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SUPEROXIDE DISMUTASE ACTIVITY OF GALACTOSE OXIDASE

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Summary

The copper protein galactose oxidase is now shown to function as a superoxide dismutase as well as an oxidase: At a concentration of about $4 \cdot 10^{-7}$M, it shows its superoxide dismutase activity by a fifty percent inhibition of the reaction of ferricytochrome *c* with the superoxide anion generated by the xanthine—xanthine oxidase system. The dismutase activity is not due to a minor impurity in the enzyme preparation, as this activity, the galactose oxidase activity, and the protein all have the same mobility on disc gel electrophoresis.

D-Galactose oxidase from *Polyporus circinatus* (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) [1] is a Cu(II)-protein of molecular weight 42400 [2], which catalyzes the reaction

D-Galactose + O_2 → galactohexodialdose + HOOH

The oxidation occurs on the primary alcohol group at carbon six of galactose or galactosides [3, 4]. Superoxide anion (O_2^-) has been proposed as an intermediate in the enzymic reaction [5], and support for this intermediate comes from the inhibition of oxygen consumption in the galactose—galactose oxidase–oxygen system by superoxide dismutase [6]. However, this inhibition is a function of the substrate used as well as the composition of assay mixtures and the dismutase concentration, and the inhibition is not observed if peroxidase is present in the assay mixture [7].

As a step in the elucidation of the mechanism of galactose oxidase, we have attempted to detect superoxide anion in the galactose—galactose oxidase system by trapping it with ferricytochrome *c*. In addition, we have investigated the possibility that galactose oxidase, as a Cu(II) metalloenzyme, has super-

oxide dismutase activity, since Joester et al. [8] have already shown that some Cu(II)-amino acid chelates have superoxide dismutase activity.

Galactose oxidase was obtained from Miles Laboratory, and its homogeneity was checked by disc gel electrophoresis. Xanthine, xanthine oxidase, catalase and ferricytochrome *c* were obtained from Sigma Chemical Co. All solutions were prepared in deionized redistilled water.

Superoxide dismutase activity was assayed using the xanthine—xanthine oxidase reaction [9]: Reduction of ferricytochrome *c* was monitored at 550 nm in a reaction mixture containing xanthine, ferricytochrome *c* and xanthine oxidase in the presence and absence of catalase and/or galactose oxidase. Inhibition of the reduction of ferricytochrome *c* was indicative of superoxide dismutase activity. Superoxide dismutase activity was also assayed following disc gel electrophoresis by the gel staining technique of Beauchamp and Fridovich [10]: The gels were immersed first in nitro blue tetrazolium, then in a solution containing riboflavin and tetramethylethylenediamine buffered to pH 7.8. On exposure to light, O_2^- generated by reaction of riboflavin and oxygen reacts with nitro blue tetrazolium to produce a blue precipitate in the gel. Where this reaction is inhibited by superoxide dismutase, a colorless band is obtained.

Galactose oxidase was assayed using 0.033 M galactose as substrate by measuring oxygen consumption with an oxygen electrode in a Model 53 biological oxygen monitor from Yellow Springs Instrument Co.

Enzyme preparation

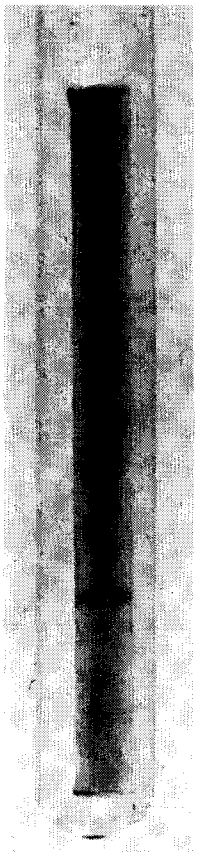
Galactose oxidase obtained from Miles Laboratory was found to give a single band on disc gel electrophoresis, as shown in Fig. 1. Since *Polyporus circinatus* secretes galactose oxidase into the culture medium, the enzyme can be readily purified to homogeneity after removal of mycelium by two ammonium sulfate precipitations and DEAE-Sephadex chromatography [2].

Assay for superoxide anion in galactose oxidase—galactose system

In an effort to detect O_2^- in a galactose oxidase reaction, the ability of a mixture of galactose and galactose oxidase to reduce ferricytochrome *c* was investigated, as shown in Fig. 2. When galactose oxidase is added to a mixture of galactose and ferricytochrome *c* in phosphate buffer containing EDTA, no increase in ferrocytochrome *c* absorption at 550 nm is observed: Ferricytochrome *c* is not reduced under these conditions. This is not due to inhibition of galactose oxidase as it can still oxidize galactose under these conditions.

