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THE PURIFICATION AND PROPERTIES OF SUPEROXIDE DISMUTASE FROM *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Superoxide dismutase has been isolated from *Saccharomyces cerevisiae*, a unicellular eucaryote, and has been found to be a blue-green, copper and zinc containing enzyme similar to those already described from a number of multicellular eucaryotes.

INTRODUCTION

Superoxide dismutase catalyzes the reaction: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (refs. 1–3). This enzyme was found to be present in a variety of oxygen-tolerant organisms and to be absent from obligate anaerobes and has been proposed to play a central role in protecting cells against the deleterious actions of superoxide radicals[4]. This enzyme has been isolated from several organisms, both procaryotes[5,6] and eucaryotes[1,7,8], and in the cases thus far examined the procaryotic enzymes (*Escherichia coli* and *Streptococcus mutans*) were reddish mangano-proteins, whereas the eucaryotic enzymes (mammalian, plant, bird and fungal) were blue-green and contained Cu^{2+} and Zn^{2+}. From an evolutionary standpoint it seemed important to obtain the enzyme from a unicellular eucaryote, such as a yeast, to see whether it was a mangano or a cupro–zinc enzyme and thus to extend the basis for the generality stated above.

There was an entirely independent reason for undertaking the isolation of superoxide dismutase from a yeast. Thus, increasing the nutritional supply of Cu^{2+} has been reported to enhance the radioresistance of aerobically grown yeast[9]. Since oxygen enhances the radiosensitivity of a variety of cells[10–12] and since ionizing radiation, in passing through oxygenated aqueous solutions, would certainly generate O_2^- (ref. 13), one may propose that the observed[9] effect of Cu^{2+} was due to an increase in the intracellular level of superoxide dismutase and that this, in turn, caused the enhanced radioresistance. It was necessary, in pursuing this proposal, to demonstrate first that the superoxide dismutase of yeast is a copper enzyme and then that enriching the growth medium with Cu^{2+} does raise the level of this enzyme in the yeast. This report, which deals with the isolation and characterization of the superoxide dismutase of *Saccharomyces cerevisiae*, establishes the first of these points.

MATERIALS AND METHODS

S. cerevisiae was obtained from the Fleischmann's Yeast Co. Cytochrome *c*, type III, and xanthine were products of the Sigma Chemical Co. Xanthine oxidase (EC 1.2.3.2) was prepared from unpasteurized cream by Mr Ralph Wiley by a procedure which avoided exposure to proteolytic agents[14]. Superoxide dismutase was assayed as previously described[1] but with the modification that $2 \cdot 10^{-5}$ M CN^- was used to inhibit peroxidases, which might otherwise interfere with assays of crude cell extracts. This level of CN^- was observed to inhibit the activity of the purified yeast superoxide dismutase by 28% and assays in the presence of CN^- were corrected for this inhibition. Spectrophotometric assays were performed at 25 °C in a Gilford Model 2000. Disc gel electrophoresis was performed as described by Davis[15] and by Panyim and Chalkley[16]. Absorption spectra were recorded with a Cary Model 15. The molecular weight of the holoenzyme was determined by sedimentation equilibrium using a synthetic boundary cell[17] in a Beckman Model E Analytical Ultracentrifuge. The molecular weight of subunits of the enzyme was estimated by sedimentation equilibrium[18] in 6.0 M guanidinium chloride and by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate and β-mercaptoethanol[19]. Amino acid analyses were performed by the accelerated method of Hubbard[20] on a Beckman Model 120B Amino Acid Analyzer equipped with high sensitivity cuvettes. Metal analyses were done with a Perkin Elmer Model 303 Atomic Absorption Spectrophotometer. Electron paramagnetic resonance spectra were obtained with a Varian Model E-9HF equipped with a 9.5 GHz microwave bridge assembly and operated at a modulation frequency of 100 kHz. Attempts were made to demonstrate carbohydrate in yeast dismutase by staining gels on which the enzyme had been electrophoresed with the periodic acid–Schiff base reagent according to the method of Zacharius *et al.*[21], with the modification that after staining the gels were washed overnight in deionized water and then destained in 50% methanol for 8 h.

