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Expression, High Cell Density Culture and Purification of Recombinant EC-SOD in *Escherichia coli*

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Abstract Superoxide dismutase (SOD) catalyzes the dismutation of the biologically toxic superoxide anion into oxygen and hydrogen peroxide and is deployed by the immune system to kill invading microorganisms. Extracellular SOD (EC-SOD) is a copper- and zinc-containing glycoprotein found predominantly in the soluble extracellular compartment that consists of ~30-kDa subunits. Here, we purified recombinant EC-SOD3 (rEC-SOD) from *Escherichia coli* BL21(DE3) expressing a pET-SOD3-1 construct. Cells were cultured by high-density fed-batch fermentation to a final OD_{600} of 51.8, yielding a final dry cell weight of 17.6 g/L. rEC-SOD, which was expressed as an inclusion body, comprised 48.7% of total protein. rEC-SOD was refolded by a simple dilution refolding method and purified by cation-exchange and reverse-phase chromatography. The highly purified rEC-SOD thus obtained was a mixture of monomers and dimers, both of which were active. The molecular weights of monomeric and dimeric rEC-SOD were 25,255 and 50,514 Da, respectively. The purified rEC-SOD had 4.3 EU/mg of endotoxin and the solubility of rEC-SOD was more than 80% between pH 7 and 10. In 2 L of fed-batch fermentation, 60 mg of EC-SOD (99.9% purity) could be produced and total activity was 330.24 U. The process established in this report, involving high-cell-density fermentation, simple dilution refolding, and

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purification with ion-exchange and reverse-phase chromatography, represents a commercially viable process for producing rEC-SOD.

Keywords rEC-SOD · Fermentation · Refolding · Purification · MALDI-TOF

Introduction

Superoxide dismutase (SOD, EC 1.15.1.1) catalyzes the dismutation of the superoxide anion, which is biologically quite toxic, into oxygen and hydrogen peroxide; it is also deployed by the immune system to kill invading microorganisms [1–3]. Three basic forms of mammalian SOD enzymes, distinguished by their size and localization, are known [4]. SOD1 (Cu,Zn-SOD) is a homodimer consisting of two ~16-kDa subunits found in cytosolic and nuclear compartments of the cell, while SOD2 (Mn-SOD) is a tetramer consisting of four ~24 kDa subunits primarily localized to the mitochondria. The third SOD, SOD3, identified in 1982, is a copper- and zinc-containing glycoprotein termed extracellular SOD (EC-SOD) [5]. As its name suggests, it is found predominantly in the extracellular matrix, but can also be found in extracellular fluids such as plasma and lymph and synovial fluid [6, 7]. Individual, native EC-SOD subunits contain 222 amino acid residues and have a calculated molecular weight of 26.7 kDa. EC-SOD exists primarily as a tetramer, but monomeric, dimeric, and larger multimeric forms are also found [8, 9]. The sequence of the central region of EC-SOD is homologous with that of Cu,Zn-SOD [10]. The N-terminal region of EC-SOD is responsible for the formation of tetramers [11], and the C-terminal region mediates binding to heparin/heparin sulfate [12, 13].

Alone among SOD forms, EC-SOD can scavenge the toxic superoxide radical and preserve nitric oxide bioactivity in the extracellular environment; it is thus associated with a number of inflammation-related diseases in tissues in which it is highly expressed, such as blood vessels, heart, lungs, kidney, and placenta [14]. For example, EC-SOD in blood vessels is associated with atherosclerosis, hypertension, diabetic vasculopathy, and coronary artery disease [15–17]. In the lung, EC-SOD plays an important role in hyperoxic lung injury, bleomycin toxicity, viral pneumonia, and asbestosis [18–24]. A protective effect of EC-SOD in ischemia/reperfusion injury has been reported in the brain and the kidney [25], and EC-SOD is necessary for hypoxic activation of erythropoietin in the kidney [26]. Therefore, EC-SOD can potentially be developed as a therapeutic drug to treat these disorders.

Human recombinant EC-SOD has been expressed in CHO cells [27], insect cells [6], *Pichia pastoris* [28] and *Escherichia coli* [6, 29]. Only limited amounts of EC-SOD can be produced in CHO cells, and the amount of EC-SOD expressed in insect cells is not sufficient for production on an industrial scale. A previous study described the expression of recombinant EC-SOD (rEC-SOD) in *E. coli*, showing that it is expressed in the form of an inclusion body and must be refolded to exhibit EC-SOD activity. However, this process involved on-column refolding to restore activity and affinity chromatography to purify EC-SOD [30], a process that is difficult to scale up for industrial production.

