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PURIFICATION OF COPPER-ZINC SUPEROXIDE DISMUTASE FROM HUMAN ERYTHROCYTES AND PARTIAL CHARACTERIZATION

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ABSTRACT

Copper-zinc superoxide dismutase (CuZnSOD; E.C:1.15.1.1) catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen. In this study, CuZnSOD was isolated from human erythrocytes using DEAE-cellulose chromatography and copper chelate affinity chromatography. The enzyme was purified 196.3 fold with 33.8% efficiency. The molecular weight of CuZnSOD was determined as 20 kDa by SDS-PAGE.

Vmax and Km values were determined as 5000 U/mg protein and 3.10^{-3} mM Xanthine, respectively. Maximum CuZnSOD activity was observed at 15°C. Activation energy was calculated as 16.856 kj/mol and initiation of denaturation temperature was calculated as 19°C. Turnover number (k_{cat}) and catalytic efficiency (k_{cat}/Km) were found to be 1667 s^{-1} and 5.6 x10^{5} $s^{-1}.mM^{-1}$ Xanthine^{-1}, respectively.

The enzyme was found to have good storage stability as 93.6% of initial activity after 28 days of storage in 50% glycerol solution at -20°C.

Keywords: Superoxide Dismutase, purification, Immobilized Metal Affinity Chromatography, erythrocyte, Iminodiacetic Acid Agarose

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Introduction

In 1938, Mann and Keilin described a blue-green protein containing copper (*haemocuprein*) that they had isolated from bovine blood. In 1953, a similar protein was isolated from horse liver and named *hepatocuprein*. Other proteins of this type were later isolated, such as *cerbrocuprein* from brain. In 1970, it was discovered that the erythrocyte protein contains zinc as well as copper. No enzymic function was detected in any of these proteins, so it was often suggested that they served as a metal store (1). However, in 1969 McCord and Fridovich reported that the erythrocyte protein is able to remove the superoxide radical catalytically, i.e. it functions as a superoxide dismutase enzyme (SOD) (2, 3). SOD, catalyzes the dismutation of the superoxide anion to hydrogen peroxide (5). Thus, SOD has anti-inflammatory effects (4).

In ion exchange chromatography proteins were reversibly bound to ion exchanger by ionic interaction. The adsorbed proteins are then eluted through an increase in the ionic strength of the buffer or change of pH of the buffer (in anion exchanger a decrease of pH and in cation exchanger an increase of pH, respectively) (6).

In Immobilized-Metal Affinity Chromatography (IMAC) the adsorption of proteins is based on the coordination between an immobilized metal ion and electron donor groups from the protein surface. Although many residues, such as Glu, Asp, Tyr, Cys, His, Arg, Lys and Met, can participate in binding, the actual protein retention in IMAC is based primarily on the availability of histidyl residues. Adsorption of a protein to the IMAC support is performed at a pH at which imidazole nitrogens in histidyl residues are in the nonprotonated form, normally in neutral or slightly basic medium. Usually relatively high-ionic-strength buffers containing 0.1 to 1.0 M NaCl are used to reduce nonspecific electrostatic interactions. Elution of the target protein is achieved by protonation, ligand exchange or extraction of the metal ion by a stronger chelator, like EDTA (7).

In this study IMAC was used to purify CuZnSOD from human erythrocytes due to its high efficiency in purification of metallo enzymes.

Materials and Methods

Chemicals

DEAE-cellulose (E-2145), iminodiacetic acid agarose (I-4758), xanthine (X-7375), xanthine oxidase (X-4500), nitrotetrazolium blue chloride (N-6876), dialysis tubing (D-9777), cytochrome c (C-2037), chymotrypsinogen (C-4879), ovalbumin (O-4757) were obtained from Sigma. Albumin was obtained from Merck. All other chemicals used were of analytical grade purity.

Protein measurements

Protein concentration was determined by the method described by Lowry et.al. using bovine serum albumin as standard (8).

CuZnSOD activity assay

This method is based on generation of superoxide radicals by xanthine and xanthine oxidase, which react with nitrotetrazolium blue to form blue formazan dye. The

superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of CuZnSOD is defined as the amount of CuZnSOD that brings 50% inhibition of the rate of reduction of nitrotetrazolium blue under the conditions of the assay (9).

