponding to a subunit molecular weight of 58,000. Specific activity was high (Kat. f. = 66,000); catalytic and spectroscopic properties were similar to those of catalases from other species. The enzyme is present in commercial yeast and in a variety of haploid and diploid wild type strains.

The catalase of bakers' yeast, Saccharomyces cerevisiae, has been the object of considerable research, since it was first investigated by Issajew in 1904 (1). It was studied intensively in the laboratory of von Euler (2,3) and was used by Keilin and Hartree (4) in their classic paper on the inhibitory action of sodium azide. Its biosynthesis was shown to be inducible by oxygen (5,7), and it was found to be stable but diluted out during anaerobic growth of catalase-rich, originally aerobic yeast (8). The catalase activity of intact yeast, that is, its ability to decompose exogenous H2O2, was shown to be due to a relatively heat-stable fraction (9) localized at the cell surface (10). Cell fractionation was used to demonstrate that part of the catalase of yeast was localized in the microbodies (11) or peroxisomes of de Duve (12). Kinetic studies of crude enzyme preparations (13), and the assumption that the specific activities of yeast and beef liver enzymes were similar, permitted calculation of the number of molecules of catalase in aerobic yeast cells (14).

Several unsuccessful attempts to purify the enzyme to homogeneity have been reported (15,16); the most recent, by Drews and Moeller (17), reported achieving a purity of approximately 50%. We now report purification of yeast catalase to homogeneity; this should permit a more systematic study of the regulation, intracellular function, and localization of this much investigated enzyme.

EXPERIMENTAL PROCEDURE

Materials
Commercial dried bakers' yeast was from Standard Brands. Wild type strains were obtained from F. Lacroute (FL-90) and from F. Sherman (D 585-11c and D 587-4b). Special chemicals were obtained as indicated: bovine serum albumin and sodium dodecyl sulfate (SDS) from Sigma; pyruvate kinase from Boehringer-Mannheim; myokinase from Calbiochem; Sephadex from Pharmacia; hydroxylapatite from Bio-Rad; partially hydrolyzed starch from Connaught Laboratories; Tris-barbital buffer from Gelman.

Methods
Enzyme Assay—Catalase activity was based on the titration sulfate procedure of Patti and Bonet-Maury (18) and Chantrenne (6), a pseudo-first order rate constant being calculated by the method of Bonnichsen et al. (19). Specific activity is expressed as Kat. f., after von Euler and Josephson (20). H2O2 was diluted into 5 x 10⁻³ M phosphate buffer, pH 7.0, to yield a final substrate concentration of 0.01 M. The reaction, at 0°C, was begun by adding 30 µl of enzyme to 10 ml of buffered substrate, agitating rapidly by means of a Vortex mixer, and returning to crushed ice.

From 0 min, and thereafter at intervals of 1 min, 1-ml aliquots were withdrawn and rapidly pipetted into 2 ml of 2.0 N H2SO4, saturated with TiSO4, and agitated. At least six aliquots were withdrawn per run, the last usually at 5 min, at which time approximately 30% of the substrate remained; purified preparations contained about 1 µg of protein, and impure preparations proportionately more. Subsequently, tubes were read at 410 nm in the Zeiss PMQ spectrophotometer. The rate of substrate decomposition was proportional to enzyme concentration, and the reaction was linear with time.

Sedimentation Measurements—Sedimentation velocity measurements were made at 48,000 rpm in a Spinco model E analytical ultracentrifuge equipped with schlieren optics. Sedimentation constants were calculated as described by Schachman (21) and Chervenka (22). Pictures were taken at intervals of 8 min, after reaching speed. The sedimentation boundary was followed until it disappeared.

Gel Electrophoresis—Electrophoresis was run in 5% polyacrylamide gels as described by Davis (23), except that the enzyme was layered directly onto the gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (24), except that preincubation was for a duration of 1, rather than 2 hours, since additional dissociation occurred with the longer incubation time. Stained gels were scanned at 570 nm with a Gilford recording spectrophotometer equipped with a gel scanning accessory with recorder. For quantitation of tracings, the area beneath the peaks was estimated by counting of squares. Electrophoresis in starch gels utilized 15% starch in 0.05 M Tris-barbital buffer, pH 8.6, at 5°C; runs were for 17 hours at 600 volts.