The purpose of the present study was to develop a scalable fermentation and purification process for the production of rEC-SOD. We established high-density fed-batch fermentation conditions, a simple dilution-refolding procedure, and a purification process that uses ion-exchange and reverse-phase chromatography. The resulting process represents a commercially viable method for the production of rEC-SOD.

Materials and Methods

Bacterial Strain and Plasmid Construction

The pET-SOD3-1 plasmid for expression of the EC-SOD gene in *E. coli* was constructed from pHIS8. Amino acids 1 to 209 of EC-SOD were amplified by polymerase chain reaction (PCR) from a pUC18 plasmid containing the EC-SOD gene (kindly provided by Prof. Stefan L. Marklund, Umea University Hospital, Sweden) using the sequence-specific DNA primers, TCG GAA TTC TGG ACG GGC GAG GAC (forward) and CCG CTC GAG TCA CTC TGA GTG CTC CCG CGC (reverse), where the underlined sequences indicate introduced restriction sites for *Eco* RI and *Xho* I, respectively. This EC-SOD construct lacks a 13-amino-acid C-terminal region that corresponds to the heparin-/heparin sulfate-binding domain. PCR reactions were performed on a PTC-200 DNA Engine (MJ Research, Waltham, MA, USA) using 30 cycles of 94 °C for 30 s (denaturation), 65 °C for 30 s (annealing), and 72 °C for 30 s (polymerization). After digestion of the amplified EC-SOD gene and plasmid pHIS8 [31] with *Eco* RI and *Xho* I, the DNA fragments were ligated, and the resulting pET-SOD3-1 plasmid was used to transform the *E. coli* BL21 (DE3) strain following a standard protocol.

Fed-batch Fermentation

Luria broth (LB) containing 50 mg/L kanamycin (added to the initial fermentation medium through a 0.2 μm syringe filter) was used as a growth medium for stock and seed cultures. Stock cultures, stored at −70 °C, were prepared by growing *E. coli* BL21(DE3)/pET-SOD3-1 cells to the mid-exponential phase in LB medium containing 50 mg/L kanamycin, followed by harvesting and resuspending cells in LB medium with 15% glycerol. Seed cultures for fermentation experiments were prepared by subculturing the stock culture with 10 mL LB medium in a flask at 30 °C.

The composition of the fed-batch fermentation medium was as follows: 10 g/L trypton, 5 g/L yeast extract, 5 g/L NaCl, and 5 g/L K_2HPO_4. After sterilization, 10 mM $MgSO_4$, 1 mL/L of a trace metal solution [32], and 40 g/L glucose were added. The feeding solution was supplemented with glucose as a carbon source. Fed-batch cultures were initiated by transferring seed cultures directly to a 2-L fermenter (B. Braun Biotech, Allentown, PA, USA). The pH of the culture was controlled at 6.8 by the addition of 15% ammonia. The dissolved oxygen concentration was maintained at 30% of air saturation by automatically increasing the agitation speed to 500 rpm and replacing ambient air with oxygen-enriched air. After the glucose concentration of fermentation medium reached close to 0.05%, the feeding solution was added by the linear-increase method to maintain the glucose concentration at 0.05%. Protein expression was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM.

Purification and Refolding of Inclusion Bodies Containing Recombinant EC-SOD

The cells were harvested by centrifugation at 12,000×*g* using a high-speed centrifuge. The cells were resuspended in resuspension buffer (20 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, pH 7.9) and lysed at a pressure of 13,000 psi with a high-pressure homogenizer (Thermo Spectronic, Rochester, NY, USA). The disrupted cells were centrifuged at 12,000×*g*, the supernatant was discarded, and the precipitate was collected. The collected inclusion bodies containing rEC-SOD were washed

with inclusion body washing solution (20 mM Tris, 1% Triton X-100, 0.02% lysozyme), collected by centrifugation at 12,000×*g*, and then solubilized with solubilization buffer (8 M urea, 10 mM Glycine, pH 10.6). The solution containing soluble inclusion bodies was then diluted to a final protein concentration of 0.3 g/L with a refolding buffer solution (0.5 M urea, 10 mM Glycine, pH 10.6) containing 0.1 mM β-mercaptoethanol and incubated for 16 h at 4 °C. The refolded solution was concentrated using an ultrafiltration apparatus (Millipore, Bedford, MA, USA).

