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A New Method for the Purification of Cu-Zn Superoxide Dismutase from Human Erythrocytes

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Abstract

The human erythrocyte is a rich raw material for the purification of Cu-Zn superoxide dismutase (SOD). We applied a simple and rapid procedure for the purification of SOD from human erythrocytes by ion exchange chromatography. The purified SOD had a specific activity of 2285.6 u/mg protein and gave a single band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and each of its to subunit has a molecular weight about 18600 daltons (SOD molecular weight is 37200 daltons).The physicochemical properties of the enzyme obtained by this method are identical to those of the native protein.This procedure appears, therefore, to be a convenient and easily method for isolating this enzyme.

Keywords: *Superoxide dismutase (SOD), Purification, Erythrocytes, SDS-PAGE, Cu-Zn Superoxide, Iran*

Introduction

Copper, zinc superoxide dismutase (Cu-Zn SOD: EC.1.15.1.1) also known as erythrocuprein, catalyzes the dismutation of the superoxide radical to O2 and H2O2 (1, 2).

SODs are classified into four groups depending on their metal cofactors: MnSOD (3), Cu-ZnSOD (4), FeSOD (5) and NiSOD (6, 7). The Cu-ZnSODs are typically found in the cytosol of eukaryotes, while FeSODs are mainly found in prokaryotes and chloroplasts and MnSODs are found in prokaryotes and in mitochondria. The entomopathogenic fungus metarhizium anisopliae contains three SOD. One of these enzymes was purified and partially characterized as a Cu-Zn SOD (8). Cu-Zn SOD of human erythrocyte enzyme has a molecular mass of 32000 and consists of two identical subunits each containing one cu^{2+} and one Zn^{2+} in the active site (9). Cu-ZnSODs are generally very stable enzymes, tolerating exposure to organic solvents and retaining activity in 8.0 M urea (10) or in 2% SDS (11). Cu-Zn SOD from some sources are not stable in the presence of these denaturants (12, 13). SOD have been remarkably resistant to evolutionary modifications, and enzymes obtained from plants, fungi, birds and mammals are very similar (14- 16).

The superoxide radical can result in many deterious physiological effects and numerous studies have focused on the therapeutic value of Cu-ZnSOD in counteracting these effects (2, 17). Reactive oxygen species are associated with many different diseases and for some of them the administration of antioxidants is recommended (18).

It is now widely accepted that SODs are able to weaken, or to eliminate altogether, a wide range of toxic effects produced by the exposure to O2 generating systems and it appears that the strug-

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gle against superoxide radical anion became a pharmaceutical necessity. The use of the isolated or coupled antioxidant enzymes SOD has been studied in relation to the treatment of coronary arteriosclerosis (19), pancreatitis (20), inflammation (21) and ischaemic heart disease (22, 23).

As these applications for SOD emerge, new technics will be required to produce and purify the enzymes in a way that will be acceptable for wide pharmaceutical use.

These enzymes were usually purified by a conventional technique involving precipitation by ammonium sulphate, ion exchange chromatography and gel filtration. However the yields and extents of purification are relatively low. The present study describes a combined methodology extraction by organic solvent and ion exchange chromatography for the purification of Cu-ZnSOD from human erythrocytes.

Materials and Methods

Erythrocytes (packed cells) from outdated human blood were washed 3 times by centrifugation with 3 volumes of cold 0.15 M NaCl at 4000g for 5 min. The cells were frozen at -20º C and lysed by thawing. The hemolysate was diluted by addition 3 volumes of cold deionized water. The temperature was maintained at 0-2º C by means of an ice water mixture while ethanol was added with adequate stirring to a final concentration of 25% (v/v). Cold chloroform was then added to the mixture to a concentration of 12% (v/v). Strirring was continuated for 30 min during which time the hemoglobin was rendered insoluble. The supernatant was decanted and additional solution in the precipitate recovered by centrifugation for 10 min at 2000 g in a centurion centrifuge.

The resulting supernatant (0.218 ml) was tested for SOD activity and allowed to warm at room temperature and 65.4 g of solid K2HPO4 (300g/l) were added resulting in the separation of two liquid phases. The denser phase was essentially aqueous and contained most of the salts, the lighter phase was ethanol and contained SOD and little salts.

The upper phase was collected (72.5 ml) and centrifuged. The supernatant contained essentially all of the SOD activity. This solution was cooled at 4º C and 0.75 volume of cold acetone was added to precipitate the SOD. After 15-30 min incubation, the precipitate was centrifuge for 10 min at 4000g. The precipitate was then dissolved in an equal volume of 2.5 mM potassium phosphate buffer (PBS) at pH 7.4 and dialyzed against the same buffer. The resulting dialysate (13 ml) was assayed for SOD activity and filtered by a 0.45 um Millipore filter. The filtered solution was processed on ion exchange chromatography.