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after which the starch blocks were cut in half. One-half was stained for protein with Amido black, and the other for catalase activity, according to Scandalios (25).

Other Measurements—Iron determinations utilized the phenanthroline procedure (20), the purified enzyme was exhaustively dialyzed against distilled water, and was acid-digested prior to reaction with the iron reagent. For the spectrophotometric measurements, the purified enzyme was dissolved in 0.005 M phosphate buffer, pH 7.0. Protein was determined according to Lowry et al. (27), with bovine serum albumin as standard. All manipulations during purification were carried out at room temperature, except the centrifugations which were at 5°C in the Sorvall RC-2B at 39,000 × g, except where otherwise indicated.

RESULTS

Purification

All of the previous attempts to purify this enzyme started by incubating yeast with buffer at room temperature for several hours; the resulting supernatant, after centrifugation, was the crude extract (15-17). Our crude extracts were prepared by homogenizing the yeast with techniques used in this laboratory to purify other enzymes (28); such crude extracts had specific activities higher by a factor of 40 to 100 times those obtained with nonhomogenized, partly autolyzed preparations. A typical purification is shown in Table I.

Step 1: Crude Extract—Thirty grams of dry yeast were added to 100 ml of 0.03 M veronal-HCl buffer, pH 8.2, and stirred for 30 min. Aliquots (25 ml), together with 20 g of glass beads (0.45 to 0.50 mm) were shaken in the Braun MSK homogenizer for three periods of 30 s, with cooling by CO2. Unbroken cells and debris were removed by centrifugation at 12,000 × g for 30 min, and the supernatant was recentrifuged at 39,000 × g for 20 min. The resulting supernatant was designated the crude extract.

Step 2: Ethanol-Chloroform Treatment—One volume of a mixture of ethanol and CHCl3 (1:1, v/v) was added to 5 volumes of crude extract in a 250-ml separatory funnel. The stoppered funnel was then subjected to three periods of vigorous shaking for 15 s; the stopper was opened between periods. After 20 min at room temperature, the contents were spun at 39,000 × g; the top, aqueous layer, which contained 80% of the catalase activity of the crude extract, was removed, and the dense, middle layer of denatured protein and bottom layer of organic solvent was discarded.

Step 3: First Ammonium Sulfate Fractionation—Solid (NH4)2SO4 was added to 40% saturation, using the nomogram of Dixon (20). The precipitate was discarded, and the supernatant was brought to 60% saturation. The precipitate was collected and suspended in 0.005 M phosphate buffer, pH 7.0. The final concentration was approximately 15 mg of protein per ml.

Step 4: Second Ammonium Sulfate Fractionation—Solid salt was added to 45% saturation, assuming (no doubt incorrectly) that none was carried over when the second precipitate of Step 3 was resuspended in buffer. The precipitate was discarded, and the supernatant again was brought to 60% saturation. The precipitate was dissolved in 1 to 2 ml of the same buffer as in Step 3 and reserved. This second fractionation was found to cause about a 2-fold purification without much loss of catalase.

