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Original Article

Human Erythrocyte Superoxide Dismutase Encapsulated in Positively Charged Liposomes

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Abstract

Superoxide dismutase (SOD) is an important antioxidant that protects many types of cells from the free radical damage. One of the possible ways for the use of SOD is its incorporation in liposomes. The aim of this study was to investigate the effect of cationic phospholipids on the entrapment of human erythrocyte superoxide dismutase (Cu/Zn SOD) in liposomes. Also, in the present study, we examined the effect of this formulation on the permeability of these liposomes for SOD at two different temperatures (4 °C and 37 °C). Cu/Zn SOD was purified from human erythrocytes. Several methods, including, precipitation by acetone, chloroform, centrifugation and also ion exchange chromatography on DEAE-32 were applied. In this study, encapsulated SOD in liposomes was prepared using the film hydration method. The results obtained from the prepared human erythrocyte SOD showed that at the end of the last stage, the purification was 21 times the result of the first stage, with a specific activity of 3000 U/mg. The enzyme activity and the retained enzymatic activity in liposome solution were 74±0.2 U/mg and 45%, respectively. Incubation of SOD-liposomes at 4 °C and 37 °C for 8 hours, caused the enzyme activity to decrease to 66±0.2 U/mg and 31±0.2 U/mg, respectively. The present study showed that preparation of the liposomes with cationic phospholipids was an important role in the increase of the entrapped enzyme.

Keywords: Cu/Zn superoxide dismutase; Human erythrocytes; Liposomes; Purification.
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1. Introduction

The enzyme superoxide dismutase (SOD. EC 1.15.1.1) catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide. As such, it is an important antioxidant defense in nearly all cells exposed to oxygen [1, 2]. Several common forms of SOD exist. They are proteins co-factored with copper and zinc, manganese, or iron [3]. The cytosol of virtually all eukaryotic cells contain an SOD enzyme with copper and zinc (Cu/Zn SOD). Chicken liver (and nearly all other) mitochondria and

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many bacteria (such as *E. coli*) contain a form with manganese (Mn SOD) [3]. *E.coli* and many other bacteria also contain a form of the enzyme with iron (Fe SOD) [4, 5]. In human, three types of SOD are recognized. SOD1 is located in the cytoplasm, SOD2 in the mitochondria and SOD3 is extracellular. SOD1 is dimmer, while the others are tetramers. SOD1 and SOD3 contain copper and zinc, while SOD2 has manganese in its reactive center. The genes are located on chromosomes 21, 6 and 4, respectively [6]. Human erythrocyte superoxide dismutase is Cu/Zn SOD type. This enzyme is a homodimer with a total molecular weight of about 36 KDa, that contain one atom of zinc and one atom of copper per subunit. Two subunits joined by a disulfide bond [6]. Cu/Zn SOD possesses a very compact structure that is highly resistant to denaturing agents such as urea and sodium dodesyl sulfate (SDS) and attack by proteolytic enzymes. Several factors are thought to contribute to the enzyme stability, including the prosthetic metal ions [7], the intrasubunit disulfide bond [8] and the close packing of the hydrophobic interface between the subunits and the two halves of the β-barrel core [9].

The presence of SOD has been shown to protect many types of cells from the free radical damage that is important in aging, senescence and ischemic tissue damage. SOD also protects cells from DNA damage, lipid peroxidation, ionizing radiation damage, protein denaturation and many other forms of progressive cell degradation [10]. Furthermore, SOD has been studied for use in the treatment of several diseases in which the superoxide radical is involved [11]. A major limitation of the therapeutic use of SOD is its rapid elimination from the circulation via the kidney, with a plasma half life of 6 min [12]. In order to improve its therapeutic efficiency, attempts have been made to prolong the half life of SOD. For example it has been incorporated in liposomes [13]. Corvo et al. indicated that liposomal encapsulation of the enzyme increased the plasma terminal half life of the enzyme after intravenous administration up to 5-10 folds [14]. Also, intravenously administered SOD containing liposomes were reported to be therapeutically superior to the free enzyme [15]. Also, It has been reported that difference in the charge of liposome and drug had an important effect in the entrapment of drug [14]. SOD has an isoelectric point of 5.86, therefore, it bears a negative charge at the physiological pH [16].

