Increased Yield of High-Purity and Active Tetrameric Recombinant Human EC-SOD by Solid Phase Refolding

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Superoxide dismutase (SOD) removes damaging reactive oxygen species from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen. Extracellular superoxide dismutase (EC-SOD) is a tetramer and is present in the extracellular space and to a lesser extent in the extracellular fluids. Increasing therapeutic applications for recombinant human extracellular superoxide dismutase (rEC-SOD) has broadened interest in optimizing methods for its purification, with a native conformation of tetramer. We describe a solid phase refolding procedure that combines immobilized metal affinity chromatography (IMAC) and gel filtration chromatography in the purification of rEC-SOD from *Escherichia coli*. The purified rEC-SOD tetramer from the Ni^{2+}-column chromatography is refolded in Tris buffer. This method yields greater than 90% of the tetramer form. Greater than 99% purity is achieved with further purification over a Superose 12PC 3.2/30 column to obtain the tetramer and specific activities as determined *via* DCFH-DA assay. The improved yield of rEC-SOD in a simple chromatographic purification procedure promises to enhance the development and therapeutic application of this biologically potent molecule.

Keywords: Extracellular superoxide dismutase, solid phase refolding, chromatographic purification procedure

Three isoenzymes of superoxide dismutase (SOD) have been identified in mammals. Cu/Zn-SOD, which is found in the cytoplasm and nucleus, Mn-SOD found in the mitochondria, and Cu/Zn-containing EC-SOD found in the extracellular matrix of tissue and to a lesser extent in extracellular fluids [11]. EC-SOD is a secretory, tetrameric, glycoprotein containing one copper and one zinc atom. It

has a molecular mass of about 135 kDa and a subunit molecular mass of around 30 kDa [10]. The N-terminal region of EC-SOD is likely to form an amphipathic α-helix, which is responsible for the formation of tetramers [17, 20]. Prior to the secretion of EC-SOD, the ECM-binding region can be proteolytically removed by a two-step event [1, 4, 13]. Consequently, both intact and cleaved EC-SOD subunits are produced. The mature tetramer can be composed of cleaved subunits only (type A), both intact and cleaved subunits (type B), or of intact subunits only (type C), producing EC-SOD with no, intermediate, or high affinity for the ECM, respectively [8, 16]. The ratio between intact and cleaved subunits is tissue specific, indicating that the proteolytic events are regulated [4]. In addition, it has been shown that the ratio can be modulated by oxidative stress [6, 14, 19]. Recent studies have reported that EC-SOD is likely to play an important role in mediating nitric oxide-induced signaling events, since the reaction of superoxide and nitric oxide can interfere with nitric oxide signaling [5]. EC-SOD is highly expressed in blood vessels, particularly arterial walls. EC-SOD protects against the oxidation of low-density lipoprotein (LDL) and abrogates hypertension caused by angiotensin [18]. EC-SOD has an important role in a number of lung diseases, where it modulates oxidant injury, inflammation, and fibrosis. In hyperoxic lung injury, EC-SOD expression and activity is disrupted, potentially contributing to oxidant damage and inflammation [14].

It is difficult to obtain enough EC-SOD by purification because the amount of EC-SOD in mammalian tissue is limited. In the *E. coli* system, recombinant EC-SOD is expressed in the form of an inclusion body. In order to generate an active form of EC-SOD, a previous study introduced a refolding system. However, their results got the dimer form of EC-SOD from inclusion bodies [21].

In this study, we generated recombinant EC-SOD, except for having an N-terminal signal peptide region and C-terminal heparin-binding domain. The expression product

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as inclusion bodies in *E. coli* was purified by affinity chromatography on a Ni^{2+} and GFC column. We present a technique whereby hEC-SOD solubilized from genetically engineered *E. coli* inclusion bodies are chromatographically purified utilizing a rigid, monosized combination of immobilized metal affinity and gel filtration chromatography columns in a process that enhances the separation of the tetramer and monomer after the refolding step.

MATERIALS AND METHODS

Materials

Plasmid pET-28a (+) and *E. coli* host cells BL21 (DE3) were obtained from Novagen (Darmstadt, Germany). HisTrap FF crude Columns and Superose 12 PC 3.2/30 were all from GE Biosciences (Buckinghamshire, England). Ultrafiltration membrane cartridges (VIVASPIN, MWCO=5,000) were purchased from Sartorious (Hannover, Germany). The BioLogic Duo-Flow chromatography system used for column purification was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Trypton, yeast extract, isopropyl-β-*D*-thiogalactoside (IPTG), and trypsin were from Sigma-Aldrich (St. Louis, Mo, U.S.A.). All other common chemicals were of analytical grade.