Step 5: Sephadex G-75 and Hydroxylapatite Chromatography—The solution was passed over a column (1.5 cm × 30 cm) of Sephadex G-75 pre-equilibrated with distilled water. Elution was also with distilled water, utilizing a slow flow (approximately 1 ml per 20 min) and collecting 1.5 ml aliquots. Protein peaks were monitored by their absorption at 280 nm; the catalase usually appeared with the first protein peak. Tubes with catalase activity were pooled, and the enzyme was precipitated with (NH4)2SO4 (80% saturation) and taken up in a small volume (1.5 to 2.0 ml) of distilled water. The resulting brown solution was desalted by passing over a column (0.9 cm × 15 cm) of Sephadex G-25, coarse mesh, and eluting with distilled water. Aliquots (1.5 ml) were collected, and those with catalase activity were pooled and placed on a column (2 cm × 5 cm) of hydroxylapatite which had been pre-equilibrated with 0.1 M phosphate buffer, pH 7.0. The column was then washed with approximately 10 ml of the same buffer, and the eluate was discarded. Next, the column was washed with 20 ml of 0.1 M phosphate buffer, pH 8.0. The column was again washed with 30 ml of 0.15 M phosphate buffer, pH 8.0; the eluate contained a protein with catalase activity to be described elsewhere. The enzyme to be described in this communication was eluted from the hydroxylapatite column by means of vacuum dialysis and then stored in the freezer at −15°C; under these conditions, its activity was stable for several months. Its activity declined after several cycles of freezing and thawing; it also declined rapidly at 4°C, unlike the beef liver catalase.

Properties

Starch Gel Electrophoresis—Fig. 1 shows a starch block cut in half after electrophoresis; one-half was then stained for protein (a) and the other for catalase (b). Only one band was noted in each case and these corresponded in position.

Analytical Ultracentrifugation—Fig. 2 shows that the purified catalase sediments as a single, symmetrical boundary with a sedimentation constant s20,w of 11.5 × 10−13, which corresponds to a molecular weight of 248,000, assuming that the values of v = 0.715 and D = 4.1 × 10−13 for partial specific volume and diffusion constant obtained for the beef liver enzymes (30) apply to yeast catalase. When the yeast and beef liver enzymes were run together in the analytical centrifuge, they traveled as a single, homogeneous boundary.

Polyacrylamide Gel Electrophoresis—Fig. 3a shows that the purified enzyme migrates as a single band at the usual pH of 8.6; the same is true when it was run at pH 6.3 (Fig. 3b). Fig. 3c shows a run after incubation of the sample with 1% sodium

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a Prepared from 30 g of commercial yeast.
Fig. 1 (left). Starch gel electrophoresis of yeast catalase. Protein in gel is 0.1 mg. Conditions of run were described under "Experimental Procedure." a, protein stain; b, activity stain.

Fig. 2 (center). Sedimentation velocity pattern of yeast catalase. Protein concentration is 45 mg in 5 x 10^{-4} M phosphate buffer pH 7. Photographed at 52 min after reaching speed of 48,000 rpm, with bar angle at 65°. Sedimentation is from right to left.

Fig. 3 (right). Polyacrylamide gel electrophoresis of yeast catalase. a, 50 μg of enzyme were applied to a 5% polyacrylamide gel containing 5 x 10^{-2} M Tris-barbital buffer, pH 8.6, and subjected to 100 volts and 5 ma for 3 hours at room temperature. Protein was stained with amido black. b, 30 μg of enzyme were run under identical conditions as in a, except pH of the buffer was 6.3 instead of 8.6. c, 100 μg of enzyme were preincubated in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. Running buffer was 0.1 M boric acid-sodium acetate, pH 8.5, containing a final concentration of 0.1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol. Running time was 3 hours at 60 volts and 5 ma per gel at room temperature. Protein subunits were stained with 0.25% Coomassie brilliant blue.

Fig. 4. Densitometric tracing of the gel shown in the previous figure. Peak area was calculated as that beneath the curve and above the extrapolated baseline (---).

dodecyl sulfate for 1 hour; one intense band is noted, preceded by a faint band of lighter material. Tracing the gel, as shown in Fig. 4, revealed that the yield of the major component was approximately 90% of the total protein. When the native catalase was incubated with sodium dodecyl sulfate for 2 hours or longer, two additional faint bands of lighter material preceded the intensely stained main band; we noted a similar phenomenon in the case of crystalline beef liver catalase. The major subunit had a molecular weight of 61,000, as determined by interpolation from the migration of the marker proteins bovine serum albumin, pyruvate kinase, and myokinase; yeast catalase thus dissociates principally into quarter-molecules, as does the beef liver enzyme under these conditions (24).