Cation-exchange and Semi-preparative Reverse-phase Chromatography

Cation-exchange chromatography was performed on a BioLogic™ chromatography system (Biorad, Hercules, CA, USA) using a UNOsphere S column (40×12.6 mm, Biorad, USA). Equilibrium buffer and elution buffer A were 6 M urea and 0.25 M acetic acid, pH 4.0; elution buffer B was 6 M urea, 0.25 M acetic acid, and 1 M sodium chloride, pH 4.05. The gradient for elution started with 100% elution buffer A for 5 min, and was followed by a step to 70% elution buffer A/30% elution buffer B for 10 min, and a linear gradient of 30–100% elution buffer B for 50 min. The column was eluted at a flow rate of 1 mL/min, and the rEC-SOD eluant was collected in 10 mL tubes. After elution, the column was washed with 100% elution buffer B for 20 min and equilibrated with 100% equilibrium buffer for 20 min. Elution was monitored at 280 nm.

The cation-exchange chromatography fractions containing rEC-SOD were further separated by semi-preparative liquid chromatography (Waters, Milford, MA, USA) using a 250×10 mm column (Grace Vydac, Columbia, MD, USA). Solvent A contained 0.05% trifluoroacetic acid (TFA) and solvent B contained 0.05% TFA and 90% acetonitrile. The elution gradient started with 100% solvent A mobile phase, which was linearly increased to 100% solvent B over 40 min. The column was eluted at room temperature at a flow rate of 3 mL/min, and the rEC-SOD eluant was collected in 10-mL tubes. Elution was monitored at 225 nm.

In vitro Assay of EC-SOD Activity

The activity of rEC-SOD was determined using the NBT method [33], which measures the rate of NBT reduction to blue formazan (monitored at 560 nm) by O_2^- generated by the xanthine oxidase reaction. EC-SOD converts O_2^- to oxygen and hydrogen peroxide, thus reducing the amount of substrate available for xanthine and xanthine oxidase. Accordingly, as the concentration of rEC-SOD in the reaction solution is increased, the rate of NBT reduction is increasingly inhibited. The reaction mixture, containing 0.05 M potassium phosphate buffer, 1 mM DETAPAC, 0.13 mg/mL BSA, 1.0 U catalase, 56 μM NBT, and 50 μM BCS, was incubated for 20 min at 25 °C in the cell compartment of the spectrophotometer. The reaction was initiated by adding 100 μM of a xanthine oxidase solution, and the absorbance change at 560 nm was monitored at 25 °C after 20 min. EC-SOD activity in a given blank-subtracted sample (blank: 0.05 M potassium phosphate buffer, pH 7.8) was determined by reference to a standard curve generated during the same experiment from known concentrations of EC-SOD.

Analytical HPLC Conditions

rEC-SOD was analyzed at room temperature on an Alliance 2695 HPLC system (Waters) using a Protein & Peptide™ C8 analytical column (250×4.6 mm i.d.; particle size, 5 μm;

Grace Vydac, Hesperia, CA, USA). The column was eluted at a flow rate of 1 mL/min using solution A (0.05% TFA) and B (0.05% TFA with 90% acetonitrile) as the two mobile phases. The elution gradient started with a mobile phase consisting of 100% solution A and 0% solution B and was programmed to increase the amount of solution B at a rate of 2.5% per minute over 40 min, ending with a final composition of 0% solution A and 100% solution B. After injection of a 20-μL sample, absorbance was monitored at a wavelength of 225 nm using a UV detector.

Dimeric and monomeric forms of rEC-SOD were also analyzed by HPLC using the Alliance 2695 HPLC system equipped with a PROTEIN PAK 300™ analytical column (300×7.5 mm i.d., Waters). The column was eluted at room temperature at a flow rate of 1 mL/min using 10 mM potassium phosphate monobasic with 0.15 M sodium chloride (pH 7.4) as the mobile phase. After injection of a 100-μL sample, absorbance was monitored at a wavelength of 225 nm using a UV detector.