RESULTS

Purification of the enzyme

Four pounds of yeast cake were frozen and thawed and suspended in 190.4 ml of 0.1 M $NaHCO_3$ per 100 g of yeast. Ethanol–chloroform (5:3, v/v) was then added (109.6 ml/100 g of yeast) and the mixture was stirred at 25 °C for 2 h after which it was clarified by centrifugation at 13 000 × *g* for 10 min. Unless stated otherwise all of these manipulations were performed at 25 °C. Solid K_2HPO_4 (300 g/l) was added slowly under stirring to the clear supernatant, and the organic phase, which was salted out was allowed to rise for 15–30 min, was separated, and then clarified by centrifugation at 13 000 × *g* for 10 min at −15 °C. During centrifugation and chilling a small amount of dense phase separated out and collected in the centrifuge bottles. This dense phase was removed by aspiration before the supernatant was decanted. If the precipitate was dispersed during the aspiration of the dense liquid phase, then the centrifugation was repeated before the supernatant was decanted. The supernatant was chilled and all subsequent steps were performed at 4 °C. Chilled acetone (0.75 vol.) was added slowly with stirring. The precipitate which formed was collected

by centrifugation at 13 000 × *g* for 10 min and was dissolved in 80 ml of 0.025 M potassium phosphate, pH 7.8. Microgranular diethyl aminoethyl cellulose (DE-32), which had been equilibrated with 0.0025 M potassium phosphate buffer at pH 7.8 and then collected on a buchner funnel, was added (2.0 g DE-32/100 g of yeast) to the enzyme solution, and the mixture was stirred for 30 min. The DE-32 adsorbed brownish impurities and was removed by filtration. The pale green filtrate was dialyzed overnight against changes of 0.0025 M potassium phosphate, pH 7.8, and, after clarification by centrifugation, it was applied to a 2.5 cm × 22 cm column of DE-32, which had been equilibrated with 0.0025 M potassium phosphate, pH 7.8. The sample was washed onto the column with approx. 60 ml of the equilibration buffer and a linear gradient of potassium phosphate (0.0025 → 0.050 M) at pH 7.8 was applied in a total volume of 1 l. The flow rate was 45 ml/h and 6.0 ml fractions were collected.

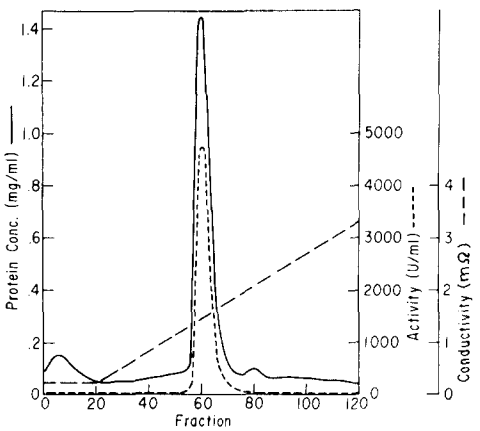

Fig. 1. Chromatography on DE-32. The filtrate from the DE-32 batch step was dialyzed against 0.0025 M potassium phosphate, pH 7.8, and centrifuged. The supernatant was loaded onto a 2.5 cm × 22 cm column of DE-32 equilibrated with the same buffer. The column was washed with about 60 ml of 0.0025 M potassium phosphate, pH 7.8, and a linear gradient (0.0025 M–0.05 M) of this buffer was then applied. 6-ml fractions were collected. ———, protein concentration of the fractions collected, determined by the method of Murphy and Kies[19]; ---------, superoxide dismutase activity of the fractions collected; — — —, conductivity. If the absorbance at 260 nm of the column eluent is followed, one sometimes finds a small peak eluting before the dismutase peak. The nature of this material is unknown.

The elution profile obtained from this DE-32 column is illustrated in Fig. 1. Superoxide dismutase was eluted as a well defined peak, clearly separated from other protein components. The active fractions were pooled and found to have a specific activity of 3330 units/mg, essentially identical to the activity of the bovine enzyme[1,22]. The results of this purification procedure are summarized in Table I. The protein concentrations of the relatively impure fractions, obtained before the column chromatography, were determined by the biuret method[23], whereas purer protein fractions were quantitated on the basis of absorption in the short ultraviolet[24].

