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Expression and Characterization of Recombinant Human Cu,Zn-Superoxide Dismutase in *Escherichia coli*

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Abstract : Expression of human Cu.Zn-superoxide dismutase (SOD) with activity comparable to human erythrocyte enzyme was achieved in *E. coli* B21(DE3) by using the pET-17b expression vector containing a T7 promoter. Recombinant human SOD was found in the cytosol of disrupted bacterial cells and represented > 25% of the total bacterial proteins. The protein produced by the *E. coli* cells was purified using a combination of ammonium sulfate precipitation, Sephacryl S-100 gel filtration and DEAE-Sephacel ion exchange chromatography. The recombinant Cu,Zn-SOD and human erythrocyte enzyme were compared using dismutation activity, SDS-PAGE and immunoblotting analysis. The mass of the subunits was determined to be 15,809 by using a electrospray mass spectrometer. The copper specific chelator, diethyldithiocarbamate (DDC) reacted with the recombinant Cu,Zn-SOD. At 50 μM and 100 μM concentrations of DDC, the dismutation activity was not inhibited for one hour but gradually reduced after one hour. This result suggests that the reaction of DDC with the enzyme occurred in two distinct phases (phase I and phase II). During phase I of this reaction, one DDC reacted with the copper center, with retention of the dismutation activity while the second DDC displaced the copper, with a loss of activity in phase II.
Key words : copper, zinc-superoxide dismutase, diethyldithiocarbamate, expression, purification.

Superoxide anion radical ($O_2\cdot^-$) is a potentially toxic byproduct of oxidative metabolism, and aerobic organisms have evolved enzymatic defenses to overcome oxygen radical-mediated toxicity. Cu.Zn-superoxide dismutase (SOD) (EC 1.15.1.1) is a class of metalloenzyme which catalyzes the dismutation of two superoxide anions into an oxygen and a hydrogen peroxide, $2O_2\cdot^- + 2H^+ \rightarrow O_2 + H_2O_2$ and thus is involved in protecting cells from oxidative damage (Fridovich, 1995). Since every biological macromolecule can serve as a target for the damage action of the abundant oxygen radical, interest has grown over the therapeutic potential of SOD. A wide range of clinical applications has been suggested. These include prevention of oncogenesis and tumor promotion, reduction of the cytotoxic and cardiotoxic effects of anticancer drugs (Oberley and Buettner, 1979), anti-inflammatory action (Huber and Menander-Huber, 1980) and protection against repercussion damage of ischemic tissues (Fridovich, 1983).

The overexpression and purification of human Cu,Zn-SOD might be helpful for clarifying the physiological roles and catalytic function of this enzyme. The human Cu.Zn-SOD cDNA has been cloned and sequenced (Hallewell *et al.*, 1985; Sherman *et al.*, 1993). The sequence indicates that the enzyme is a dimeric protein composed of 16 kDa subunits and each subunit is composed of 153 amino acids (Hallewell *et al.*, 1985). It has been reported that human Cu.Zn-SOD was expressed in *E. coli* by using the *tacI* (Hallewell *et al.*, 1985) and P_L (Hartman *et al.*, 1986) promoter systems respectively.

Diethyldithiocarbamate (DDC) reacts with Cu.Zn-SOD leading to the removal of copper from the enzyme and inactivation (Misra, 1979; Cocco *et al.*, 1981). This compound has been used to inactivate the Cu.Zn-SOD *in vivo* (Kelner *et al.*, 1989) and to distinguish between the Cu.Zn-SOD and Mn-SOD in eukaryotes (Iqbal and Whitney, 1991). DDC is also used in treatment of infectious diseases (HIV and AIDS) (Deneke and Fanburg 1980; Mansour *et al.*, 1986; Lang *et al.*, 1988; Brewton *et al.*, 1989; Kaplan *et al.*, 1989; Buhl *et al.*, 1990; Reisinger *et al.*, 1990). However, it has been shown that DDC could have toxic effects on cellular systems. One of its main properties is an inhibition of Cu.Zn-SOD probably by copper chelation (Halliwell and Gutteridge, 1986). Therefore, the effect of DDC on the Cu.Zn-SOD may provide some information for the

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use of DDC as a copper chelator and therapeutic agent.