Endotoxin assay of Purified EC-SOD

To determine the amount of the remaining endotoxin, ToxinSensor™ chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA) was used. Endotoxin standard, 100 μL, and samples, 100 μL, were added into endotoxin-free vials. The pH of standard and samples were adjusted at pH 7.0. Limulus amebocyte lysate (LAL) reagent was added into each vial and mixed thoroughly. The vials were incubated at 37 °C for 45 min. After incubation, 100 μL of chromogenic substrate solution was added to each vial and incubated at 37 °C for 6 min. Five hundred microliters of stop solution and 500 μL of color-stabilizer (#2 and #3) were added into each vial and mixed well. The absorbance of each reaction at 545 nm was analyzed by spectrophotometer. After analyzing the absorbance, we calculated endotoxin unit with the standard curve obtained by standard solution.

Solubility Test of Purified EC-SOD

Freeze-dried EC-SOD was solubilized by Tris buffer (pH 8.0) at 0.5 mg/mL. The solution was adjusted from pH 3 to 10 and incubated at room temperature for 30 min. After incubation, the solution was centrifuged for 5 min at 12,000×*g* and each supernant was collected. The protein concentration was analyzed by Bradford method. The solubility was calculated by the formula (solubility (%)=the protein concentration after centrifugation/0.5).

MALDI-TOF Mass Spectrometry

Purified rEC-SOD was analyzed by MALDI-TOF MS using a Voyager-DE Biospectrometry Workstation (Applied Biosystems, Carlsbad, CA, USA). The analysis was conducted in linear mode, and data for 2-ns pulses of the 337-nm nitrogen laser were averaged for each spectrum. Linear and positive-ion TOF detection were performed using an acceleration voltage of 20 kV. The grid and guide wire voltages were chosen for each spectrum to achieve the optimal signal-to-noise ratio. Spectra were obtained by summing over 200 laser shots. A saturated solution of α-CHCA in 50% acetonitrile in deionized water containing a final concentration of 0.05% (v/v) TFA was used as a matrix solution. The sample was thoroughly mixed, and 1 μL of the sample-matrix solution was deposited onto the sample plate and dried by rapid vacuum evaporation.

Results

Preparation of the pET-SOD3-1 Vector and Expression of rEC-SOD as an Inclusion Body

EC-SOD was amplified by PCR from a pUC18 plasmid containing the EC-SOD gene using primers that excluded the C-terminal heparin-/heparin sulfate-binding domain [9], and cloned into the pHIS8 vector to construct the pET-SOD3-1 bacterial expression plasmid, as described in the "Materials and Methods" section (Fig. 1). The *E. coli* BL21 (DE3) strain was transformed with pET-SOD3-1 by the calcium chloride method, and kanamycin-resistant, transformed strains were selected and stored at −70 °C.

After induction with IPTG, rEC-SOD was expressed as an inclusion body in transformed *E. coli* BL21(DE3)/pET-SOD3-1 cultured in LB medium supplemented with 50 μg/mL kanamycin at 37 °C. The apparent molecular weight of expressed rEC-SOD lacking the C-terminal heparin-/heparin sulfate-binding domain as determined by SDS-PAGE was approximately 26 kDa (data not shown).

rEC-SOD Production in Fed-batch Fermentation

Transformed *E. coli* BL21(DE3)/pET-SOD3-1 cells were cultured at 37 °C by fed-batch fermentation to increase the productivity of rEC-SOD. rEC-SOD expression was induced with IPTG after fermenting for 11 h. The expression pattern of rEC-SOD during fermentation was analyzed by SDS-PAGE of crude protein extracts. The production of rEC-SOD increased dramatically after induction and reached a plateau 6 h later, after which there was essentially no further increase in rEC-SOD content. Cell growth, however, continued, reaching a final OD_{600} of 51.8 and a final dry cell weight of 17.6 g/L; rEC-SOD ultimately comprised 48.7% of total protein (Fig. 2).