Cloning and Expression of EC-SOD

The EC-SOD cDNA was amplified from the pUC18 plasmid, provided by Prof. Stefan L. Marklund (Umea University Hospital, Sweden), harboring a 1,396 bp cDNA fragment encoding the human EC-SOD with EcoRI, using the following primers: 5'-TA*GAATTC*GGACGGGC-GAGGA-3' (upstream) and 5'-TA*CTCGAG*TCACTCTGAGTGCT-3' (downstream). An EcoRI site was introduced into the upstream primer, and a XhoI site was introduced into the downstream primer. The polymerase chain reaction (PCR) product was inserted into the EcoRI and XhoI sites of the linearized pET-28a (+) plasmid 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).

Cell Growth and Harvesting

A Luria-Bertani (LB) medium (100 ml containing 50 μg/ml kanamycin) was inoculated with 50 μl of glycerol stock of *E. coli* host cells and cultured overnight at 37°C, 250 rpm. The overnight culture was inoculated into 1 l of LB with 50 μg/ml kanamycin and distributed aseptically into 2-l flasks (500 ml per flask). The cells were cultured at 37°C at 250 rpm. The culture was then induced, after reaching an optical density of 0.6 at 600 nm, by the addition of IPTG to a final concentration of 1 mM and cultured at 37°C, 250 rpm for 3 h. The culture was centrifuged at 6,000 ×g for 30 min, the supernatant was carefully removed, and the cell pellet was washed by gently suspending in Tris buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 100 mM NaCl). The washed cell mass was collected by centrifuging at 14,900 ×g, 4°C for 30 min and stored at -20°C.

Cell Lysis, Inclusion Body Purification, and Solubilization

The cell pellet was resuspended in TE buffer (50 mM Tris-HCl, 0.5% Triton X-100, 1 mM EDTA, pH 8.0) for sonication on ice (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 a washing buffer (20 mM Tris-HCl, 1% Triton X-100, pH 8.0) and incubated at room temperature for 30 min. After another centrifugation at 9,600 ×g for 15 min, the pellet was washed with TE buffer and distilled water twice, respectively, to completely remove the Triton. About 5 mg of purified inclusion body extract was solubilized in 50 mM Tris-HCl (pH 8.0) containing 8 M urea and 0.1 M NaCl for 2 h at room temperature. After centrifugation at 14,600 ×g for 30 min, the supernatant was collected and the protein concentration was determined. The protein solution was stored at 4°C.

Immobilized Metal Affinity Chromatography

The immobilized metal affinity chromatography (IMAC) was performed with HisTrap FF crude columns (GE Biosciences). The protein sample above was loaded onto the IMAC column, which was pre-equilibrated with equilibration buffer (50 mM Tris-HCl, pH 8.0, 3 mM GSH, 0.5 mM GSSG, 8.0 M urea) at a flow rate of 1 ml/min, and then the column was washed again with 10 ml of the equilibration buffer 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 M, starting with the equilibration buffer and finishing with a buffer containing 50 mM Tris-HCl, pH 8.0, 3 mM GSH, 0.5 mM GSSG, 50 μM $ZnCl_2$, 100 μM $CuSO_4$, and 1 M urea. The total gradient volume was 30 ml and the flow rate was 0.6 ml/min. The refolded rEC-SOD was eluted using renaturation buffer incorporating 1 M urea and 1 M imidazole, pH 8.0. We pooled the eluae and concentrated it by ultrafiltration using Vivaspin (MWCO=5,000).

Gel Filtration Chromatography

After the refolding step, the concentrated rEC-SOD was purified on a Superose 12 PC 3.2/30 column equilibrated with PBS buffer. Conalbumin (Mw=75,000), ovalbumin (Mw=43,000), carbonic anhydrase (Mw=29,000), and ribonuclease (Mw=13,700) were also loaded onto the column as calibration standards. The profile of rEC-SOD was then compared with the standards for molecular weight confirmation and the determination of multimer formation.