In this study we report the overexpression of human Cu.Zn-SOD in *E. coli* by using pET-17b expression vector containing a T7 promoter and the purification of recombinant enzyme. The purified recombinant human Cu.Zn-SOD has similar properties as the authentic enzyme. We also investigated the effect of a copper specific chelator. diethyldithiocarbamate (DDC) on the dismutation activity of this enzyme.

Materials and Methods

Isolation and sequencing of human Cu,Zn-SOD cDNA

A human Cu.Zn-SOD cDNA fragment was isolated using polymerase chain reaction (PCR) technique (Friedman *et al*., 1990) from the λzap human placenta cDNA library. On the basis of the cDNA sequence of human Cu.Zn-SOD. two oligonucleotides were synthesized. The forward primer. 5'-GGATCCCATATGGCGACGAAGC-CGTGTGC-3' contained *Nde*I restriction site and the reverse primer. 5'-CGAGAATTCTTATTGGGCGATC-CCAATTA C-3' contained *Eco*RI restriction site. The PCR reaction was performed in a thermal cycler (Perkin-Elmer. model 9600). Library aliquot was placed in 600 μl siliconized reaction and then brought to a final volume of 74 μl with distilled sterile water. Phage particles were disrupted by incubation at 70°C for 5 min and then cooled on wet ice. The PCR master mixture was made up in a 600 μl reaction tube. The final solution was 192 mM KCl. 38.5 mM Tris-HCl (pH 8.3). 5.8 mM $MgCl_2$. 0.038% (w/v) gelatin. 0.77 mM in each dNTP. 3.8 μM in each oligonucleotide primer and 0.5 μl (2.5 U) of Taq polymerase. Library aliquots were mixed with the master mixture and then the reaction mixture heated at 94°C for 5 min. The program for library PCR consisted of 25 cycles of extention at 72°C for 45 seconds. denaturation at 94°C for 20 seconds and annealing at 45°C for 2 min and the final extension at 72°C for 7 min. The PCR products were purified by preparative agarose gel electrophoresis. The purified products were ligated into pCRII TA cloning vector (Invitrogen. Sandiego. USA) and then transformed into INV F' competent cell (Invirogen. Sandiego. USA). White colonies were selected on plates containing ampicillin and X-gal. The plasmid was purified by using a plasmid purification kit (Qiagen. Chatsworth. USA) and then the *SOD1* sequence was confirmed by Taq dye primer method on an automated fluorescent dye DNA sequencer (Applied Biosystem, model 37A).

Construction of human Cu,Zn-SOD expression vector, transformation and growth

The purified pCRII vector containing human *SOD1* cDNA was digested with *Nde*I and *Eco*RI. and purified by preparative agarose gel electrophoresis. The purified insert was ligated into *Nde*I and *Eco*RI cleaved pET-17b expression vector (Novagen. Madison. USA). The host *E. coli* BL21(DE3) (Novagen. Madison. USA) was transformed with pET-hSOD and then the transformants were selected on LB plate containing ampicillin. The selected colonies were cultured in LB medium containing ampicillin and the production of protein was induced by addition of IPTG (final concentration of 0.4 mM). $CuSO_4$ (1 mM) was also added to growing cultures. The production of recombinant SOD was checked by Coomassie blue staining the SDS-PAGE gel and by Western blotting analysis.

Purification of recombinant human Cu,Zn-SOD

Induced bacterial cells (2 L cultures) were suspended in 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA and disrupted by sonication. The lysate was centrifuged at 50.000×*g* for 1 h and the precipitate was dicarded. The ammonium sulfate was added to this supernatant fraction to 60% of saturation. After 2~3 h, the precipitate was removed by centrifugation at 15.000×*g* for 30 minutes and the ammonium sulfate was added to the supernatant fraction to 95% of saturation. The precipitate was collected after 20 h by centrifugation at 30.000×*g* for 1 h and was dissolved in a minimal volume of 2.5 mM potassium phosphate (pH 7.8), 0.1 mM EDTA (buffer I) and loaded onto Sephacryl S-100 (2.5×100 cm). Proteins were eluted with buffer I and then active fractions were absorbed onto DEAE-Sephacel (2.5×20 cm) column preequilibrated with buffer I. After washing the column with 5 volumes of buffer I, bound proteins were eluted with a linear gradient of potassium phosphate from 2.5 to 50 mM. Active fractions were dialyzed against Chelex 100-treated 10 mM potassium phosphate (pH 7.8). 0.1 mM EDTA. Dialysate containing SOD activity was concentrated to 1~2 ml by using a Amicon YM10 ultrafilter.