SDS-Polyacrylamide gel electrophoresis

The molecular weight of CuZnSOD was determined with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) performed according to the Laemmli method using a vertical slab gel apparatus (10). Electrophoresis was performed according to the discontinuous method with 5% stacking gel and 15% separation gel at 200V for about 35 min. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 dye solution (0.05% dye, 45% methanol, 10% acetic acid and 45% distilled water) for 3 hours. The stained gel was then destained with washing solution (45% methanol, 10% acetic acid and 45% distilled water) for about 24 hours. After removing the gel from the washing solution, it was immersed to 10% glycerol to obtain clear protein bands.

Isolation of CuZnSOD

1. Centrifugation, Haemolysis, Dialysis

Fresh human blood containing citrate taken from Cukurova University, Medicine Faculty Blood Centre, was centrifuged at 906 x g for 15 min and the plasma was then removed with a Pasteur pipette. The erythrocytes were washed with isotonic NaCl solution. A volume of 40 ml packed red cells was haemolysed by addition of 60 ml cold destilled water at 4°C.

The haemolysate was dialysed against distilled water. Cl^- passing through dialysis tubing were tested by 0.1 M $AgNO_3$. After exhaustion of Cl^- dialysis ended.

2. DEAE-Cellulose ion exchange chromatography

DEAE-Cellulose was equilibrated with 1.5 mM pH 6.8 phosphate buffer at 4°C, then filtered and equilibrated with the same buffer again. Dialysed haemolysate was then added to DEAE-Cellulose mixture and stirred by a magnetic bar. The enzyme was adsorbed on DEAE-Cellulose. The ion exchanger-enzyme mixture was packed in a column (2.6 x 10.5 cm). This column was then washed thoroughly with equlibration buffer and CuZnSOD was eluted with 20 mM phosphate buffer pH 6.8 at a flow rate of 3.1 ml/minute (11). Fractions of 10 ml were collected (**Fig. 1**) and the fractions with CuZnSOD high activity were brought together for copper chelate affinity chromatography.

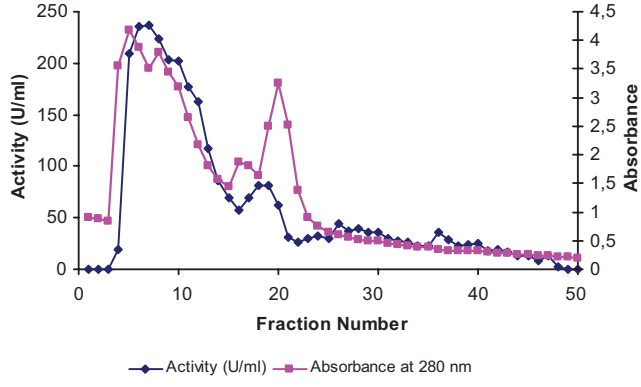


Fig. 1. Elution profile of DEAE-Cellulose ion exchange chromatography

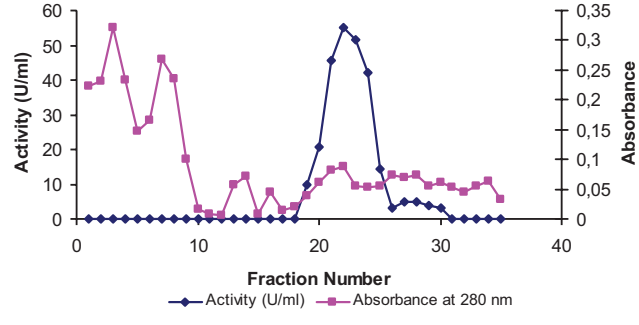


Fig. 2. Elution profile of affinity chromatography

3. Copper chelate affinity chromatography

Iminodiacetic acid-agarose suspension was packed in the occupied column (2.3 x 4.4 cm). The column was washed with 3 bed volumes of water. Afterwards the matrix was charged with 3 bed volumes 1 mg/ml $CuSO_4.5H_2O$. Then the column was washed with 5 bed volumes chromatography buffer (50 mM phosphate buffer pH 7.0 containing 500 mM NaCl) (12). Eluate was tested with 3 M KI whether there was leakage of Cu^{2+} ions or not. Enzyme solution was diluted 1:1 with chromatography buffer. 10 ml of diluted enzyme solution was applied to copper-iminodiacetic acid-agarose column which

TABLE 1

Purification table for CuZnSOD

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Haemolysate	100	95.852	9585.2	735.95	73595	7.68	100	1
DEAE cellulose Ion exchange chromatography	380	0.687	261.1	106.77	40573	155.41	55.1	20.2
Copper-chelate Affinity chromatography	532	0.031	16.5	46.74	24866	1507.74	33.8	196.3

had been equilibrated with chromatography buffer. Firstly, column was washed with 20 ml of chromatography buffer to elute nonbinding proteins. After that CuZnSOD was eluted with 50 mM pH 8.0 Tris HCl buffer containing 500 mM NaCl at a flow rate of 1 ml/minute (Fig. 2). In each step, fractions of 2 ml were collected. The fractions with high activity were brought together.