SDS-PAGE and Western Blotting

Recombinant protein expression and purification were evaluated by SDS-PAGE and Western blot. SDS-PAGE was performed in pre-casting gradient gel (Nupage 4–12% Bis-Tris Gel; Invitrogen). Samples were boiled for 10 min in the presence of 2% SDS and 5 mM DTT before electrophoresis. The protein concentration was determined according to the method described by Bradford [2], by using bovine serum albumin (BSA) as the standard. SDS-PAGE was performed according to the method described by Laemmli [9]. Coomassie brilliant blue R-250 was used for spot staining. For Western blots, after electrophoresis the samples and molecular weight standards were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (0.2 μm pore size) from Invitrogen (Carlsbad, CA, U.S.A.). After transfer, the PVDF membrane was incubated in blocking buffer (Pierce, Rockford, IL, U.S.A.) for 30 min at room temperature. The transferred rEC-SOD was probed with a rabbit polyclonal anti-EC-SOD antibody (1:1,000; Abcam, Cambridge, U.K.) for 16 h at 4°C, followed by incubation with specific secondary anti-rabbit antibodies in blocking buffer for 1 h at room temperature. Three washes with TBS-T (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) were performed. The SuperSignal West Pico Chemiluminescent

Substrate (Pierce, Rockford, IL, U.S.A.) was used for detection and the results were recorded using a LAS3000 image detection system.

Cross-Linking Analysis

Interchain cross-linking of rEC-SOD was performed using disuccinimidyl suberate (DSS) (Pierce, Rockford, IL, U.S.A.). For reaction, 40 μl of purified rEC-SOD (0.6 mg/ml in PBS) and 10 μl DSS (20 mM in DMSO) were mixed and incubated for 5 min at room temperature. The reaction was stopped by the addition of 25 μl of 0.5 M Tris, pH 8.7.

Superoxide Dismutase Activity Assay

In order to measure SOD activity, the refolded rEC-SOD was determined using a Dojindo SOD Assay Kit (Dojindo Laboratories, Kumamoto, Japan) [15]. In this assay, water-soluble tetrazolium (WST), the sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, was used as a detector for the superoxide radicals generated by xanthine oxidase and hypoxanthine, in the presence of a range of concentrations of an SOD standard (Sigma Co., Saint Louis, Mo, U.S.A.) and patient erythrocyte SOD preparations. SOD activity was assessed by the ability to inhibit the reaction of the superoxides with WST to form a yellow (440 nm) formazan dye. After a 20 min incubation, the reaction was stopped, the absorbance was read in a spectrophotometer plate reader (Dynatech MR4000), and the SOD activity was calculated by relating to inhibition by the SOD standard measured at the same time.

Measurement of Intracellular ROS

Serum-starved cells were seeded at 3×10^5 cells/well in 6-well plates, stabilized in Hanks' Balanced Salt Solution (HBSS, BioWhittaker, U.S.A.) for at least 30 min, and exposed to UVB after pretreatment with EC-SOD protein (20 μg). Four hours later, 10 μM DCFH-DA was added for 30 min and the cells were immediately observed with a laser scanning confocal microscope (Carl Zeiss).

RESULTS

Construction of rEC-SOD for Expression in *E. coli*

The eukaryote-derived signal peptide can associate onto membrane or produce an insoluble protein. The N-terminal
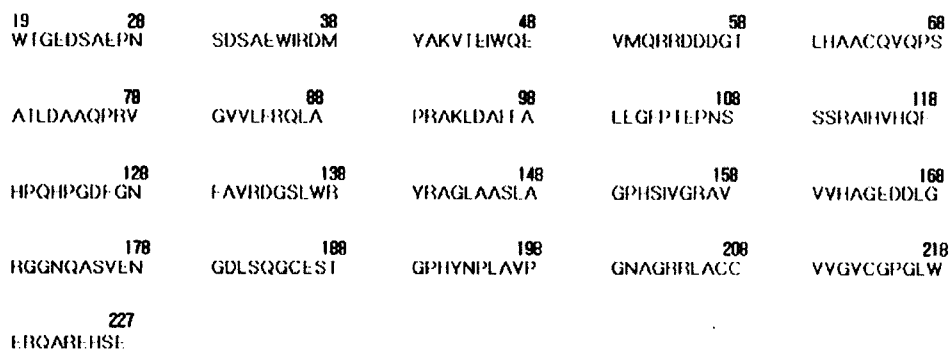
sequence of native human EC-SOD is preceded by a 18-residue signal peptide region. It has been reported that the C-terminal heparin-binding domain of mature EC-SOD can penetrate into the cell and target the nuclei. In order to overcome these features, we generated an rEC-SOD protein in which an N-terminal signal peptide region (N-MLALLCSCLLLAAGASDA-C) and a C-terminal heparin-binding domain (from R210 to A222, N-RKKRRRESECKAA-C) were removed (Fig. 1). It has been reported that a free N-terminus signal peptide is required for the formation of the proper structure of the tetramer [7]. The rEC-SOD expressed in the transformed and induced *E. coli* cells was demonstrated to be about half of the total cell proteins and was harvested as inclusion bodies. These inclusion bodies were then purified and renatured to yield biologically active rEC-SOD.