In this study, we investigated the effect of cationic phospholipids on the entrapment of Cu/Zn SOD in liposomes. Moreover, the effect of this formulation on the permeability of these liposomes for SOD at two different temperatures was also studied.

2. Materials and methods

2.1. Materials

Riboflavin, nitro blue tetrazolium (NBT), Triton X-100, distearoyl phosphatidyl choline and distearoyl phosphtidyl amine were obtained from Sigma (Germany). Bovine serum albumin and cholesterol were purchased from Merck (Darmstadt, Germany).

2.2. Purification of Cu/Zn SOD from human erythrocyte

The content of a bag of 500 ml of human packed cells (obtained from blood transfusion organization) was lysed by the addition of an equal volume of deionized water. Then hemoglobin was precipitated from the 1000 ml of hemolysate by chloroform-ethanol treatment. With stirring, 0.25 volume (250 ml) of ethanol and 0.15 volume (150 ml) of chloroform were added to the precipitant in the cold temperature of 4 °C [17, 18]. This mixture was allowed to stir for 15 min, during which the mixture became very thick. It was diluted with 0.1 volume (140 ml) of water, and the precipitate was removed by centrifugation (10 min, 5000 x g). The resulting supernatant

(790 ml) was pale yellow and contained Cu/Zn SOD. The solution was warmed to the room temperature, and 270 g of solid K_2HPO_4 (0.342 g/ml) was added, resulting in the separation of the two liquid phases. The denser phase was essentially aqueous and contained most of the salt. The lighter phase was water-ethanol and contained little salt. A brownish precipitate was present in the upper phase. The upper phase was collected and centrifuged (10 min, 12000 x g). The pale yellow supernatant contained essentially all of the SOD. This solution was cooled to 4 °C, and 0.75 volume of cold acetone was added to the precipitate of dismutase. The light blue precipitate was dissolved in water and dialyzed against 0.0025 M potassium phosphate at pH 7.4, then applied to a diethylaminoethyl cellulose (DEAE-32) column (2 x 17 cm) equilibrated with the same buffer [17, 18]. The column was eluted with a gradient of potassium phosphate ranging from 0.0025 to 0.2 M, pH 7.4 [18]. The protein amounts in the column fractions were observed via absorbance variations at 280 nm. The protein concentration was measured by Lowry method, using bovine serum albumin as the standard [19], and the purified enzyme was electrophoresed on SDS-PAGE [20].

2.3. Cu/Zn SOD assay

SOD activity was measured using the method of Winterbourn [21]. This method is based on the ability of SOD to inhibit the reduction of NBT by superoxide. One unit is defined as that amount of enzyme causing half the maximum inhibition of NBT reduction. One hundred tubes were chosen. The amount of 0.2 ml EDTA (0.1 M, containing 0.3 mM sodium cyanide), 0.1 ml NBT (1.5 mM) and SOD solution ranging from 0 to 100 µl obtained from human erythrocytes were added to the series of the tubes. Then the volumes were made up to 3 ml with potassium phosphate buffer (0.067 M, pH 7.8). The tubes were placed in a light box providing uniform light intensity. Then they were incubated for 5-8 min to achieve a standard temperature. At zero time and at timed intervals, 0.05 ml riboflavin were added (0.12 mM). All tubes were incubated in the light box for 12 min and at timed intervals, the absorbance at 560 nm (include tube with no enzyme as control) were measured. The percentage of inhibition of NBT reaction was determined. The plot of the percentage inhibition versus the amount of the enzyme in the tests were used for determination of the amount of enzyme resulting in one half of maximum inhibition or U/mg=1000/µg enzyme resulting in 1/2 maximum inhibition [21].

