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Short Communication

On-column Refolding of an Insoluble His_6-tagged Recombinant EC-SOD Overexpressed in *Escherichia coli*

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Abstract The EC-SOD cDNA was cloned by polymerase chain reaction (PCR) and inserted into the *Escherichia coli* expression plasmid pET-28a(+) and transformed into *E. coli* BL21(DE3). The corresponding protein that was overexpressed as a recombinant His_6-tagged EC-SOD was present in the form of inactive inclusion bodies. This structure was first solubilized under denaturant conditions (8.0 M urea). Then, after a capture step using immobilized metal affinity chromatography (IMAC), a gradual refolding of the protein was performed on-column using a linear urea gradient from 8.0 M to 1.5 M in the presence of glutathione (GSH) and oxidized glutathione (GSSG). The mass ratio of GSH to GSSG was 4:1. The purified enzyme was active, showing that at least part of the protein was properly refolded. The protein was made concentrated by ultrafiltration, and then isolated using Sephacryl S-200 HR. There were two protein peaks in the A_{280} profile. Based on the results of electrophoresis, we concluded that the two fractions were formed by protein subunits of the same mass, and in the fraction where the molecular weight was higher, the dimer was formed through the disulfide bond between subunits. Activities were detected in the two fractions, but the activity of the dimer was much higher than that of the single monomer. The special activities of the two fractions were found to be 3475 U/mg protein and 510 U/mg protein, respectively.

Key words refolding; His_6 tag; recombinant EC-SOD; inclusion body; protein subunit structure

Superoxide dismutase (SOD) is one family of antioxidant enzymes whose function is to remove damaging reactive oxygen species (ROS) from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen. There are three isozymes of SOD in mammals, including the cytoplasmic or nuclear Cu,Zn-SOD [1], the mitochondrial Mn-SOD [2] and the Cu,Zn-integrated extracellular SOD. The Cu,Zn-integrated extracellular SOD was purified by Marklund *et al.* in 1982 and termed extracellular superoxide dismutase (EC-SOD), as it was shown to be the predominant SOD in extracellular fluids such as lymph, synovial fluid and plasma [3,4]. Most studies concerning SOD have focused on Cu,Zn- and Mn-SOD, and therefore less is known about the biochemical properties of EC-SOD.

Previous studies have suggested that human EC-SOD is a secretory glycoprotein with an apparent molecular weight of about 135 kDa [5]. The human EC-SOD exists in tetramers, and each tetramer is composed of two dimers linked by disulfide bridges formed between carboxy-terminal cysteine residues located in each monomer [6,7]. The rat EC-SOD has been reported to consist of one dimer, and larger multimers of EC-SOD can also be formed [8]. Each EC-SOD is composed of the same monomer, and the molecular weight of the monomer is about 30–35 kDa.

At least one of the physiological functions of EC-SOD is to regulate the vascular tone [9]. Nitric oxide (NO) is necessary to stimulate smooth muscle relaxation, but it is exquisitely sensitive to inactivation by superoxide. A high concentration of EC-SOD may be important in maintaining low superoxide concentration and preserving the NO function. In addition, EC-SOD may be important in vascular-related diseases [10]. EC-SOD is highly ex-

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pressed in blood vessels, particularly arterial walls, which can offer protection against the oxidation of low density lipoprotein (LDL) and abrogate hypertension caused by angiotensin [11]. The EC-SOD activity is decreased in both anterior and posterior tibial arteries of diabetic patients. Furthermore, EC-SOD is the primary extracellular antioxidant in lung tissue. As airways are uniquely exposed to relatively higher levels of oxygen than most other tissues, EC-SOD is important in modulating or preventing the pathogenesis of many pulmonary diseases, such as acute lung injury, pulmonary fibrosis and lung inflammation, etc. [12].

Although EC-SOD has many important functions, the amount of EC-SOD in mammalian tissue is limited, so it is difficult to obtain enough EC-SOD by purification. Gene cloning and expression is one effective means to establish a production system for large amounts of EC-SOD. We inserted the gene encoding the mature human EC-SOD protein into the plasmid pET-28a(+) and expressed the product in *Escherichia coli* [13]. The product that was expressed in the form of inclusion bodies was a His_6-tagged recombinant protein.

In this paper, we propose an on-column method of protein refolding to recover active EC-SOD from highly insoluble inclusion bodies and analyze the structure of refolded proteins .