Inclusion Body Purification

The crude inclusion body preparation of rEC-SOD was washed with urea and Triton X-100 to remove native *E. coli* proteins and other soluble components. The procedure also removed significant amounts of endotoxins (to less than 5 EU/mg protein) and DNA from the host cells (to less than 100 pg/mg of protein). The inclusion body preparation was then washed with 0.1 M sodium chloride and centrifuged at low speed to remove low-density protein contaminants. This final inclusion body isolate was about 50% pure.

First Step of Solid Phase Refolding Procedure Using Immobilized Metal Affinity Chromatography

Solubilized IB of rEC-SOD was loaded onto a Ni^{2+}-Sepharose column. In order to produce an active form of rEC-SOD, the urea was removed by washing the column with urea washing buffer (50 mM Tris-HCl, pH 8.0, 3 mM GSH, 0.5 mM GSSG, 50 μM $ZnCl_2$, 100 μM $CuSO_4$, and 1 M urea). The mass ratio of the GSH to GSSG was 6:1. At this step, the urea gradient and the presence of a reduced form of glutathione (GSH) and oxidized glutathione (GSSG),

|				
19 WIGEDSAEPN	28 SDSAEWIRDM	48 YAKVIEIWQE	58 VMQRRDDDGT	68 LHAACQVQPS
78 AILDAAQPRV	88 GVVLHRQLA	98 PRAKLDAITA	108 LLGPPIEPNS	118 SSRAIHVHQI
128 HPQHPGDFGN	138 FAVRDGSLWR	148 YRAGLAASLA	158 GPHSIVGRAV	168 VVHAGEDDLG
178 RGGNQASVEN	188 GDLSQGCEST	198 GPHYNPLAVP	208 GNAGRRLACC	218 VVGVCGPGLW
227 ERQARFHSF				

Fig. 1. Protein sequence of the recombinant human extracellular superoxide dismutase.

The amino acid residues of the human EC-SOD are numbered from the N-terminus methionine of the native enzyme after processing the signal peptide [3]. The cloned rEC-SOD protein is identical to the native EC-SOD, except for having an N-terminal signal peptide region (from M1 to A18, N-MLALLCSCLLLAAGASDA-C) and a C-terminal heparin-binding domain (from R228 to A240, N-RKKRRRESECKAA-C).

including metal ions (Cu^{2+} and Zn^{2+}), were necessary for higher activity recovery. As a protein without a His tag cannot combine IMAC, the process of renaturation allows the purification of the His 6-tagged recombinant EC-SOD. During this step, the adsorbed fusion protein was solid-phased refolded as attached to the resin surfaces by the dilution effect of the denaturant (urea) and the reconstitution of the metal ions. When used with a high concentration of metal ions, over 200 μM, the bound rEC-SOD was released to the refolding buffer from Ni^{2+}-Sepharose resin. A previous study using column refolding was performed without metal ions and produced active forms of rEC-SOD [21]. However, when we followed this procedure, we did not get the active form of rEC-SOD or we just got the aggregated form. This showed that metal ions were needed for the