Purification and Refolding of Inclusion Bodies Containing rEC-SOD

Escherichia coli cells, collected by centrifugation of the fermentation broth, were resuspended in buffer solution and lysed using a high-pressure homogenizer. A subsequent centrifugation step collected inclusion bodies and removed soluble proteins and a portion of

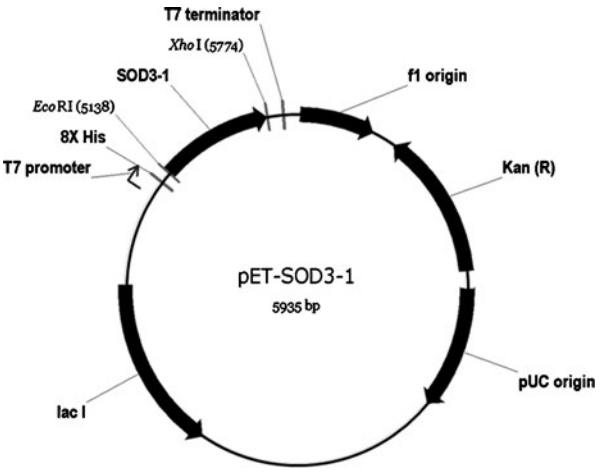


Fig. 1 Map of the rEC-SOD expression vector, pET-SOD3-1, which contains a T7 promoter, a pUC origin, a lac I gene, and a kanamycin resistance gene

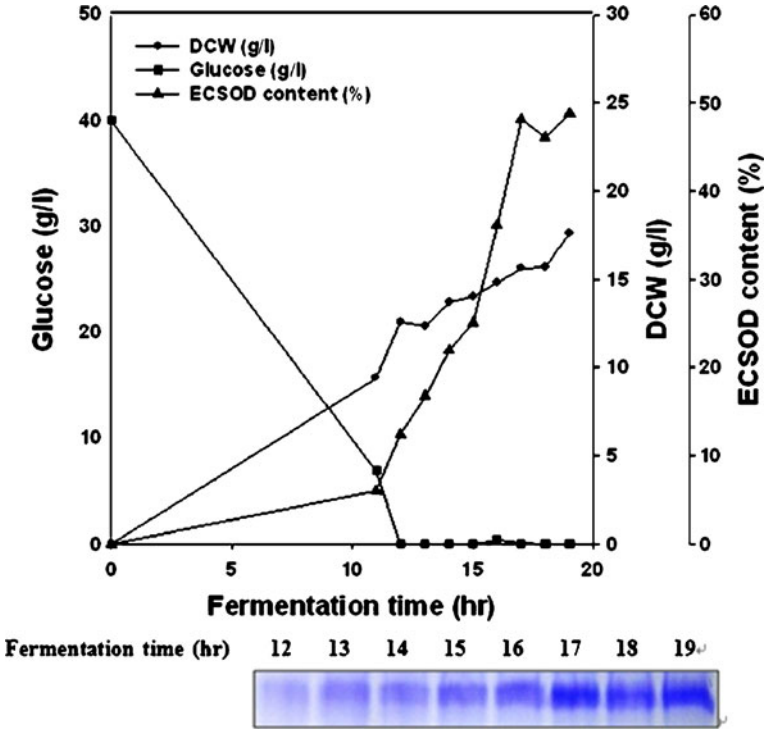


Fig. 2 Bacterial density, glucose concentration, rEC-SOD content, and SDS-PAGE of rEC-SOD expressed during the growth of *E. coli* BL21(DE3). *Closed squares*, glucose concentration; *closed circles*, dry cell weight; and *closed triangles*, rEC-SOD content. The crude extracts of transformed *E. coli* BL21(DE3)/pET-SOD3-1 cells grown in high cell density culture were analyzed by SDS-PAGE

the cell debris [34]. The separated inclusion bodies containing rEC-SOD were washed to remove other cell wall and membrane components, as described in the "Materials and Methods" section. The washing steps increased the purity of inclusion bodies sufficiently to allow the rEC-SOD refolding step to be performed.

rEC-SOD refolding was carried out at a protein concentration of 0.3 g/L in the presence of 0.1 mM β-mercaptoethanol, as described in the "Materials and Methods" section. After the refolding step, the solution containing refolded rEC-SOD was concentrated by ultrafiltration. Analytical reverse-phase chromatography showed that the purity of concentrated rEC-SOD was about 78.5% (Fig. 4a).