Experimental Procedures

All primers were synthesized by BioAsia Company Limited. The UV-2100 spectrophotometer was supplied by Unico; the J2-21M production drive centrifuge was from Beckman; Vivaspin (2 ml) was from Sartorius; Ni^{2+} chelating Sepharose fast flow was from Amersham; Sephadex G-25, the fast performance liquid chromatography (FPLC) system and Sephacryl S-200 HR were all from Pharmacia. Trypton, yeast extract, isopropyl β-*D*-thiogalactoside (IPTG) and trypsin were from Oxoid, UK. All other common chemicals were of analytical grade.

The EC-SOD cDNA was amplified from the pUC18 plasmid from Prof. Stefan L. MARKLUND (Umea University Hospital, Sweden) harboring a 1396 bp cDNA fragment encoding the human EC-SOD with *Eco*RI, using following primers: 5′-TA*CATATG*TGGACGGGCGAGGACTCGGCGGA-3′ (upstream) and 5′-AT*AAGCTT*TCAGGCGGCCTTGCACTCGCTC-3′ (downstream). An *Nde*I site (in italic) and a start codon were introduced into the upstream primer, and a *Hin*dIII site (in italic) was introduced into the downstream primer. The polymerase chain reaction (PCR) product was inserted into the *Nde*I and *Hin*dIII sites of the linearized pET-28a (+) plasmid from Prof. Hui-Zhan ZHANG (Ecust, Shanghai, China) to construct the pET-EC-SOD expression plasmid in *E. coli*.

We then transformed the plasmid pET-EC-SOD into the *E. coli* strain BL21(DE3). The bacterial cells were cultured overnight in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) containing 50 μg/ml kanamycin, 500 μM Cu^{2+} and 100 μM Zn^{2+}. The bacterial culture was diluted to 1:100 using fresh LB medium and the cells grown at 37 °C until A_{600}=0.5–0.8, and then IPTG was added to obtain a final concentration of 0.5 mM for induction of the T7 promoter activity. After growing at 37 °C for 5 h, the induced bacterial cells were harvested by centrifugation at 9600 *g* for 10 min, and then resuspended in TE buffer (20 mM Tris-HCl, 0.5% Triton, 1 mM EDTA, pH 8.0) for sonication (30 s per pulse with a 2 s interval between two pulses). The lysate was centrifuged for 30 min at 14,600 *g*. After centrifugation the pellet containing the inclusion bodies was resuspended in washing buffer (20 mM Tris-HCl, 1% Triton, pH 8.0) and incubated at room temperature for 30 min. After another centrifugation at 9600 *g* for 15 min, the pellet was washed with TE buffer and distilled water twice respectively to remove Triton completely.

About 50 mg of purified inclusion body extract was solubilized in 50 mM Tris-HCl (pH 8.0) containing 8 M urea and 0.5 M dithiothreitol (DTT) for 2 h at room temperature. After centrifugation at 14,600 *g* for 10 min, the supernatant was collected and the protein concentration was determined. The protein solution was stored at 4 °C.

We used the Sephadex G-25 system with a 15 cm×1.0 cm column containing 5 ml Sephadex G-25 beads to remove DTT. The equilibration buffer was 50 mM Tris-HCl (pH 3.0) containing 8.0 M urea. A 1 ml protein solution (protein concentration of at least 10 mg/ml) was applied onto the Sephadex G-25 column and the column was eluted with the equilibration buffer. Fractions of the first peak containing protein were collected and the protein concentration was determined.

The immobilized metal affinity chromatography (IMAC) was performed with the 1.5 cm×1.0 cm column (Amersham). The column was pre-treated as follows. The IMAC column containing 1.5 ml chelating Sepharose fast flow was washed with 10 ml of distilled water, followed by 10 ml of 10 mg/ml $NiSO_4$, and then washed with 10 ml of distilled water again. The protein sample above (5 ml, 0.68 mg/ml) was loaded onto the IMAC column, which

was pre-equilibrated with equilibration buffer (50 mM Tris-HCl, pH 8.0, 4 mM GSH, 1 mM GSSG, 8.0 M urea) at a flow rate of 0.5 ml/min, and then the column was washed with 10 ml of the equilibration buffer again until the UV absorbance baseline was reached. Then, the refolding of the bound protein was performed on-column by the use of a linear urea gradient from 8.0 M to 1.5 M, starting with the equilibration buffer and finishing with a buffer containing 50 mM Tris-HCl, pH 8.0, 4 mM GSH, 1 mM GSSG, and 1.5 M urea. The total gradient volume was 30 ml and the flow rate was 0.6 ml/min. The refolded EC-SOD was eluted using renaturation buffer incorporating 1.5 M urea and 100 mM histidine, pH 8.0.