TABLE I

PURIFICATION

Fraction	*Protein concn (mg/ml)*	*Total volume (ml)*	*Total protein (mg)*	*Units per ml*	*Total units*	*Yield* (%)*	*Specific activity (units/mg)*	*Fold purification*
Chloroform–ethanol extract of yeast	1.56	5354	8350	62.5	33 500	100	40.1	—
Salted out organic phase	3.34	1515	5060	179.0	271 000	80.9	53.6	1.34
Resuspended acetone precipitate	6.36	80.0	509	3400	272 000	81.2	535	13.3
DE-32 batch step filtrate	2.64	77.5	205	2460	191 000	57.0	932	23.2
Dialyzed and spun DE-32 batch step filtrate	1.73	83.5	144	2080	174 000	51.9	1200	29.9
Pooled fractions from DE-32 column	1.08	48.0	52	3600	173 000	51.6	3330	82.3

* All yield data are relative to the organic extract.

Polyacrylamide gel electrophoresis

A crude extract of *S. cerevisiae* was prepared by ultrasonication and centrifugation and was compared with the purified superoxide dismutase by disc gel electrophoresis. The gels were run in duplicate and were stained both for protein[15] and for superoxide dismutase activity[25]. The results of these manipulations are shown in Fig. 2A. The soluble extract of whole, sonicated yeast exhibited at least a dozen bands of protein and one major band and two minor bands of superoxide dismutase activity. The purified enzyme showed one major and one minor band of protein: both of which were enzymatically active; the major band accounted for at least 90% of the total protein and activity in the purified enzyme. The gel showed no electrophoretically detectable protein components which were inactive. Electrophoretically separable bands of superoxide dismutase have been seen previously[6,25]. It is clear that one of the forms of the enzyme, present in the whole yeast extract, failed to survive the purification procedure.

Disc gel electrophoresis of the purified enzyme at low pH (3.2) and in the presence of urea (6.25 M), according to Panyim and Chalkley[16], demonstrated only one band of protein. This result is illustrated in Fig. 2B.

Absorption spectra

The purified enzyme had a blue-green color and exhibited a relatively weak absorption band at 670 nm ($\varepsilon = 231$). The ultraviolet absorption spectrum of the yeast enzyme, like the comparable spectra of the superoxide dismutase from bovine tissues[1,22], *Neurospora crassa*[8] and garden peas[7], exhibited a maximum at 258 nm ($\varepsilon = 11\,300$) and resembled the spectrum of phenylalanine. This unusual spectrum reflects the absence of tryptophan and the paucity of tyrosine in these enzymes[1,7,8,22]. The visible and ultraviolet absorption spectra of the yeast enzyme are illustrated in Fig. 3.

