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Purification and Partial Characterisation of Superoxide Dismutase from Chicken Erythrocytes

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Superoxide dismutase (SOD), which plays a very important role in protecting organisms from oxygen toxicity, was purified from chicken erythrocyte and partially characterised. Erythrocyte membranes were disintegrated via freeze-thaw methods in the presence of Triton X-100. Following ethanol precipitation, SOD-containing solution was applied to DEAE-cellulose and then Sephadex G-100 gel columns. Chicken erythrocyte SOD was purified 508-fold with a specific activity of 8,480 units per mg. The molecular weight was estimated to be 30.6 kDa ± 0.4 by gel filtration. The enzyme was composed of two subunits of equal size and contained one atom of copper and one atom of zinc per molecule. Maximum SOD activity was observed between pH 7.0 to 9.0 at 25°C. The enzyme has high thermal stability.

Key Words: SOD, purification, characterisation, chicken, erythrocytes.

Introduction

The superoxide radical is an intermediate reduction product of oxygen produced by a variety of biological reactions. The superoxide radical ($O_2^{\cdot -}$) and other reactive derivatives have received recent attention as agents of oxygen toxicity in cells. Most organisms, therefore, have defense systems, such as metallo-enzymes, to protect themselves from toxic oxygen species. Metallo-enzymes that catalyze the disproportionation of superoxide free radicals ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2), are known as superoxide dismutases (SODs). SODs play an important role in the protection of cells from the oxidative damage of superoxide radicals. Cell damage may also be due to the superoxide itself or, indirectly, even more reactive oxygen species, such as hydroxyl radicals ($^{\cdot}OH$), formation of which, via the Fenton reaction, is favoured by excess superoxide[1−10]. SODs have subunit molecular weights ranging from 16 kDa to 26 kDa and are divided into three classes on the basis of their active site metals: copper and zinc (CuZn-SOD), manganese (Mn-SOD), and iron (Fe-SOD). CuZn-SOD were found widely in the cytoplasm and certainly in the mitochondrial intermembrane space of the eukaryotic cells and chloroplasts of plants. Mn-SOD are located in procaryotes and in the mitochondria of eucaryotes[11], while Fe-SOD has been found in bacteria, blue-green algae and

protozoa[12,13]. Recent reports also indicated that the enzyme was present in higher land plants[5]. In addition, Ni-SOD have been isolated from some microorganisms[14].

We described a new purification procedure for the preparation of highly purified Cu-Zn superoxide dismutase from chicken erythrocyte. In addition, purified superoxide dismutase was characterized partially according to the 6-OHDA method modified by us[15,16].

Materials and Methods

Materials

6-Hydroxydopamine (6-OHDA; 2,4,5-trihydroxyphenylethylamine), β-mercapto ethanol, Coomassie brilliant blue G, gel filtration and electrophoresis molecular weight marker kits, DEAE-cellulose, and Sephadex G-100 were purchased from Sigma, St. Louis USA. All other chemical reagents used were of analytical grade.

Optical measurements were achieved with a spectrophotometer (UV-1601,Shimadzu, Japan) at 490 nm. Electrophoresis was performed with an Atto Model AE6220, Mixer-mill MM2 (Retsch, Germany) was employed and atomic absorption was measured with aVarian, SpectrAA-300 plus during experimental studies.

Isolation and Purification of Superoxide Dismutase

Chicken (Ross PM3) blood samples (570 ml) containing 15% (w/v) citrate were centrifuged at 3,500 rpm for 10 min and the plasma was then removed completely. The packed red cells were lysed by the addition of 2 volumes of cold water containing Triton X-100 and 2-mercaptoethanol at 0.3% and 2 mM final concentration respectively. Cell suspension was stirred for 10 min and then frozen. The precipitation of the proteins in the haemolysates were carried out in a refrigerated bath maintained at -2°C with 4 volumes of ethanol and then separated by centrifugation at 5,000 rpm and 4°C. The resulting pellet was resuspended in 250 ml 0.05 M phosphate buffer (pH 7.4) and stirred for 115 min and then centrifuged again. Supernatant containing SOD activity was concentrated with 10,000 NMWC ultrafilter and applied to a DEAE-cellulose column (40×1.6 cm), which was equilibrated with 0.05 M phosphate buffer (pH 7.4) at 4°C. Elution of the enzyme was achieved by establishing a linear gradient with a 0.06-0.140 M phosphate buffer (pH 7.4) at a flow rate of 0.5 ml/min. Fractions containing SOD activity were concentrated by an 10000 NMWC ultrafilter and then loaded onto a Sephadex G-100 (90×1.6 cm) column equilibrated with 0.05 M phosphate buffer pH 7.4 and eluted with the same buffer at a flow rate of 0.21 ml/min.