Ion exchange chromatography was performed on a column (7x0.7 cm) of DEAE-Sepharose-CL-6B (Pharmacia) using a protocol as described earlier (24). DEAE-Sepharose was equilibrated with 2.5 mM PBS pH 7.4 at 4º C at a flow rate of 12 ml/h. Following sample application, the DEAE-Sepharose was washed with equilibration buffer and the column was eluted with a gradient of potassium phosphate buffer ranging from 2.5 mM to 200 mM, pH 7.4. SOD was eluted with 50 mM PBS and the fractions 35-47 containing SOD were collected (20ml). These fractions were assayed for SOD activity. Their volume was then reduced to 1.5 ml by freeze dryer. The molecular mass of purified components were determined by SDS PAGE using standard protein of 116 kDa-14.4 kDa.

The protein content of the crude isolates and the final SOD preparation was measured by the commassie blue G250 protein assay procedure of Bradford (25).Bovine serum albumin was used as the standard for all samples.

SDS-PAGE SDS lab gel electrophoresis was performed using a modification (26) of the Lammeli procedure (27). Gel were stained with 0.05% (w/v) commassie Brilliant Blue R250.

SOD activity measurement SOD activity was determined by measurement of the inhibition by SOD, of the photo catalyzed reduction

of nitroblue tetrazolium (NBT).

Assay principle The role of superoxide dismutase (SOD) is to accelerate the dismutation of the toxic superoxide radical (O2) produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen.

This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4Iodophenyl)-3-(4nitrpphenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye.

The superoxide dismutase activity is then measured at 505nm by the degree of inhibition of this reaction (One units of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the condition of the assay (28)). Enzyme activity was expressed as SOD U/grHb. The different steps of purification are summarized in Fig. 1.

Results

The purification steps for SOD are summarized in Table1.

All of the hemoglobin (10.214 g) was eliminated from the hemolysate at the first step by the ethanol- chloroform treatment.

SOD from human erythrocyte was purified 7606 fold with a yield of 73% (Table 1).and its specific activity was 2285.6 u/ml of protein.

In Ion-exchange chromatography, the 35-47 eluted fraction detected at 280 nm presented an SOD activity and corresponds therefore to the SOD fractions (Fig. 2).

The pooled fraction had a volume of 20 ml and was lyophilized after reconstitution to a 1.5 ml volume. The final sample was applied on SDS-PAGE to determine its molecular weight (Fig 3). The MW of SOD by SDS-PAGE was 37200 dalton. Fig 4 shows the electrophoretic pattern of SOD obtained during different steps of SOD purification.

Table 1: Purification steps of human erythrocytes SOD

Sample	Volume	Total SOD (U)	Total Protein (mg)	Specific Activity (u/mg)	Recovery %	purification factor	Total hemoglobin (g)
Hemolysate	200	8646.7	28770.5	0.3005	100	1	10.214
After ethanol-chloroform treatment	218	8066.55	316.6	25.48	93.3	84.8	Undetectable
After K2HPO4	72.5	7738.8	66.13	117.022	89.5	389.4	Undetectable
After aceton	13	7090.3	6.75	1049.67	82	3493.1	Undetectable
After ion exchange chromatography	20	6312.1	2.76	2285.6	73	7606	Undetectable