We pooled the IMAC eluate and concentrated it by ultrafiltration using Vivaspin, and then applied it onto a Sephacryl S-200 HR column pre-equilibrated with 20 mM sodium phosphate (pH 7.4) and 50 mM NaCl at a flow rate of 0.8 ml/min. The outflow fractions containing proteins were pooled and concentrated using Vivaspin, and then analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine whether the proteins in the different fractions were the same. The protein fraction with the higher molecular weight was further analyzed by non-reduced electrophoresis.

EC-SOD activity was measured according to the method described by Xie *et al.* [14] with pyrogallol as a substrate, and one unit of enzyme activity was defined as the amount of the enzyme that restrains the self-oxidation speed of pyrogallol by 50% per min in a 1 ml solution at 25 °C.

The protein concentration was determined according to the method described by Bradford [15], by using bovine serum albumin (BSA) as the standard. SDS-PAGE was performed for 1 h at 80 V in condensing gel and 2 h at 100 V in 12% separating gel according to the method described by Laemmli [16]. Coomassie brilliant blue R-250 was used for spot staining.

Results and Discussion

His_6-tagged EC-SOD was expressed under the control of the T7 promoter and was not excreted into the culture medium. Soluble protein fractions from bacterial lysates and purified product were analyzed by SDS-PAGE under reducing conditions (**Fig. 1**). Compared with the soluble protein fraction obtained from bacteria containing the expression plasmid without an insert, the EC-SOD extracts showed a novel minor band at the predicted subunit molecular weight of 28 kDa [13]. The expression strain efficiently produced the recombinant human EC-SOD as inclusion bodies. SDS-PAGE analysis revealed that the recombinant human EC-SOD accumulated up to 26% of the total protein of *E. coli* cells.

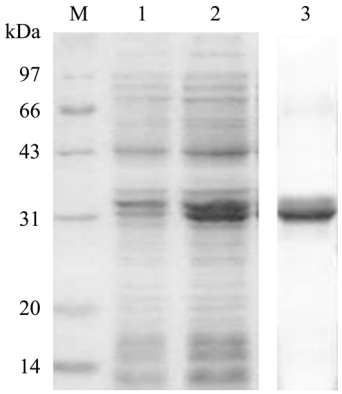

Fig. 1 SDS-PAGE analysis of EC-SOD expressed in *E. coli*
M, protein marker; 1, sample without induction; 2, sample induced with 0.5 mM IPTG for 5 h; 3, purified expression product.

The inclusion bodies were solubilized with 8.0 M urea and loaded onto the column and, subsequently, the majority of them bound with Ni^{2+}-Sepharose. The denatured EC-SOD was subjected to refolding on the IMAC column using a descending concentration gradient from 8.0 to 1.5 M urea (**Fig. 2**). The urea gradient was necessary for higher activity recovery.

As the protein with no His tag can not combine IMAC,

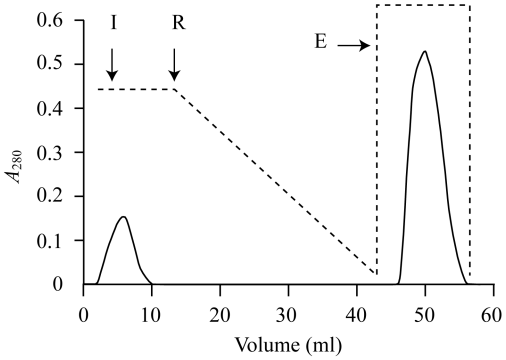

Fig. 2 Refolding and purification of recombinant EC-SOD during IMAC
I, pass through peak of sample in binding buffer containging 50 mM Tris-HCl (pH 8.0), 4 mM GSH, 1 mM GSSG, and 8 M urea; R, linear 8.0–1.5 M urea gradient in binding buffer; E, elute buffer containing 100 mM histidine, 50 mM Tris-HCl (pH 8.0). The flow rate was 1 ml/min during sample loading, equilibration and elution, and 0.6 ml/min during refolding. The detection was performed at 280 nm.