Molecular Weight Determination

The apparent molecular weights of the purified SOD were determined by gel exclusion on a Sephadex G-100 column[17]. In the column, BSA (66 kDa), carbonic anhydrase (29 kDa) aprotinin (6.5 kDa) were used as molecular weight markers with blue dextran (200 kDa) for determining the void volume. The molecular weight for purified SODs was estimated from the calibration curve.

SOD Activity Assay

The SOD activity assay system was based on the inhibitory effect of SOD on the spontaneous autoxidation of 6-hydroxydopamine (6-OHDA)[15,16,18]. A total of 1 U of superoxide dismutase caused a 50% inhibition in

the initial rate of 6-hydroxydopamine autoxidation.

The autoxidation rate of 6-OHDA (4.10^{-4} M) in 0.1 M phosphate buffer (pH 7.4) which was saturated by air-O_2 (8.2mg/L) was determined by observing the absorbance changes at 490 nm at 15 s intervals at 25°C [15,16]. A native SOD activity assay was carried out under the same conditions by adding enzyme solution required to drop half of the initial absorbance value of 6-OHDA autoxidation at the 90^{th} s mark.

Protein Determination

During the purification steps, protein levels were determined spectrophotometrically according to the Bradford method, using bovine serum albumin as the standard[19]. Protein amounts in column fractions were observed via absorbance variations at 280 nm.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the Laemmli method using a vertical slab gel apparatus[20]. The following proteins were used as SDS-PAGE electrophoresis molecular weight standards: BSA (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa). Molecular weight markers and chicken erythrocyte SOD were dissolved in 0.0625 M Tris HCl buffer (pH 6.8) containing 5% β-mercaptoethanol and denatured by 4 min incubation in boiling water.

Electrophoresis was performed according to discontinuous methods with 12% separating and 4% stacking polyacrylamide gels at room temperature at 125 volts for 4 h Gel was stained with Coomassie brilliant blue R-250 dye reagent overnight.

Determination of Metal Content

The metal content of purified chicken erythrocyte SOD was determined by micro cuvette atomic absorption methods after the enzyme was dialysed extensively against 0.01 M phosphate buffer (pH 7.4) containing 1 mM EDTA and followed by buffer lacking EDTA[21,22].

The Effect of pH on SOD Activity

The pH activity profile of SOD was studied at standard assay conditions by using two different buffer systems in two different pH ranges. A 0.05 M phosphate buffer was used for pH 6.0-8.0 intervals and a 0.05 M borate buffer was used for pH 9.0-10.0 intervals.

Estimation of pH Stability

Enzyme was added to different buffers having pH values between 6.0 and 8.0 and incubated for 2 h at 25°C. After incubation, the activity of enzyme preparations was measured under standard assay conditions with pH stability being expressed as a percentage of retained activity.

Estimation of Temperature Stability

Enzyme was incubated for 2 h in 0.05 M phosphate buffer (pH 7.4) at temperature values between 45 and 75°C. After incubation, the activity of enzyme preparations was measured under standard assay conditions. Temperature stability was expressed as a percentage of retained activity.

The Effect of Ionic Strength on SOD Activity

Activity changes were determined in standard assay conditions depending on the ionic strength in the 0.02-0.200 M concentration of buffer at various pH values.

Storage Stability

The activity values of purified SOD were determined in standard assay conditions depending on the time at 4°C pH 7.4.

Results

SOD Purification

The isolation of SOD from chicken erythrocyte was performed by the disintegration of the membranes by mechanical, non-ionic detergent solubilisation and freeze-thaw methods.

The erythrocyte cells were packed in 1.5 ml plate vials together with 0.4 g glass beads (0.3 mm in diameter) and 5 min grinding was applied with a Retch Mixer Mill. Cell debris was removed by centrifugation at 3,500 rpm (2,800 g) and the activity of SOD in haemolysate was 14.71 U/mg.