2.4. Preparation of SOD-liposomes

Liposomes were prepared by thin film hydration method [14]. Briefly, for the synthesis of positively charged liposomes, the lipids incorporated were distearoyl phosphatidyl choline, distearoyl phosphatidyl amine and cholesterol in the molar ratio of 4:0.1:0.1 in diethyleter was dried under a nitrogen stream until a homogeneous film was formed. This film was dispersed in a solution of 0.5 mg/ml SOD in 0.145 M NaCl/10 mM citrate buffer at pH 5.6. Non-encapsulated SOD was separated by dialysis with a molecular weight cut off of 40 KDa. at

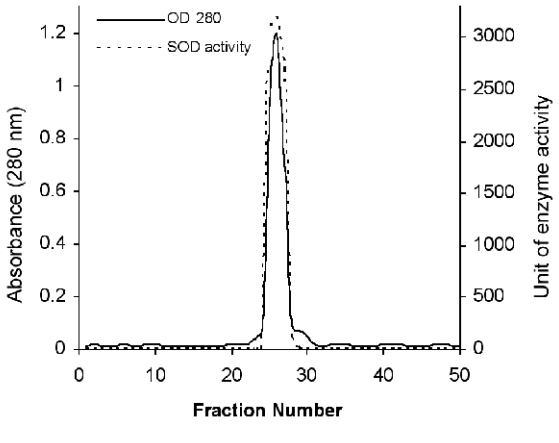

Figure 1. Variation of protein amounts in the elution fragments from DEAE-32 column Cu/Zn SOD activity. DEAE-32 column (2x17 cm) equilibrate with 2.5 mM potassium phosphate, pH 7.4 and eluted with a linear gradient of 2.5 to 200 mM of the same buffer. The flow rate was 1 ml/min.

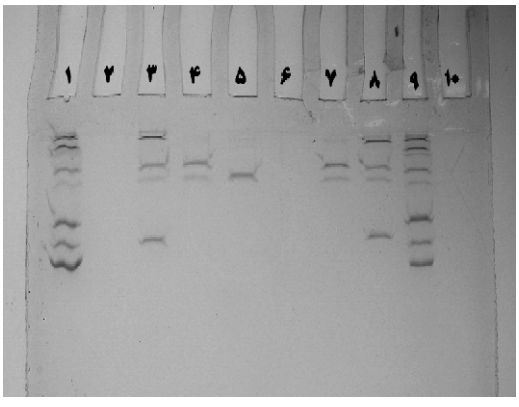

Figure 2. 15% SDS-PAGE analysis of the purified human erythrocytes Cu/Zn SOD. Sample of hemolysate step (1, 7), sample of ethanolic phase (2, 6), sample of after chromatography on precipitate were dissolved in water (3, 5), and sample of after chromatography on DEAE-32 (4).

4 °C for 15 h against two changes of at least 1000 volumes of the same buffer (0.145 M CaCl/10 mM citrate buffer, pH 5.6) [22].

Protein was determined with Lowry method [18] with prior disruption of liposomes with Triton X-100 and solution of sodium dodecylsulphate [21].

The SOD activity assay was based on the ability of the enzyme to inhibit the reduction of NBT by superoxide [21]. In the case of determination of liposome encapsulated enzyme, the enzyme first was released from the liposomes by the addition of 5% Triton X-100 to 1 ml SOD-liposome [14]. Previously, Corvo et al. reported that Triton X-100 did not affect the SOD activity [14], therefore, in this study 5% Trition X-100 were added to 1 ml SOD liposome for releasing enzyme from the prepared liposome. The retained enzymatic activity also, was calculated as follows [14]:

Retained Activity % = (Final Activity/Initial Activity) x 100

2.5. Measurement of the stability of SOD-liposomes at 4 °C and 37 °C

Eight samples containing 1 ml SOD-liposome solution were incubated at 4 °C. Each sample was taken out in 1 h intervals [23], and then the enzyme activity was measured for samples as previously described. Also, for the study of the stability of SDS-liposomes at 37 °C, all of the above stages were repeated at 37 °C. Data were expressed as mean (±SD) values.

3. Results and discussion

Phagocyte cells, such as macrophages and neutrophils are responsible for the generation of reactive oxygen species (ROS) *in vivo*. Antioxidant defense may be overcame by excessive ROS formation by hyperactive neutrophils and macrophages. Such an oxidative stress may result in damages such as observed in diabetes, rheumatoid arthritis, and several degenerative neurological diseases (such as Alzheimer and Parkinson's diseases) [24, 25]. One of the key regulatory mechanisms in these events involves the dismutation of superoxide anion radical via the enzyme SOD. The activity of the enzyme is curtailed in some disorders [24, 25]. Therefore, controlled manipulation of SOD at the cellular level could be of importance as a

Table 1. Purification steps of Cu/Zn SOD from human erythrocytes.