Analysis of the recombinant human SOD

The identity and purity of the recombinant human Cu, Zn-SOD were analyzed by SDS-PAGE, immunoblotting, activity staining, and the assay of dismutation activity. The enzyme activities were measured by monitoring the inhibition of ferricytochrome c reduction by xanthine/xanthine oxidase reaction described by McCord and Fridovich (1969). The standard assay was performed in 3 ml of 50 mM potassium phosphate buffer at pH 7.8 containing 0.1 mM EDTA in a cuvette at 25°C. The

reaction mixture contained 10 μM ferricytochrome c, 50 μM xanthine and sufficient xanthine oxidase to produce a rate of reduction of ferricytochrome c at 550 nm of 0.025 absorbance unit per min. Under these defined conditions, the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome c by 50% (to a rate of 0.0125 absorbance unit per min) is defined as 1 unit of activity. Immunoblotting analysis was performed according to the method of Kang (1995) using a sheep-anti-human Cu,Zn-SOD antibody (Calbiochem, Sandiego, USA). The activity staining was described previously (Yim *et al.*, 1996). Protein concentration was determined spectrophotometrically using the extinction coeffient $\varepsilon_{265}=1.51\times10^{4}\ M^{-1}cm^{-1}$ (Beyer, Jr. *et al.*, 1987). Molecular weight of the purified protein was determined with a electrospray mass spectrometer (VG, model Qurtto). N-terminal amino acid sequencing was performed on an automatic protein sequencer (Applied Biosystem, model 476A).

The effect of DDC on Cu,Zn-SOD

The enzyme (10 μM) was allowed to react with various concentrations of DDC (10~200 μM) at 25℃ in Chelex 100-treated 50 mM potassium phosphate buffer (pH 7.8). The absorbance for enzyme/DDC complex reached a maximum at 450 nm. It was necessary to assay this enzyme in the presence of DDC not exceeding 1×10^{-4} M in order to avoid the direct reduction of cytochrome c by DDC, which could interfere with this assay (Misra, 1979).

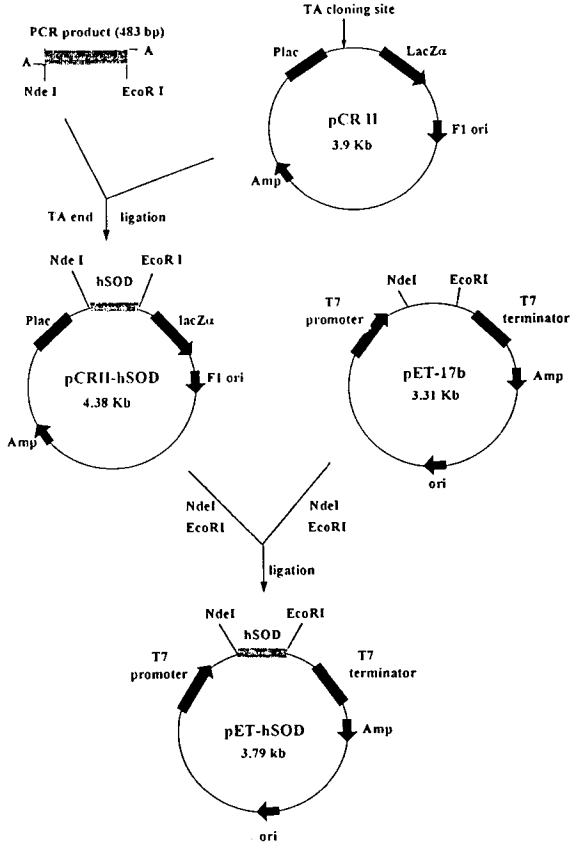

Fig. 1. Construction of human Cu,Zn-SOD expression vector, pET-hSOD.