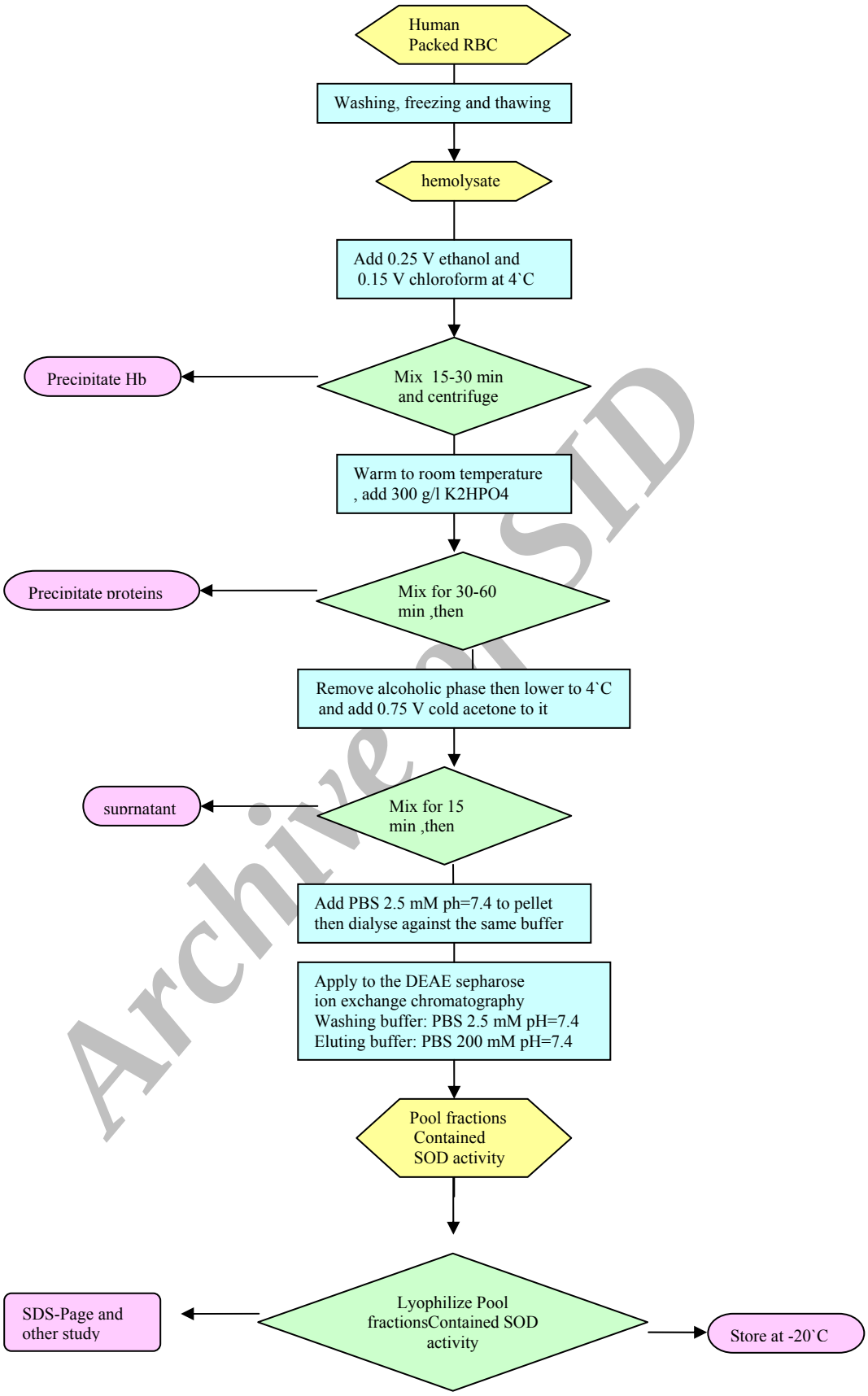

Fig.1: Flow chart for purification of SOD from hemolysate

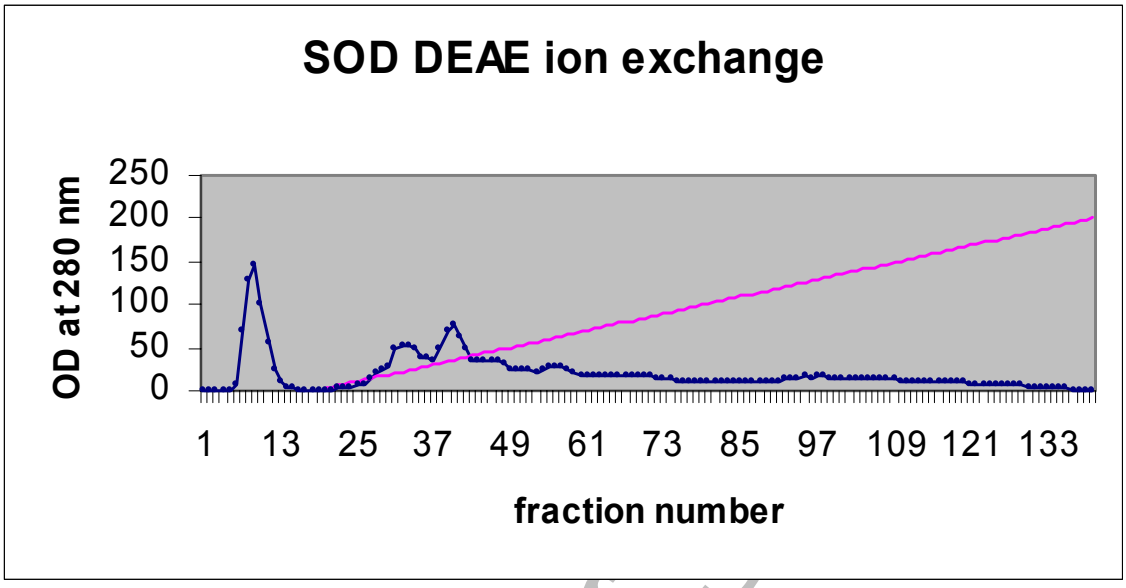

Fig. 2: Chromatogram of fractions absorbance at 280 nm after DEAE ion exchange chromatography