activation of rEC-SOD. The active site of EC-SOD was similar to that of the dimeric intracellular Cu/Zn-SOD, which contains a disulfide bond in the mature protein structure [12]. The cysteine residues forming this bond are conserved in human EC-SOD and are similarly responsible for the intrasubunit disulfide bond in human EC-SOD. The refolded protein was eluted with a linear gradient of the elution buffer (50 mM Tris-HCl, pH 8.0, 1 M urea, and 1 M imidazole). The refolding profile is shown in Fig. 2A. The major peak was collected in fractions at the elution step. The fractions Were then analyzed by SDS-PAGE (Fig. 3A, lanes 1–3) and western blot (Fig. 3B, lanes 1–3). These fractions proved to be uncontaminated and were pooled for the refolded form of the protein (Figs. 3A and 3B, lane 3).
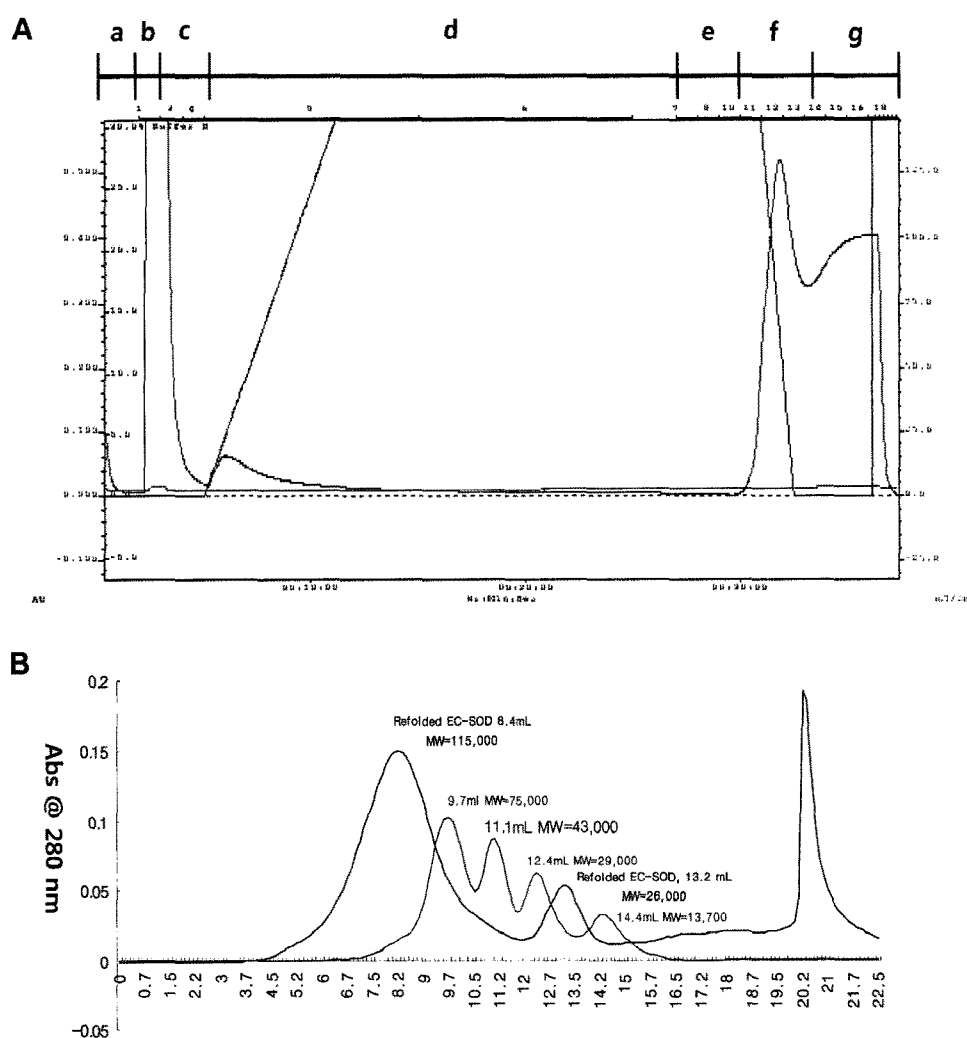
Fig. 2. Purification profile of solid phase refolding of the rEC-SOD by chromatography.

Refolding of the rEC-SOD from solubilized inclusion body was performed with HisTrap FF crude columns (GE Biosciences). A. Refolding profile by FPLC (a: pre-equilibrium step, b: sample injection, c: washing step, d: refolding step, e: refolding equilibrium step, f: elution step, g: column cleaning step) B. Purification profile of the rEC-SOD by gel filtration chromatography. The tetrameric rEC-SOD (retention volume=8.4 ml) eluted before the retention volumes of conalbumin and monomeric rEC-SOD (retention volume=13.2 ml) was eluted between carbonic anhydrase and ribonuclease, indicating that it was a tetrameric rEC-SOD of >>112 kDa. Properly assembled tetramers were assessed against conalbumin (Mw=75,000, retention volume=9.7 ml), ovalbumin (Mw=43,000, retention volume=11.1 ml), carbonic anhydrase (Mw=29,000, retention volume=12.4 ml), and ribonuclease (Mw=13,700, retention volume=14.4 ml) standards using gel filtration chromatography on a Superose 12 PC 3.2/30 fast protein liquid chromatography column.