Characterization of CuZnSOD

The molecular weight of CuZnSOD, optimum temperature, activation energy, initiation of denaturation temperature, Vmax and Km, turnover number (k_{cat}), catalytic efficiency (k_{cat}/Km) and storage stability were all determined.

Results and Discussion

Purification of CuZnSOD

The elution profiles of DEAE-Cellulose ion exchange chromatography and copper chelate affinity chromatography were shown in **Fig. 1** and **Fig. 2**, respectively.

A purification table was constructed using the data obtained after each purification step (**Table 1**). As shown in **Table 1** specific activity of CuZnSOD increased from 7.68 U/mg to 1507.74 U/mg by copper-chelate affinity chromatogpraphy.

Determination of molecular weight of CuZnSOD

Molecular weight of CuZnSOD was determined by SDS-PAGE. SDS gel electrophoresis of standards and purified CuZnSOD (obtained after copper chealete affinity chromatogprahy) is shown in **Fig. 3**.

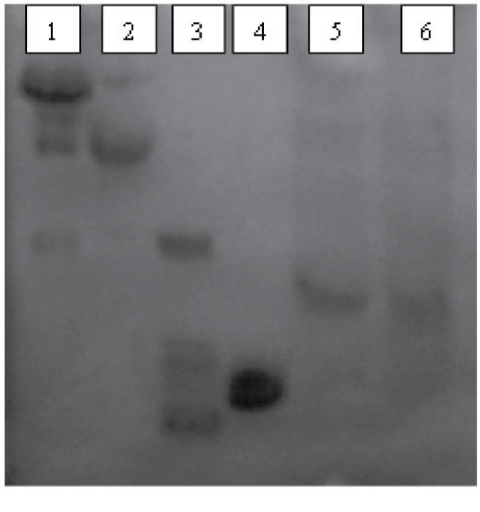


Fig. 3. SDS-Polyacrylamide gel electrophoresis of standards and purified CuZnSOD fraction. 1. Albumin (68,000 g/mol); 2. Ovalbumin (45,000 g/mol); 3. Chymotrypsinogen (25,000 g/mol); 4. Cytochrome C (12,500 g/mol); 5. Purified CuZnSOD in presence of 2-mercaptoethanol; 6. Purified CuZnSOD

As demonstrate in **Fig. 3** purified CuZnSOD in the presence 2-mercaptoethanol and purified CuZnSOD in the absence 2-mercaptoethanol both showed only one peak with the same molecular weight 20 KDa. The absence of impurities outside the region of CuZnSOD bands suggested the preparation was substantially pure.

Optimal temperature

CuZnSOD activities were measured from 5°C to 35°C and the optimum temperature was determined as 15°C (**Fig. 4**).

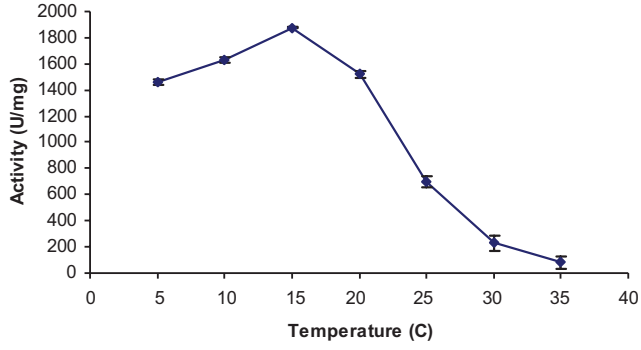


Fig. 4. Effects of temperature on the activity of CuZnSOD

Activation energy and initiation of denaturation temperature

Arrhenius plot (ln V versus 1/T) is shown in **Fig. 5**.

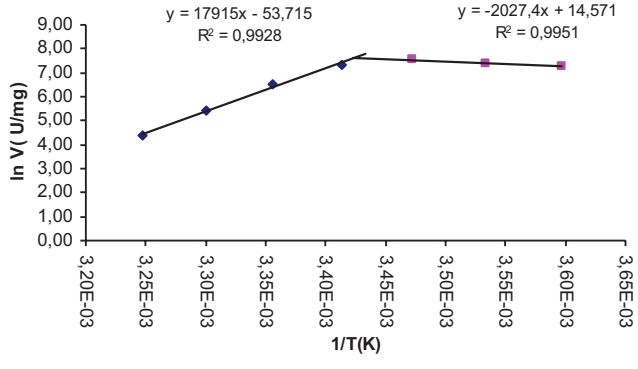


Fig. 5. Arrhenius diagram for CuZnSOD

Activation energy was calculated as 16.856 kj/mol and initiation of denaturation temperature was determined as 19°C.