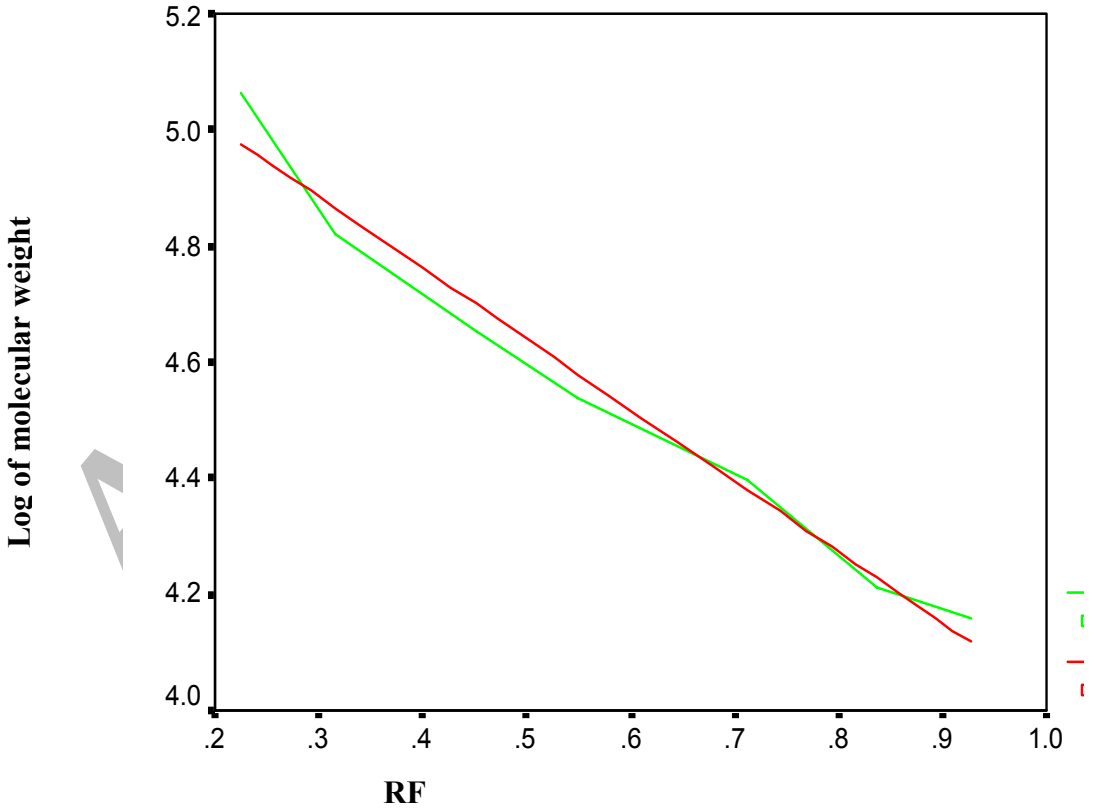

Fig. 3: Plot for determination of molecular weight of SOD

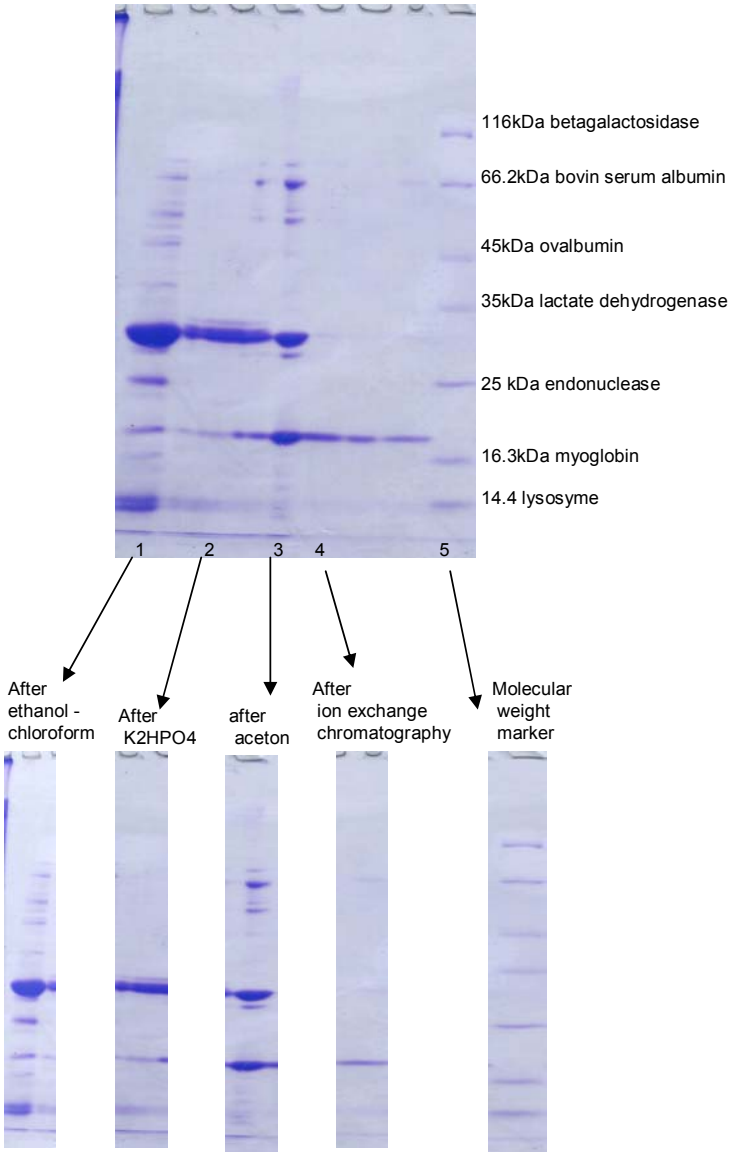

Fig. 4: Electrophoretic pattern of SOD purification steps

Discussion

The first study concerning SOD was that of Mann and Keilin in 1938 (29). They demonstrated the existence of cupper ion in this enzyme. This protein was isolated from human erythrocytes by Markowitz in 1959 (30). He used different steps of precipitation by lead, ethanol, and ammonium sulfate and purified this protein. He named it erythrocuprein. Its molecular weight was about 33000 daltons (30). Cu/Zn SOD is a stable protein that is resistant to a number of chemical treatments (9). There is a consensus that organic solvent procedures do not inactive enzyme or change its properties. The use of an ethanol-chloroform mixture in early stages of purifications of erythrocytes Cu/Zn SOD removes hemoglobin almost completely and facilitates future purification (9). The hemoglobin free extracted is typically fractionated by anion- exchange chromatography

and the enzyme can finally be purified to homogeneity by size exclusion chromatography and/ or preparative poly acryl amid gel electrophoresis (PAGE) (9).

According to the results in the first step of precipitation, the hemoglobin was undetectable (Table 1). Stansell and Deutch observed that exposure of their purified material to the chloroform-ethanol treatment lead to and increased sedimentation constant, and the loss of about 8% of the cupper (31). Such treatment also leads to an increase in the degree of heterogeneity (32). Our results suggest the loss of SOD activity about 7% after treatment by ethanol and chloroform.

Our data are a little different with the results of Mccord and Fridovich, because the bovine enzyme is more resistant to modification by treatment with ethanol and chloroform (33).

The precipitation by K2HPO4 is very important, where the purification factor increase 4 fold and specific activity 5 fold Table 1.

Utilization of K2HPO4 increases the pH of the solution and since this pH is higher than the pHi of SOD only the contaminant proteins are precipitated. In this step total protein content was 5 fold decreased.

After acetone addition to eliminate contaminants and concentrate the protein solution, the decrease of recovery was 8% which is compatible with Fridovich findings (33). The use of acetone at -20º C is important to obtain a good recovery.

After IEC, the final recovery was 73% that is better than that obtained by Fridovich (33).

The homogeneity of the superoxide dismutase preparation was analyzed by SDS gel electrophoresis. SOD appeared as a single bond upon analyses by SDS-PAGE (Fig. 4) showing the purity of the enzyme.

MW of each subunits of the enzyme was about 18600 (Fig. 3).

The physicochemical properties of the enzyme obtained by this method are identical to those of the native protein (34).

This procedure appears, therefore, to be a convenient and easily method for isolating this enzyme.

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