A   B

M   1   2   3   4   1   2   3   4
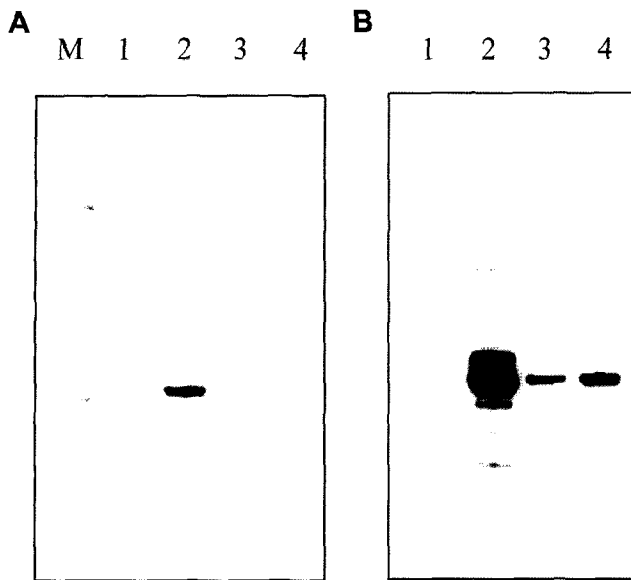
Fig. 3. SDS-PAGE and Western blot analyses of rEC-SOD during the refolding process.

Purified recombinant human EC-SOD was loaded on a pre-cast gradient (4–12%) gel. **A.** Coomassie brilliant blue stain. **B.** Western blot (M, molecular weight marker; 1, before induction; 2, after induction; 3, after refolding process by Ni-column; 4, after GFC purification).

Purification of Refolded rEC-SOD and Separation of Multimer Complex Using Gel Filtration Chromatography

The pure fractions from the Ni^{2+}-Sepharose column were introduced into the Superose 12 PC 3.2/30 column to separate tetramer and monomer. Concentrations above 1.5 mg/ml resulted in excessive protein aggregation, whereas below 1 mg/ml, suboptimal tetramer formation was observed (data not shown). There were two major peaks in the profile of the gel filtration chromatogram (Fig. 2B). The refolded rEC-SOD was then purified on the Superose 12 PC 3.2/30 to yield a greater than 90% tetramer form (in some batches even 95%). Properly assembled tetramers were assessed against conalbumin (Mw=75,000), ovalbumin (Mw=43,000), carbonic anhydrase (Mw=29,000), and ribonuclease (Mw=13,700) standards using gel filtration chromatography on the Superose 12 PC 3.2/30 fast protein liquid chromatography column. The eluted tetramer peak was collected and assessed by SDS-PAGE (Fig. 3A, lane 4) to analyze the purity, followed by Western blotting (Fig. 3B, lane 4) and a determination of ROS scavenger activity (Fig. 5). The host cell protein and DNA content were measured in the concentrated sample and were found to be absent. The identities of these bands were confirmed by Western blotting using a specific monoclonal antibody against human EC-SOD. Purified rEC-SOD was loaded on a pre-cast gradient gel and visualized using Western blots. Western blots were developed using a mouse monoclonal anti-EC-SOD antibody. Western blot results showed that the molecular mass of purified rEC-SOD was 27 kDa (Fig. 3B, lane 4).

M   1   2
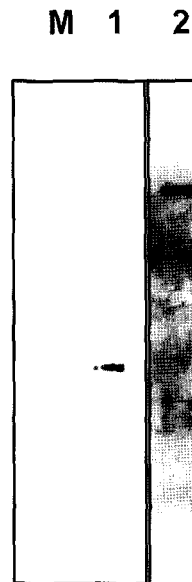
Fig. 4. SDS-PAGE analysis of cross-linked recombinant EC-SOD.