Superoxide dismutase activity of galactose oxidase

The ability of galactose oxidase to repress reduction of ferricytochrome *c* by the xanthine—xanthine oxidase—oxygen system is shown in Fig. 3. In this assay, 276 μg ($2 \cdot 10^{-6}$ M) galactose oxidase results in a 78% inhibition of the rate of ferricytochrome *c* reduction, a result independent of whether catalase is present or not. The extent of inhibition does depend on the concentration



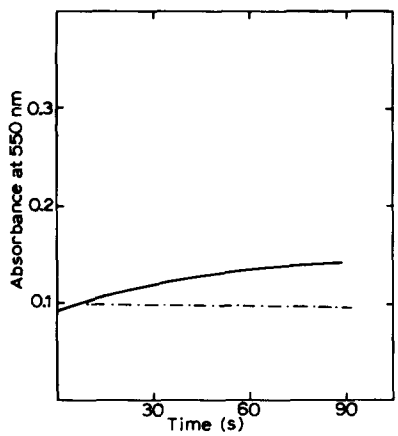


Fig. 1. Disc gel electrophoresis of galactose oxidase. Gel was stained for protein with Coomassie blue following electrophoresis on 7% gels according to procedure of Davis [11].

Fig. 2. Trapping of superoxide anion in galactose—galactose oxidase system. Reactions were performed at room temperature in a volume of 3.0 ml buffered to pH 7.8 by 0.05 M potassium phosphate containing 0.1 mM EDTA. —, reaction mixture also contains $5 \cdot 10^{-5}$ M xanthine, $1 \cdot 10^{-5}$ M ferricytochrome *c* and $2.1 \cdot 10^{-8}$ M xanthine oxidase. -·-·-·-, reaction mixture contains 0.033 M galactose, $1 \cdot 10^{-5}$ M ferricytochrome *c* and $1.4 \cdot 10^{-6}$ M galactose oxidase.

of galactose oxidase, as shown by Table I. The inhibition of cytochrome reduction in the presence of galactose oxidase is not due to inhibition of xanthine oxidase by galactose oxidase: Oxygen uptake for a mixture of xanthine and xanthine oxidase in presence of galactose oxidase is unchanged. Therefore, the inhibition is probably due to enzymatic disproportionation of O_2^- by galactose oxidase.

Superoxide dismutase activity of galactose oxidase by a gel staining technique

The superoxide dismutase activity of galactose oxidase has also been demonstrated by a disc gel staining technique [10], as shown in Fig. 4. Disc

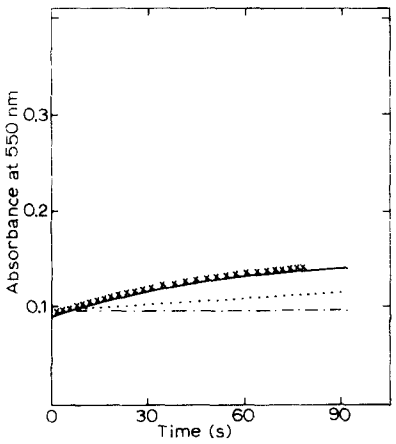


Fig. 3. Galactose oxidase as a superoxide dismutase. All reactions were performed at room temperature with a 3.0-ml reaction mixture containing $5 \cdot 10^{-5}$ M xanthine, $1 \cdot 10^{-5}$ M ferricytochrome *c* and $2.1 \cdot 10^{-8}$ M xanthine oxidase buffered to pH 7.8 with potassium phosphate buffer containing 0.1 mM EDTA. ——, reaction mixture contains no additional components; . . . . ., reaction mixture also contains $2.2 \cdot 10^{-6}$ M galactose oxidase added prior to xanthine oxidase; xxxxx, reaction mixture contains 36 units catalase added prior to xanthine oxidase; - - - - - -, reaction mixture contains 36 units catalase and $3.8 \cdot 10^{-6}$ M galactose oxidase, both added prior to xanthine oxidase.

TABLE I

ACTIVITY OF GALACTOSE OXIDASE AS A SUPEROXIDE DISMUTASE

Galactose oxidase		Rate of reduction of cytochrome *c* (A_{550}/min)	Inhibition (%)
μg	μM		
0	0	0.046	0
276	2.2	0.0097	78
414	3.2	0.0043	91
0*	0	0.045	0
140*	1.1	0.015	67
483*	3.8	0.0	100

*Contains 36 Sigma units catalase in addition to reaction mixture containing $5 \cdot 10^{-5}$ M xanthine, $1 \cdot 10^{-5}$ M ferricytochrome *c*, $2.5 \cdot 10^{-5}$ M xanthine oxidase and the listed amount galactose oxidase, in phosphate buffer (pH 7.8), containing 10^{-4} M EDTA. Total volume: 3.0 ml.

gel electrophoresis of galactose oxidase was carried out in 7% gels according to the procedure of Davis [11]. Triplicate gels were stained for protein and superoxide dismutase activity and sectioned and assayed for galactose oxidase activity. The mobility of the single homogeneous protein band coincided with the achromatic superoxide dismutase active band. A third gel was sliced into 3-mm sections and assayed; the galactose oxidase activity was shown to be associated with the same protein as the superoxide dismutase activity.