Storage stability

Storage stability of purified CuZnSOD at different temperatures and different storage conditions were tested. CuZnSOD was stored in 20 mM phosphate buffer pH 6.8 with 30% glycerol, 40% glycerol or 50% glycerol at 5°C. In addition to these CuZnSOD was also stored in 50% glycerol at -20°C (**Fig. 6**). CuZnSOD activities were measured up to 28th day of storage and the results were presented in **Fig. 6**. After 14 day of storage in 20 mM phosphate buffer pH 6.8 CuZnSOD almost lost its full activity.

Kinetic parameters of purified CuZn SOD

Michaelis-Menten kinetics were determined for purified CuZnSOD. Vmax and Km were found as 5000 U/mg protein and 3.10^{-3} mM Xanthine. Turnover number (k_{cat}) and catalytic efficiency (k_{cat}/Km) were found as 1667 s^{-1} and 5.6 x10^5 s^{-1}/ mM Xanthine, respectively.

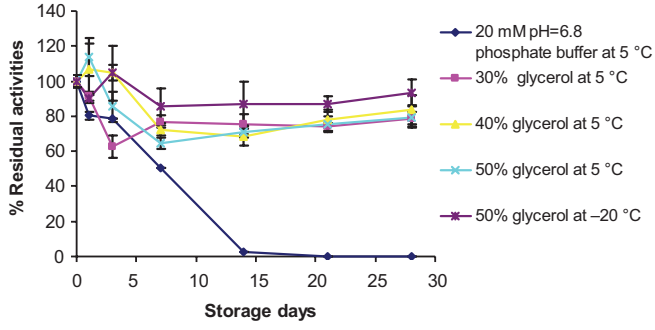


Fig. 6. Storage stability of CuZnSOD

Inouye et al. (13) purified CuZnSOD from bovine erythrocytes with acetone precipitation and high performance ion exchanger, TSK-GEL DEAE-5PW. They reported that the specific activity of CuZnSOD was 3800 units/mg protein.

Asano et al. (11) purified CuZnSOD from human erythrocytes with copper-chelate affinity chromatography. They obtained 35% yield and 2190 fold purification for CuZnSOD. They determined the molecular weight for CuZnSOD by SDS-PAGE as 17 000 ± 1 000.

Weselake et al. (14) isolated CuZnSOD from human red blood cell hemolysate by DEAE-Sepharose and copper chelate affinity chromatography. Enzyme preparations had specific activity of 3800 U/mg, 58% yield and 2000 fold purification. Molecular weight of isolated enzyme was between 18 000 and 19 000 by SDS-PAGE.

Ozturk Urek et al. (15) isolated, purified and partially characterized CuZnSOD from chicken liver by DEAE-ion exchange chromatography and Sephadex G-75 gel filtration chromatography. They reached a specific activity of 4818.2 IU/mg, corresponding to 285.8-fold purification and 7.3% yield.

Aydemir et. al. (16) purified CuZnSOD from chicken erythrocytes by DEAE-cellulose and Sephadex G-100 gel columns and partially characterised the CuZnSOD. They reached a specific activity of 8 480 units per mg, corresponding to 508-fold purification. They observed high thermal stability. Activity loss was only 13% at 45°C and 32% at 75°C after 2 hours of incubation.

Mavi et al. (17) purified CuZnSOD from human erythrocytes by DEAE-cellulose and copper-chelate affinity chromatography. They reached a specific activity of 1085 U/mg, corresponding to 837-fold purification and 12% yield. They found a molecular weight for CuZnSOD to be 18 900 by SDS-PAGE.

We purified CuZnSOD from human erythrocytes by DEAE-cellulose and copper-chelate affinity chromatography. We reached a specific activity of 1507.74 U/mg, corresponding to 196.3-fold purification and 33.8% yield. We determined the molecular weight for CuZnSOD as 20 000 by SDS-PAGE.

Conclusions

CuZnSOD was purified from human erythrocytes rapidly and efficiently by DEAE-cellulose and copper-chelate affinity chromatography. Molecular weight of purified CuZnSOD was in agreement with previous studies. Optimum temperature was 15°C. Storage stability of purified CuZnSOD was best as 93.6% of the initial activity after 28 days of storage, in 50% glycerol at -20°C.

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