Results and Dicussion

Overproduction of Cu,Zn-SOD in *E. coli*

We constructed human Cu,Zn-SOD expression vector, pET-hSOD as described in Materials and Methods (Fig. 1). Bacterial cells induced with IPTG were lysed at 4℃ in 50 mM phosphate buffer containing 0.1 mM EDTA at pH 7.8. The cells, disrupted by sonication, were centrifuged and the crude extracts obtained from the supernatant were used for electrophoresis in 15% SDS-polyacrylamide slab gels. Fig. 2A shows the protein bands visualized by staining with Coomassie brilliant blue. The bands marked by arrows in lane 2 (Cu, Zn-SOD) in comparison with that in lane 1 (pET-17b vector alone) indicate that the protein was produced at a very high level and constituted a major component of the total soluble proteins in cells. The amount of human SOD corresponds to > 25% of total bacterial proteins as determined by densitometric scanning of lane 2 in Fig. 2A. Hallewell *et al.* (1985) and Hartman *et al.* (1986) reported the expression levels of human Cu,Zn-SOD in *E. coli* were 5% and 10% of the total bacterial

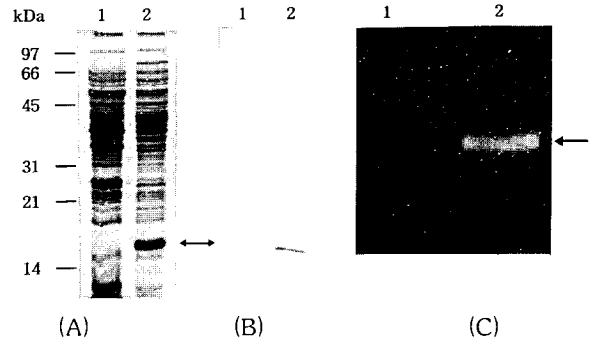

Fig. 2. Expression of human Cu,Zn-SOD in *E. coli*. Protein extracts of recombinant cells were analyzed with 15% SDS/PAGE. The gels were stained with Coomassie brilliant blue (A, 20 μg protein extracts) and subjected to immunoblot analysis an using an anti-human Cu,Zn-SOD antibody (B. 1 μg protein extracts). The gel obtained from native PAGE was used for activity staining (C. 10 μg protein extracts). Lanes in A-C are as follows: lane 1, extracts of bacteria containing expression vector (pET-17b) alone; lane 2, extracts of bacteria containing pET-hSOD. Bands marked with arrows are from human Cu, Zn-SOD.

proteins respectively. Therefore, the expression level of human Cu.Zn-SOD in our study was higher than those in other studies. This difference may derive from the expression system. Human SOD expressed in *E. coli* readily reacted with a sheep-anti-human Cu.Zn-SOD antibody (Calbiochem, Sandiego, USA) (Fig. 2B). Hence, the antigenicity of recombinant Cu.Zn-SOD is similar to that of authentic human Cu.Zn-SOD. Fig. 2C shows the activity staining of Cu.Zn-SOD on 8% native polyacrylamide gel. These results show that the recombinant Cu.Zn-SOD was active (lane 2) and the intrinsic Cu.Zn-SOD activity of *E. coli* cells (lane 1) was negligible compared to that of the recombinant enzyme.

Purification, characterization, and the dismutation activity of the recombinant Cu,Zn-SOD

The pure human Cu.Zn-SOD was obtained by using a combination of ammonium sulfate precipitation, Sephacryl S-100 gel filtration (Fig. 3A) and DEAE-Sephacel ion exchange chromatography (Fig. 3B). The purification of recombinant human Cu,Zn-SOD was summarized in Table 1. Final amounts of proteins were 20 mg of homogenous human SOD from 2 L cultures of *E. coli*. Fig. 4 shows SDS-PAGE(A) and immunoblot analysis(B) of the purified Cu.Zn-SOD (lane 1) and human erythrocyte Cu.Zn-SOD (lane 2, Sigma products) as a standard. According to the band position in the gel, the size and the mobility of the purified enzyme are similar to those of authentic human Cu.Zn-SOD.