Table 1 The activities of different protein fractions

Purification step	Protein content (mg)	Total activity (U)	Specific activity (U/mg protein)
Inclusion body	5.00	—	—
Post-on-column refolding	3.40	2709	797
Peak I	0.22	750	3475
Peak II	2.70	1376	510

the process of renaturation allows the purification of the His_6-tagged recombinant EC-SOD. The amount of renatured protein and its activity are listed in **Table 1**.

There were two major peaks in the A_{280} profile of the Sephacryl S-200 HR chromatography. The preserving time of Peaks I and II was about 20 min and 110 min, respectively (**Fig. 3**). The amount of protein at Peak I was less than that at Peak II, but the molecular weight of the protein at Peak I was much higher than that at Peak II. As the protein had been purified by IMAC before isolation with the Sephacryl S-200 HR column, the probability that Peak I was composed of mixed proteins was very small. The results of the SDS-PAGE showed that the subunit molecular weight of the two protein fractions was the same (**Fig. 4**). So, we can deduce that the protein at Peak I was the combined or aggregated product between subunits.

To determine whether the interchain disulfide bond was formed between the subunits, which resulted in the product at Peak I, we compared the band location with and without treatment with 2-mercaptoethanol. When the protein at Peak I was not reduced with 2-mercaptoethanol, the position of the band on the SDS-PAGE gel was near 43 kDa; when it was reduced by 5% 2-mercaptoethanol, the position of the band shifted nearer to 31 kDa (**Fig. 5**). The most probable explanation is that the dissociation of the interchain disulfide bonds caused the formation of a single subunit. Accordingly, this suggests that a dimer had been formed through the disulfide bonds between subunits.

We determined the activities of the post-on-column refolding, Peak I and Peak II products, and the results are summarized in **Table 1**. From the results, we found that the single subunit also displayed activity, but the activity of the dimer (the protein at Peak I) was much higher than that of the single subunit (the protein at Peak II). This might be because only the subunit that is refolded correctly can form a dimer through the interchain disulfide bonds. The protein at Peak II was not refolded correctly or only partially refolded correctly, so its activity was very much lower.

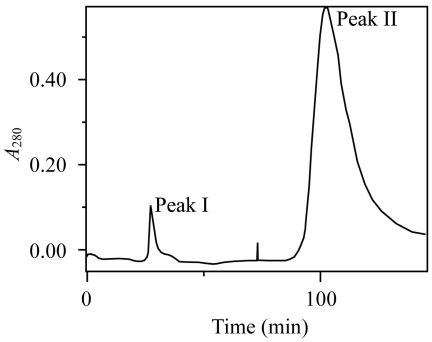

Fig. 3 Chromatogram of refolded recombinant human EC-SOD isolated on Sephacryl S-200 HR column

The loading amount of protein was about 1 mg. The protein was eluted with 50 mM Tris-HCl at a flow rate of 0.8 ml/min. Absorbance of the fraction was measured at 280 nm.

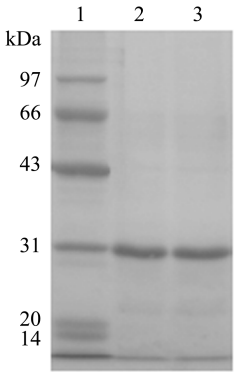

Fig. 4 SDS-PAGE analysis of proteins isolated on Sephacryl S-200 HR column

1, molecular weight markers; 2, 15 μg of protein at Peak I reduced with 30 mM 2-mercaptoethanol prior to SDS-PAGE; 3, 15 μg of protein at Peak II reduced with 30 mM 2-mercaptoethanol prior to SDS-PAGE.

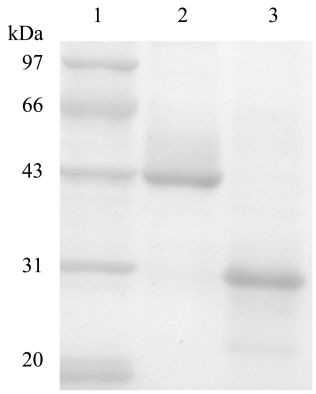

Fig. 5 Molecular mass of the monomer and dimer under reducing and non-reducing conditions

1, molecular weight marker; 2, 15 μg of non-reduced protein at Peak I; 3, 15 μg of protein at Peak I reduced with 30 mM 2-mercaptoethanol. Note the disappearance of the dimeric EC-SOD in lane 3.

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