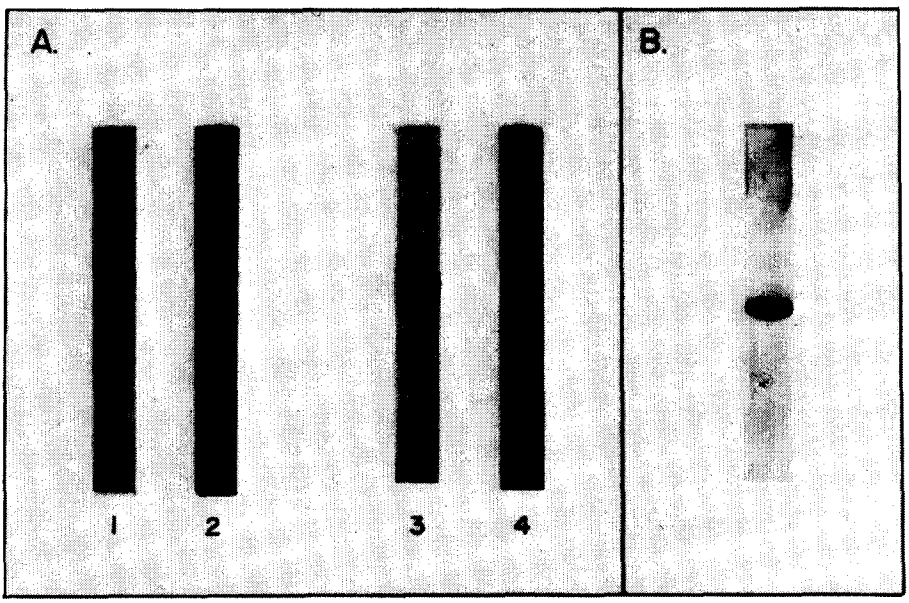

Fig. 2. Polyacrylamide gel electrophoresis. (A) Electrophoresis at high pH. Gels 1 and 2 represent a sonicate of yeast, and Gels 3 and 4 represent the purified yeast superoxide dismutase. Gels 1 and 3 are stained for protein, and Gels 2 and 4 are stained for enzymatic activity. The following amounts of material were applied to the gels: 1, 10 μl of a sonicate made by sonicating 3.3 g of yeast in 10 ml of 0.0025 M potassium phosphate, pH 7.8, for 25 min at 100 W power in the presence of 2.5 ml of 25-μm glass beads; 2, 3 μl of the sonicate; 3, 61.7 μg of protein; and 4, 1.5 μg of protein. (B) Electrophoresis at low pH. The gel is stained for protein. 10 μg of protein was applied.

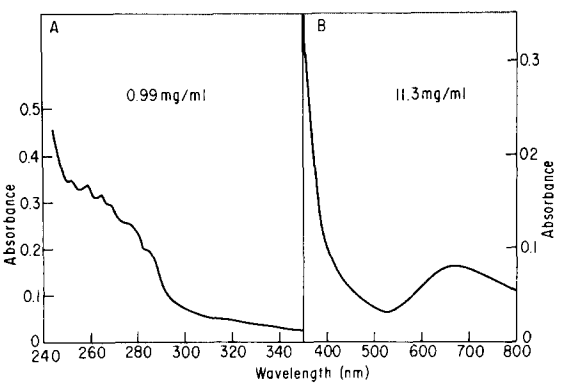

Fig. 3. Absorbance spectra of superoxide dismutase in the ultraviolet and visible. (A) Ultraviolet spectrum. The enzyme was at 0.99 mg/ml in 0.0025 M potassium phosphate, pH 7.8. The peak at 258 nm has a molar extinction coefficient of 11 300 (B) Visible spectrum. The concentration of the enzyme was 11.3 mg/ml in 0.0025 M potassium phosphate, pH 7.8. The molar extinction coefficient at the absorbance maximum at 670 nm is 231.

Molecular weight

Yeast superoxide dismutase at 0.65 mg/ml in 0.1 M NaCl–0.0025 M potassium phosphate at pH 7.8 and 21.6 °C was equilibrated in a centrifugal field at a rotor speed of 24 630 rpm (refs. 17 and 18). Interference optics were employed and ln

fringe displacement was graphed as a function of the square of the distance from the center of rotation. The data fit a straight line indicating homogeneity with respect to sedimentation properties. The slope of this line (1.24) and an assumed partial specific volume of 0.720 were used to calculate a molecular weight of 32 700.

Subunit structure

The subunit structure of yeast superoxide dismutase was probed by two independent methods. The first method involved sedimentation equilibrium[18] in the presence of 6 M guanidine hydrochloride. Superoxide dismutase at a concentration of 0.5 mg/ml was exhaustively dialyzed against 6.0 M guanidine hydrochloride and then brought to equilibrium in the analytical ultracentrifuge. When ln fringe displacement was plotted as a function of the square of the distance from the center of rotation, a straight line was obtained. Using 0.710 as the apparent partial specific volume (Φ') in this solvent[26], the molecular weight was calculated to be 15 850 or about one half of the value found for the native enzyme. This data can be interpreted to mean that the native molecule consists of two subunits of equal size which are not joined by covalent bonds. The fact that only one band was found on the polyacrylamide gels run according to Panyim and Chalkley[16] suggests that the subunits may be identical in primary structure as well as size (assuming that the conditions of low pH and high urea under which the gels were run were sufficient to dissociate the enzyme into subunits).