The N-terminal amino acid sequence of recombinant Cu.Zn-SOD turned out to be NH_2-A-T-K-A-V-COOH. Protein sequencing analysis proved that human SOD isolated from *E. coli* lacked an NH_2-terminal methionine and begins with an alanine residue. This result suggests that the methionyl residue is removed by a bacterial peptidase. It should be noted that the amino terminal of all known mammalian Cu.Zn-SODs is N-acetylated(Barra *et al.*, 1980; Jabusch *et al.*, 1990).

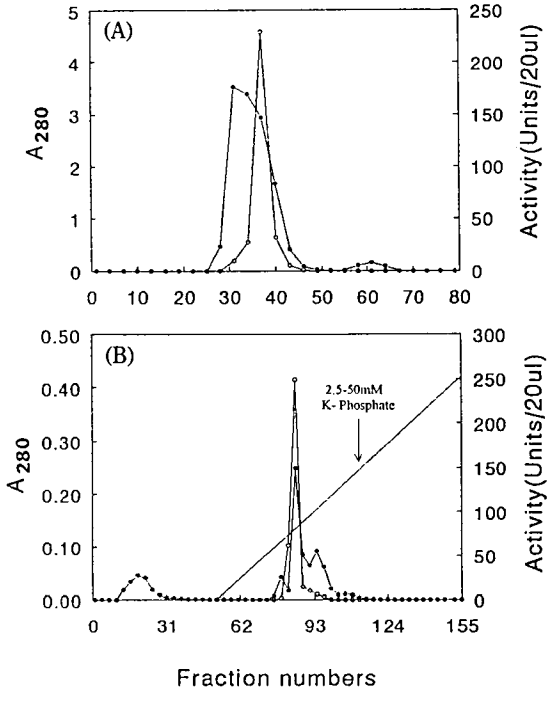

Fig. 3. Chromatographic purification of human Cu.Zn-SOD from *E. coli*. (A) Sephacryl S-100 column chromatography. (B) DEAE Sephacel column chromatography. A_{280} (●—●); Activity (○—○).

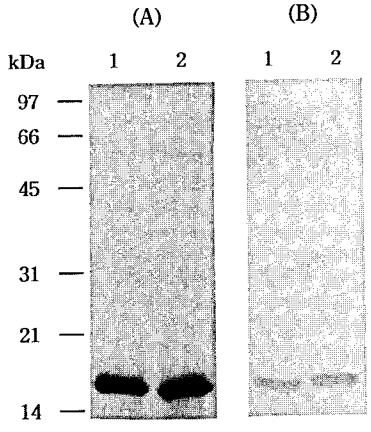

Fig. 4. SDS/PAGE and immunoblot analysis of purified human Cu.Zn-SOD. Purified proteins were analyzed by SDS/PAGE on a 15% gel and visualized with Coomassie brilliant blue (A, 5 μg protein) and subjected to immunoblot analysis with antibodies to human Cu,Zn-SOD (B, 0.5 μg protein). Lanes in A and B are as follows: lane 1, human Cu,Zn-SOD in *E. coli*; lane 2, human erythrocyte Cu,Zn-SOD (Sigma, St. Louis, USA).

Table 1. Purification of recombinant human Cu.Zn-SOD from *E. coli*

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude	428.4	159120	371	100	1
$(NH_4)_2SO_4$ (60~100%)	135	87500	648	50	1.4
Sephacryl S-100	50.4	63720	1255	40	3.4
DEAE Sephacel	20	58800	2940	37	7.9

Table 2. Dismutation activity of Cu,Zn-SOD preparation

Cu,Zn-SOD preparation	Specific activity (U/mg)
Human recombinant Cu,Zn-SOD	2630±30
Human erythrocyte Cu,Zn-SOD	2575±25
Bovine Cu,Zn-SOD	2587±38