The solubilisation of erythrocyte membrane in isotonic solution containing 0.3% Triton X-100 was performed for 5 min at 4°C. The specific activity of SOD in this solution was found to be 13.61 U/mg. SOD activity decreased after the disintegration of the erythrocyte membranes in isotonic solution at 4°C for 5 min to 6.58 U/mg.

The highest value for SOD activity was obtained after the thawing of freezed erythrocyte cells in cold water in the presence of 0.3% Triton X-100 as 16.70 U/mg.

The first purification step for obtaining SOD from the crude extract was achieved by fractional precipitation of proteins by using ammonium sulphate and (45-90%) ammonium sulphate plus ethanol (45-90% + 1/1 v/v) methods (Fig. 1). The best precipitation yield was obtained as 380 U/mg by adding four volumes of ethanol per volume of haemolysate samples in the bottom precipitate phase. As can be seen from Fig. 1, the SOD activity values in both phases were observed to be very low after precipitation with various ratios of ammonium sulphate and ammonium sulphate plus ethanol.

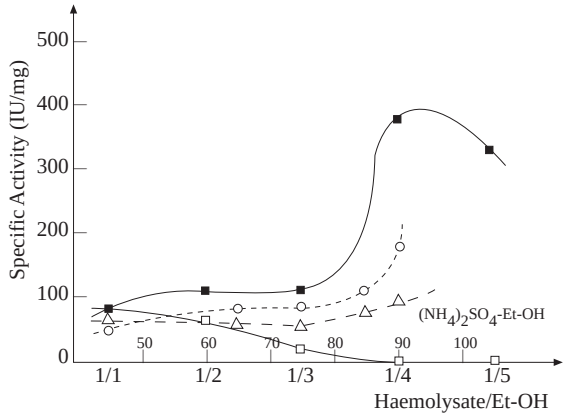

Figure 1. Various SOD precipitation methods. Ammonium sulphate-ethanol upper (o—o), lower phases (Δ—Δ), ethanol upper (□—□), lower phases (■—■).

The precipitate having the highest SOD activity was resuspended in 0.05 M potassium phosphate buffer, pH 7.4. The samples were stirred for 15 min and the insoluble material removed by centrifugation at 4,000 g for 15 min Supernatant was concentrated with a 10,000 NMWC ultrafilter and applied to DEAE cellulose columns while the unbound proteins were eluted by equilibration buffer. The bound proteins having SOD, were eluted via a linear gradient method by using 0.06-0.140 M phosphate buffers at pH 7.4.

SOD activity was observed between fractions 37 and 43 with a single peak. Thirty-five percent of SOD activity was recovered in the pooled fractions and a 403-fold purification was obtained. These fractions were concentrated by a 10,000 NMWC ultrafilter and applied to a Sephadex G-100 gel chromatography column for further purification. SOD activity was eluted between fractions$^{9-13}$ (Fig. 3). A 508-fold purification of the enzyme with a 8480 U/mg specific activity was achiev. The results of the purification procedure are summarized in Table 1. The apparent molecular weight of the chicken erythrocyte SOD was found to be 30.6 kDa ± 0.4 by gel filtration on a Sephadex G-100 column and 15 kDa ± 0.3 by SDS slab gel electrophoresis (Fig. 4).These values indicate that the enzyme is composed of dimers of equal subunit size. Atomic absorption spectroscopy revealed that purified SOD contained one atom of Cu and Zn per subunit.

Table 1. Purification steps of SOD from chicken erythrocytes.

Purification steps	Unit/ml	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg-protein)	Yield	Fold
Crude extract	95.23	51424	3078.00	16.7	100	1
Ethanol precipitation	106.96	26740	70.30	380.6	52	22.8
DEAE-cellulose chromatography	515.00	18025	2.84	6358	35	403.2
Sephadex G-100 chromatography	163.00	13370	1.56	8480	26	507.8

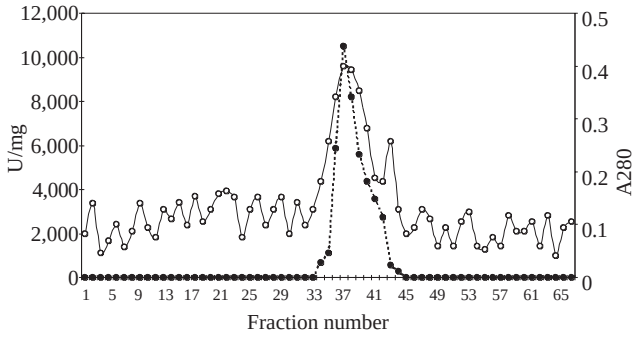

Figure 2. Variation of protein amounts in the elution fragments from DEAE-column and SOD activity . Absorbance at 280 nm. (o—o), SOD activity fractions (•—•).