Purified monomeric and tetrameric rEC-SOD was cross-linked with DSS. The tetrameric form of the rEC-SOD was loaded on a pre-cast gradient (4–12%) gel and then visualized using Western blot. Lane 1, monomeric form of rEC-SOD; lanes 2, tetrameric form of rEC-SOD (after GFC) M, molecular weight marker. The cross-linked tetrameric rEC-SOD migrated at around 115 kDa as a tetramer; however, monomeric rEC-SOD was migrating at around 27 kDa.

SOD Enzyme Activity

It was found that the concentration of metal ions (Cu^{2+} and Zn^{2+}) is very important for the process of purification. When metal ions were not added, the purified protein would be precipitated and lose its SOD activity. This may be because the Cu^{2+} is located in the SOD catalytic center and the presence of Cu^{2+} is critical for obtaining the active conformation. The enzymatic activity of the refolded rEC-SOD was 164 U/mg (after Ni^{2+} column step) and finally
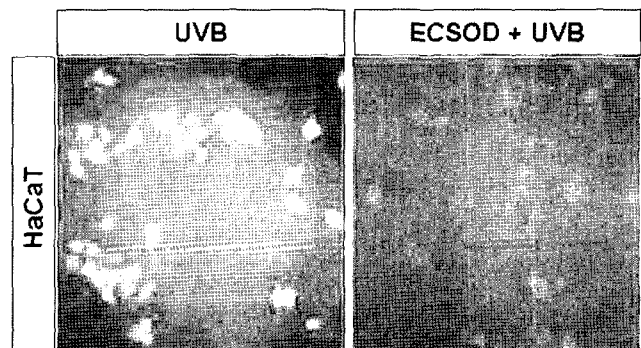
Fig. 5. Inhibition of UVB-induced ROS accumulation by rEC-SOD in HaCaT cells.

EC-SOD reduces UVB-induced ROS in HaCaT cells. The cells were preincubated for 30 min with EC-SOD (20 μg). Three hours after UVB irradiation (100 J/m²), they were labeled with 10 μM DCFH-DA for 30 min at 37°C and observed by laser scanning confocal microscopy.

Table 1. The activities on solid refolding process step.

Purification step	EC-SOD (mg)	Total activity (U)	Specific activity (U/mg)
Inclusion body	3.82	–	–
Post-Ni^{2+} column	2.73	443	164
Post-GFC column	2.32	445	192

we could get 192 U/mg (after GFC column step). The amount of refolded rEC-SOD and its activities are listed in Table 1.

Cross-Linking Analysis

Multimer formation was further confirmed by interchain cross-linking of the purified rEC-SOD using DSS as a cross-linker. The cross-linked tetramer was then analyzed by SDS-PAGE (Fig. 4). A single band corresponding to about 115 kDa was confirmed. This experiment verified that the most stable configuration of the purified product was a homotetramer; therefore, it showed different results compared with a previous report. A previous report showed that refolded rEC-SOD existed as mixed with the dimeric and monomeric forms [21]. In the previous report, the main product was the monomeric form of rEC-SOD, over 95% [21]. In our results, we observed a final refolded tetrameric rEC-SOD yield of over 98% (Figs. 3 and 4). The cross-linked tetrameric rEC-SOD migrated at around 115 kDa as a tetramer; however, monomeric rEC-SOD was migrating at around 27 kDa.

Inhibition of UVB-Induced ROS Accumulation by rEC-SOD in HaCaT Cells

UVB irradiation induces ROS formation, which causes oxidative damage to macromolecules, including nucleic acids. To determine the effect of rEC-SOD on UVB-induced ROS generation in HaCaT cells, the cells were pretreated with rEC-SOD (20 μg) for 30 min before UVB irradiation (100 J/m^2) and ROS were detected by confocal microscopy 3 h later. Since rEC-SOD was not cytotoxic, further experiments were performed with this concentration. As shown in Fig. 5, UVB caused an increase of intracellular ROS at 3 h and this effect was reduced by pretreatment with rEC-SOD (Fig. 5).

DISCUSSION

The methods described here for the preparation of rEC-SOD are simple and easily reproducible. The chromatographic purification step utilizing the Ni^{2+}-Sepharose column, followed by solid phase refolding and tetramerization, provided overall yields that were higher than those obtained by previously described methods. Our methods would be useful for making rEC-SOD and potentially for other recombinant proteins as well. rEC-SOD and other

biologically important recombinant proteins are being studied and used extensively in relation to a variety of diseases, and thus efficient production of these molecules is of significant importance.

Acknowledgment

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