The mass of subunits was determined to be 15,809 by using a electrospray mass spectrometer. This result indicates that the recombinant protein is not acetylated NH_2-terminal (on the basis of the expected mass, 15, 934.5, deduced from cDNA sequence of human Cu,Zn-SOD). This result is in agreement with the previous reports (Hallewell *et al.*, 1985; Hartman *et al.*, 1986). The dismutation activity of Cu,Zn-SOD preparations is summarized in Table 2. The purified human SOD produced in bacteria possessed a similar activity as compared to bovine (Boehringer-Mannheim, Mannheim, Germany) and human (Sigma, St. Louis, USA) erythrocyte Cu,Zn-SOD. Hence, our results indicate that NH_2-terminal acetylation is not essential for the dismutation activity.

Effect of diethyldithiocarbamate(DDC) on the dismutation activity

Diethyldithiocarbamate(DDC) is an effective chelating agent for Cu(II) which has been used in it's colorimetric estimation (Gubler *et al.*, 1952) and which has been reported to inhibit the Cu,Zn-SOD (Haniguchi, *et al.*, 1977; Misra, 1979). Fig. 5 illustrates the changes in optical spectrum of DDC/enzyme complex and the inhibition of the dismutation activity of Cu,Zn-SOD by DDC. At 50 μM and 100 μM of DDC, the optical spectra of DDC/enzyme complex reached maximum in one hour, in which the dismutation activity was not inhibited. However, the dismutation activity gradually reduced after one hour. These results suggest that the reaction of DDC with the enzyme occurred in two distinct phases (phase I and phase II). The structure of active site, as deduced by X-ray crystallography (Richardson *et al.*, 1975), places the Cu(II) in a ligand field composed of four imidazole rings. Misra (1979) has been reported that two sulfur atoms of DDC displaced two imidazole rings in phase I whereas in phase II, the second molecule of DDC displaced the remaining two imidazoles, thus severing the attachment of the Cu (II) to the active site and eliminating the dismutation activity. DDC is used in treatment of infectious diseases (Deneke and Fanburg 1980; Mansour *et al.*, 1986; Lang *et al.*, 1988; Brewton *et al.*, 1989; Kaplan *et al.*, 1989; Buhl *et al.*, 1990; Reisinger *et al.*, 1990), and as

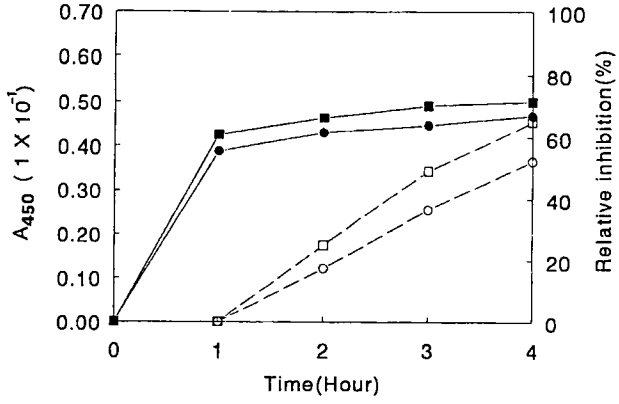

Fig. 5. Effects of DDC on the dismutation activity and visible spectrum. Enzyme, at 10 μM in 50 mM potassium phosphate at pH 7.8 and 25°C, was exposed to 50 μM and 100 μM DDC. The absorbance at 450 nm and the relative inhibition were followed as a function of time. (●—●), (■—■): A_{450} 50 μM, and 100 μM DDC. (○—○), (□—□): relative inhibition 50 μM, and 100 μM DDC.

an antidote in the treatment of heavy metal poisoning (Mankhetkorn *et al.*, 1994) and an adjunct in the treatment of alcoholism (Hellstrom-Lindhal and Weiner 1985). Unfortunately it is also very toxic. One of its toxic properties is an inhibition of Cu,Zn-SOD probably by copper chelation (Halliwell and Gutteridge, 1986). Therefore, the observation of DDC/Cu,Zn-SOD complex, without loss of the dismutation activity, will be useful in the functional analysis of this enzyme and therapeutic applications for human diseases.

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