The effect of pH on SOD activity

The pH profile of chicken erythrocyte SOD was examined using 0.02 M phosphate buffer of pH 6.0-8.0, and 0.02 M borate buffer of pH 9.0-10.0. Maximum SOD activity was observed between pH 7.0 to 9.0 (Fig. 5). The pH curve was characterized by a rapid decrease in enzyme activity above pH 9.0 and below pH 7.0.

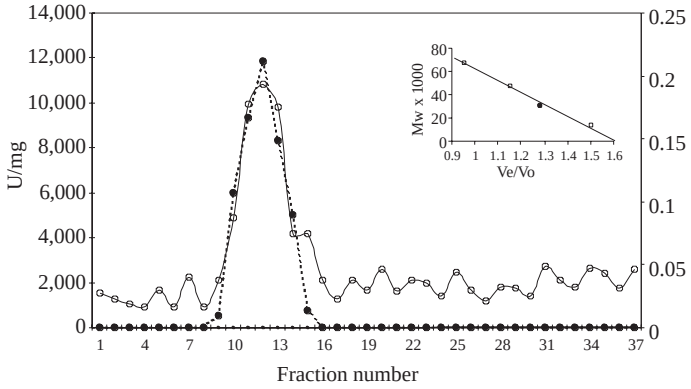

Figure 3. Gel chromatography on Sephadex G-100 of the collected chicken erythrocyte SOD fractions from the DEAE-cellulose column. (a) Absorbance at 280 nm (o—o), SOD activity fractions (•- - -•), and molecular weight calibration curve. (b) Protein markers (–o–), purified SOD (–•–).

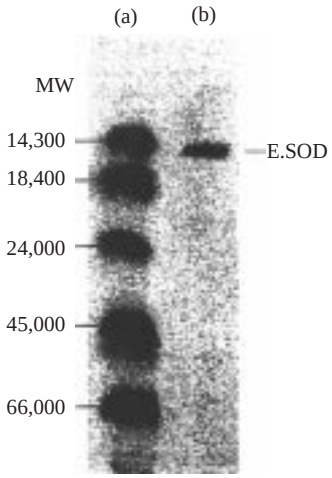

Figure 4. SDS-polyacrylamide gel electrophoresis of CuZn-SOD isolated chicken erythrocyte. (a) Protein standards. (b) Purified CuZn-SOD.

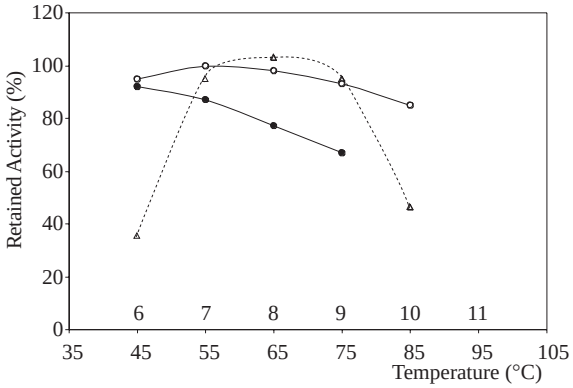

Figure 5. The effect of pH on the chicken erythrocyte CuZn-SOD activity at 25°C (Δ- - -Δ); pH-stability variations for 2 h incubation at 25°C (o—o); temperature stability variations for 2 h incubation at pH 7.4 (•—•).

pH and temperature stability profiles

The effect of pH on enzyme stability was determined under standard assay conditions after 2 h preincubation using various buffers at a concentration of 0.05 M, phosphate (pH 6.0-8.0) and borate, (pH 9.0-10.0) at 25°C (Fig. 5). The purified SOD was found to be stable at pH interval 6.0-10.0 under the conditions studied.