The second method used to probe the subunit structure was polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with and without β-mercaptoethanol[19]. This approach is open to the objection that molecular weights obtained in the absence of β-mercaptoethanol may not be accurate because of the restrictive influence of intrachain disulfide bridges. The method was attempted nonetheless and the results were satisfactory. Gels run in the absence of β-mercaptoethanol were calibrated with the following molecular weight standards: transferrin, 77 000; human serum albumin, 67 500; catalase, 60 000; ovalbumin, 43 000; pepsin, 35 000; carbonic anhydrase, 29 000; trypsin, 23 000 and bovine superoxide dismutase subunits, 16 300. The molecular weight estimated by this method[19] in the absence of β-mercaptoethanol was 17 000 and in its presence was 18 300. These results also suggest that the yeast enzyme is composed of two subunits of equal size which are not covalently bridged.

Amino acid analysis

0.1 mg samples of the enzyme were sealed *in vacuo* in tubes containing 1.0 ml of 6 M HCl, 0.1% phenol and were then hydrolyzed at 110 °C for 24, 48 and 72 h. All hydrolyses and assays were done in triplicate. The tubes were then opened, the contents evaporated to dryness, and the residues redissolved in 2.7 ml of 0.01 M HCl, 0.1% phenol. Samples for analysis of cysteic acid and tryptophan were prepared in an identical fashion except that the acid hydrolysis was carried out in the presence of 0.28 M dimethylsulfoxide[27] and 4% thioglycollic acid[28], respectively. 1 ml aliquots of the samples redissolved in dilute acid were applied on the amino acid analyzer. The results of these analyses are presented in Table II.

TABLE II

AMINO ACID COMPOSITION OF SUPEROXIDE DISMUTASE FROM *Saccharomyces cerevisiae*

Amino acid	*Residues per mole of enzyme (nearest integer)* *
Lysine	18
Histidine	11
Arginine	7
Aspartic acid**	32
Threonine***	18
Serine***	20
Glutamic acid**	25
Proline	20
Glycine	40
Alanine	24
Half cystine†	4
Valine††	28
Methionine	2
Isoleucine††	9
Leucine††	11
Tyrosine	2
Phenylalanine	10
Tryptophan	0

* All calculations were based on a molecular weight of 32 700.
** Amide content was not determined.
*** Values were extrapolated to zero time of hydrolysis.
† Measured as cysteic acid after dimethylsulfoxide oxidation.
†† 72-h hydrolysis samples were used for the calculations.

Content of Cu and Zn

Atomic absorption spectroscopy indicated that the enzyme contains 1.80 moles of zinc per mole of enzyme and 2.06 moles of copper per mole of enzyme. A copper signal was present when the enzyme was examined by EPR. The parameters of the EPR signal were $g_m = 2.071$ and $g_{\|} = 2.255$. Double integration of the EPR signal gave 1.54 moles of copper per mole of enzyme. This low EPR signal may indicate that the Cu^{2+} of the yeast superoxide dismutase has a tendency to undergo autoreduction, as does that of the chicken liver superoxide dismutase (R. A. Weisiger, personal communication).

Carbohydrate content

Acrylamide gel electrophoretograms of the yeast enzyme were stained for carbohydrate by the method of Zacharius *et al.*[21] and none was detected. Ceruloplasmin under identical conditions did give a positive indication of carbohydrate content.

The generality that all eucaryotes contain a copper and zinc containing superoxide dismutase is supported by the present studies of the enzyme from yeast. It is also clear that the catalytic and molecular properties of eucaryotic superoxide dismutases have been rigidly maintained during evolution. Thus the yeast enzyme described here and the mammalian[1,22], plant[7], and fungal enzymes[8] are strikingly similar with respect to specific activity, absorption spectra, molecular weight, subunit composition, metal content, and behavior during a purification procedure which includes an unusual extraction into a salted-out ethanol–chloroform phase.

The observation that the yeast superoxide dismutase is a copper enzyme indicates that it will be worthwhile to pursue the observation of Gesswagner *et al.*[9] on the basis of the working hypothesis that superoxide dismutase may enhance radioresistance in the presence of oxygen.

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