Inhibition of the reduction of ferricytochrome *c* by galactose oxidase indicates that galactose oxidase has superoxide dismutase activity. Since catalase has no effect on either the rate of reduction of cytochrome *c* itself or on the efficiency of galactose oxidase as a dismutase, as shown in Fig. 3, the observed inhibition by galactose oxidase is not a consequence of the production

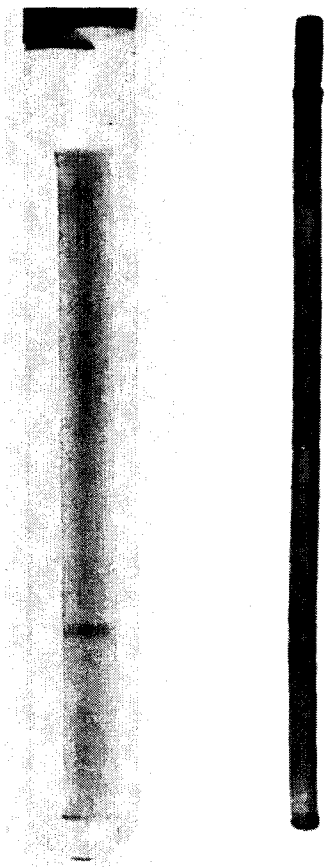


Fig. 4. Disc gel electrophoresis of galactose oxidase. Approx. 20 μg galactose oxidase per gel was electrophoresed as described by Davis [11]. The gel on the left was stained for protein with Coomassie blue; the gel on the right was stained for superoxide dismutase activity according to procedure of Beauchamp and Fridovich [10].

of hydrogen peroxide which could reoxidize reduced cytochrome *c*. The existence of superoxide dismutase activity in galactose oxidase was confirmed by the gel staining experiment, and the fact that the superoxide dismutase activity, galactose oxidase activity and protein all had the same mobility on the gel provides strong evidence that the superoxide dismutase activity is not due to a contaminant of galactose oxidase, but to the active protein itself.

Since all these experiments were performed at pH 7.8 and the pK_a of HO_2 is 4.88 [12], over 99% of the superoxide produced should be in the anion form, whose second order rate constant for dismutation is less than 10^2 $M^{-1} \cdot s$ [12]. Ferricytochrome *c* can scavenge O_2^- effectively since, at pH 8.5, the second order rate constant for this reaction is $1.1 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ [13], and is presumably of the same order of magnitude at pH 7.8. From this rate constant and the concentration of superoxide dismutase required to achieve 50%

inhibition of cytochrome *c* reduction ($3 \cdot 10^{-9}$ M superoxide dismutase), the rate constant for the reaction of bovine erythrocyte superoxide dismutase with superoxide has recently been calculated to be $2 \cdot 10^{9}$ $M^{-1} \cdot s^{-1}$ at pH 7.8 [14]. Galactose oxidase is not as effective a dismutase as superoxide dismutase, since $2 \cdot 10^{-6}$ M galactose oxidase is required to achieve a 78% inhibition in rate of cytochrome *c* reduction; by extrapolation of the data in Table I, a 50% inhibition would be achieved with approximately $4 \cdot 10^{-7}$ M. This corresponds to a rate constant for the dismutation of O_2^- by galactose oxidase of $3 \cdot 10^{6}$ $M^{-1} \cdot s^{-1}$. The efficiency of galactose oxidase as a dismutase is therefore of the same order of magnitude as that of the amino acid complexes of Cu(II) reported by Joester [8]. It may not be coincidental that the amino acids forming superoxide dismutase active Cu(II) complexes are lysine, histidine and tyrosine; on the basis of superhyperfine splitting in the EPR of galactose oxidase, coordination of the Cu(II) by four in-plane nitrogen atoms, possibly from lysine and/or histidine, is very likely (Cleveland, L. and Davis, L., unpublished). Bovine erythrocyte superoxide dismutase has three nitrogen atoms and one water molecule coordinated in the plane, but its mode of action as a dismutase appears to be substantially different from that of galactose oxidase: superoxide dismutase has been shown to undergo fast reduction and reoxidation of the copper during the dismutation of superoxide [15, 16], while no evidence of a Cu^+ species of galactose oxidase has been found in presence of either substrates or products [17].

Our failure to trap superoxide anion in the galactose—galactose oxidase system with cytochrome *c* indicates that either superoxide is not an intermediate in the oxidation of galactose, or it is decomposed more rapidly than it can react with cytochrome *c*. Since galactose oxidase is now shown to decompose superoxide, we cannot rule out a superoxide anion intermediate during oxidation of galactose, but trapping of this intermediate, if present, now seems much more difficult. Use of superoxide dismutase as a trapping agent for O_2^- has produced equivocal results, and it is not certain that even the rapid-freezing EPR technique used to detect superoxide anion in the xanthine—xanthine oxidase system [18] would fare any better.

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