The thermal stability of the SOD was also investigated between 45-75°C for a 2 h incubation period in 0.05 M phosphate buffer of pH 7.4. Chicken erythrocyte SOD showed rather high thermal stability (Fig. 5). Activity loss was only 13% at 45°C and 32% at 75°C after a 2 h incubation.

The effect of ionic strength on SOD activity

Activity variations depending on the ionic strength of phosphate and chloride in the 0.02-0.200 M concentration range were investigated by using standard activity assay conditions.

As can be seen from Fig. 6, SOD activity was decreased by the increase of ionic strength and valence of buffer ions. The decrease in activity decrease was higher at pH 10 than at 7.4, and divalent anion phosphate was found to be more inhibitory than monovalent anion chloride at pH 7.4.

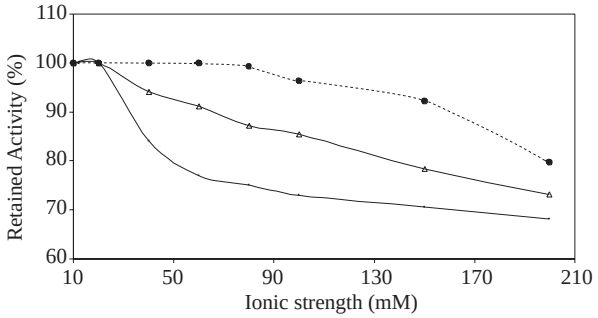

Figure 6. The effect of ionic strength on the CuZn-SOD activity. Phosphate, pH 7.4 (Δ—Δ); chloride, pH 7.4 (•—•) and borate pH 10.0 (■—■), at 25°C.

Storage stability

The purified SOD enzyme (19.2 μg/ml) was stored in 0.05 M phosphate buffer (pH 7.4) at 4°C. No loss of activity was observed over 3 months. Freeze dried enzyme was also stable when stored at 4°C for 3 months. Only 15% activity loss was observed.

Discussion

The specific SOD activity value purified from chicken erythrocytes was similar to that purified from other erythrocyte sources. The specific activities of purified human and bovine erythrocyte SOD have been reported between 3,000-6,000 U/mg[23] and 2,800-4,728 U/mg[24]. The activity of chicken erythrocyte SOD (4,800 U/mg) was observed to be higher than that of carp liver (2,100 U/mg)[25], bovine heart (3,660 U/mg)[26], mouse lung (3,020 U/mg)[27], and *Aspergillus* species (3,294-3,531 U/mg)[28].

Purified chicken SOD had some properties similar to enzymes from mammalian tissues[26,29–31], *Neurosporo crassa*[32], green pea[33] and yeast[34,35] in molecular weight, subunit structure and metal content. They

had a molecular weight of about 32 kDa composed of subunits of equal size and contained 2 atoms of copper and 2 atoms of zinc per mole enzyme.

The optimum pH value for chicken erythrocytes SOD was in wide range of 7.0-9.0. Generally, Cu-Zn SODs are stable at neutral pH[2,11,36]. The optimum pH of bovine erythrocyte SOD was essentially independent of pH between 6.0-12.0. The pH dependence of the Fe-SOD stability of *Aerobacter aerogenes*, remained comparatively stable at alkaline pH 7.0-11.0, but was rapidly inactivated below pH 7.0 [37]. The activity of Cu-Zn SODs is dependent on ionic strength and alkaline pH in a way that typically reflects the functional role of charged amino acid residues, in particular lysine[38−41]. At neutral pH, however, the radical exists mainly as $O_2^{\cdot -}$, and the dismutation reaction is relatively slow due to electrostatic repulsion of negatively charged radicals[42].

In similar conditions, the thermal, pH and storage stability of chicken erythrocyte Cu-Zn SOD is better than that of Cu-Zn SOD purified from the venom of the *H.fulvipes* scorpion[43], *Aerobacter aerogenesis*[37] and cabbage[44]. The thermal inactivation of purified bovine Cu-Zn SOD at temperatures higher than 70°C seems to be irreversible and obeys the first order process. The Cu-Zn containing SOD purified from chicken erythrocytes belongs to a class of SOD enzymes having remarkably similar properties and is distinguishable by only minor differences.

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