Purification steps	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Fold
Hemolysate	1000	2400	340000	142	100	1.00
Supernatant from chloroform- ethanol fraction	790	1580	300580	190	88	1.34
Ethanolic phase	210	420	125928	300	37	2.11
Acetone precipitate dissolved in water	15	50	109995	2200	32	15.50
After chromatography on DEAE-32	9	32	96003	3000	28	21.10

therapeutic approach to manage the diseases [26]. One of the possible ways for this controlled manipulation is the use of liposomes. Therefore, in this study, SOD-liposomes were prepared.

The results of the purification procedure are summarized in Table 1. As shown in this table, 32 mg Cu/Zn SOD displaying 96003 unit of activity with a specific activity of 3000 U/mg. The SOD activity was observed between fractions 25–27 with a single peak (Figure 1). Finally, the results of SDS-PAGE Cu/Zn SOD, showed a single protein band that confirms the purification stages (Figure 2). According to previous reports, the specific activities of the purified Cu/Zn SOD from bovine erythrocytes [27], chicken erythrocytes [18], crap liver [28], bovine heart [29] and mouse lung [30], were 2800-4728 U/mg, 4800 U/mg, 2100 U/mg, 3660 U/mg and 3020 U/mg, respectively, which were close to the results of this study.

In the second phase of this study, SOD-liposomes were prepared. After preparation of SOD-liposomes and isolation from non-entrapped enzymes (SODs) by dialysis method, SOD was released in the presence of Triton X-100 (5%), and its activity was measured [21]. The results of encapsulation test in liposomes showed that the enzyme activity in 1 ml of liposome solution, was 74.39±0.24 (n=10). The yield of enzyme encapsulation that has been often determined in these investigations, is usually expressed as the so-called retained enzymatic activity (%). In the present study, retained enzymatic activity for Cu/Zn SOD entrapped in MLV was obtained 44.65%, which is better than 30% reported by Turrens [31], Ledwozyw [32] and Regnault [33].

Finally, SOD activity assay in SOD-liposomes after 8 hours incubation at 4 °C, showed a decrease in the enzyme activity from 74.39±0.24 U/mg to 65.5±0.21 U/mg (Figure 3A), and incubation of SOD-liposome solution at 37 °C, caused a decrease in the

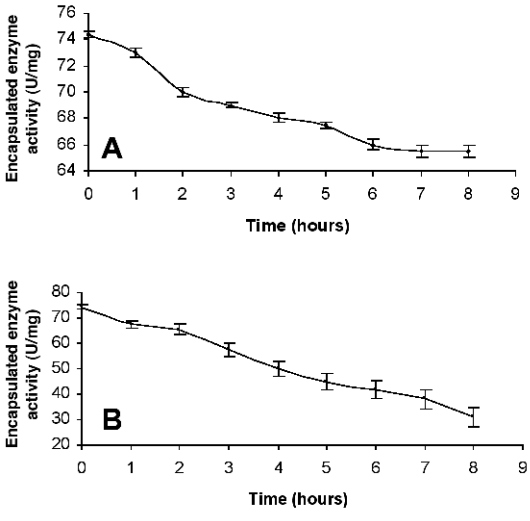

Figure 3. The effect of temperatures 4 ºC (A) and 37 ºC (B) on stability of SOD-liposomes.

enzyme activity from 74.39±0.24 U/mg to 31±0.2 U/mg (Figure 3B). Therefore, this test showed that, because of the permeability of liposomes in 4 °C and 37 °C after 8 h, the enzyme activity was decreased approximately 12% and 58%, respectively.

The present study showed that preparation of the liposomes with cationic phospholipids has an important role in the increase of the entrapped enzyme. Furthermore, study of the permeability of these liposomes for releasing the enzyme in 4 °C and 37 °C (at 8 h), indicated that these liposomes can be stored at 4 °C and also be used *in vivo*.

In summary, the findings reported here show that the encapsulation of Cu/Zn SOD in positively charged liposomes can probably be suitable for the use of the SOD as a therapeutic agent for the treatment of some disorders.

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