Fig. 5. Absorption spectrum of yeast catalase. The protein concentration is 0.8 mg per ml in 5 x 10^{-5} M phosphate buffer, pH 7.

Spectroscopy—Fig. 5 shows the absorption spectrum of the purified yeast enzyme; the location of the absorption maxima is similar to that of other catalases (31). The extinction coefficient of the yeast enzyme at 405 nm was 20.2 x 10^{4} M^{-1} cm^{-1}, as compared to 29.7 for the beef liver enzyme. The Soret band of the yeast enzyme is relatively more intense than that of the beef liver enzyme, the ratio of absorption (A_{405 nm}:A_{390 nm}) being 0.93 for the former, as against 0.78 for the latter, as determined in this laboratory.

Iron Content—The iron content of the yeast enzyme was determined to be 0.096%, which is close to that which we obtained for the beef liver enzyme (0.092%), corresponding in the case of
the former, to a subunit molecular weight of 58,200, assuming 1 atom of iron per subunit.

**Catalytic Properties**—The mean $\text{Kat. f.}$ of six different preparations was $66,500 \pm 1800$ (standard deviation); the $\text{Kat. f.}$ of our crystalline beef liver catalase was 60,000. Fig. 6 shows the variation of specific activity of the enzyme with pH. Two peaks were noted, a broad one at pH 6 to 7, and the other at around pH 0.5. The three points on Fig. 6, connected by the dotted line, represent the mean of five determinations with standard deviations (performed a day subsequent to the other assays but with the same preparation) to illustrate that the second activity peak seems not to be an artifact. Fig. 7 shows the variation in specific activity as a function of concentration of $\text{H}_2\text{O}_2$; a broad optimum at 0.01 M substrate was noted, which is the concentration often used with catalases from other sources (32). Detailed kinetic studies will be published later.

**Presence in Laboratory Strains of S. cerevisiae**—With this same procedure, we have purified to homogeneity a catalase of identical properties from wild type diploid strain FL-90 and from the haploids, D 88-11e and D 88-4b.

**Discussion**

The usual criteria for homogeneity have been met in the case of yeast catalase: analytical ultracentrifugation and electrophoresis on starch and polyacrylamide gels at varying pH. It has spectral and catalytic properties very close to those of this enzyme from other species. Under denaturing conditions, the principal subunit, accounting for 90% of the total protein, was a quarter-molecule; as in the case of the beef liver enzyme, more prolonged incubation with sodium dodecyl sulfate caused some further dissociation, resulting in the appearance of two to three minor bands of lower molecular weight than the 61,000 of the major subunit, suggesting that the latter may be a protomer consisting of more than one polypeptide chain. The iron content of the native yeast enzyme indicated a subunit molecular weight of 58,200, again suggesting that the oligomeric enzyme consists of four principal protomeric subunits, each containing 1 atom of iron. The exact subunit structure and number of individual polypeptide chains have not yet been determined, even in the case of the well characterized mammalian liver catalases. Deisseroth and Dounce (see Table 4 of Ref. 31) point out that, depending on the method of dissociation employed, one can obtain subunits which are $\frac{1}{6}$, $\frac{1}{6}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{2}$, or $\frac{3}{4}$ the molecular weight of the native enzyme.

We have isolated a catalase identical in sedimentation constant and catalytic properties from a variety of well characterized haploid and diploid laboratory wild type strains of *S. cerevisiae*. On the other hand, the enzyme was absent, or in very low concentration, in extracts of low catalase mutants, which we isolated in collaboration with Dr. Fred Sherman (to be published elsewhere); this enzyme, therefore, seems to be responsible for a major part of the catalase activity expressed by the intact cells (patent activity). However, mutants, totally deficient in this enzyme, nonetheless possessed measurable catalatic activity. This and other evidence, which we will present elsewhere, indicate that yeast possesses other proteins with catalatic activity, at least two of which we have purified from commercial yeast and from laboratory wild type strains (32). The contribution of these proteins to the catalatic activity of the yeast cell and their relationship to one another are complex questions which we hope to resolve in present research.

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**References**

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