Chromatography and Assay of Purified EC-SOD

rEC-SOD was further purified by sequential cation-exchange and reverse-phase chromatography steps. The first cation-exchange step, employing a UNOsphere S column and a linear 0.3 to 1 M NaCl elution gradient, removed a considerable amount of impurities from the concentrated, refolded rEC-SOD. Two major peaks were detected (Fig. 3a). The first peak, which had a molecular weight of 14.6 kDa based on MALDI-TOF analysis, lacked EC-SOD activity in in vitro assays (data not shown). The second peak, which was eluted at an NaCl concentration of 0.70–0.95 M (conductivity: 56–72 mS/cm), was concentrated by ultrafiltration using Amicon (Millipore) and shown to possess EC-SOD activity in in vitro EC-

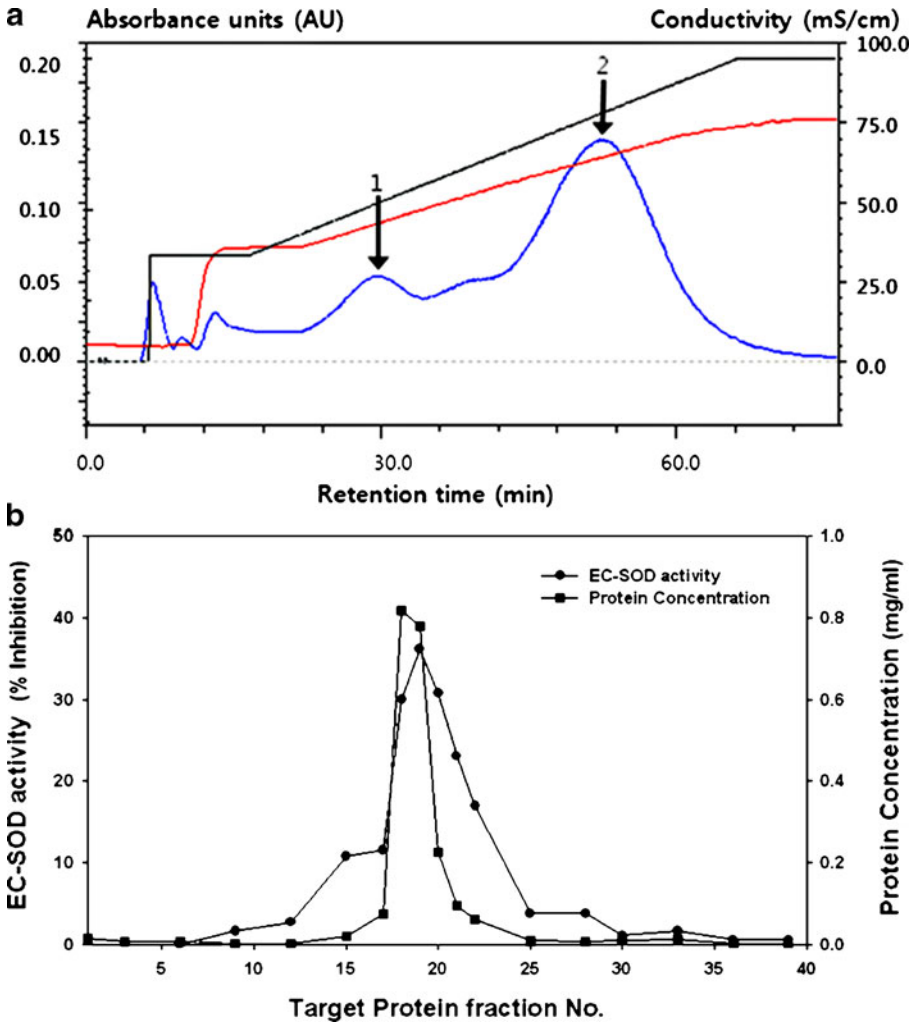


Fig. 3 Preparative chromatography of rEC-SOD. **a** Preparative cation-exchange chromatogram. Refolded rEC-SOD was applied to a UNOsphere S column and bound proteins were subsequently eluted with NaCl. The *red line* is conductivity and the *black line* is the percentage of buffer B. **b** Preparative reverse-phase chromatography. The rEC-SOD sample purified by cation-exchange chromatography was applied to a C8 reverse-phase column. The absorbance of collected fractions at 280 nm was measured to determine protein concentration, and collected fractions were analyzed in vitro for EC-SOD activity

SOD assays. Analytical reverse-phase chromatography showed that the purity of rEC-SOD in active fractions collected from the cation-exchange column was greater than 90.2% (Fig. 4b). This second, active rEC-SOD peak from cation-exchange chromatography was loaded onto a semi-preparative reverse-phase chromatography column and eluted using a linear 0–90% acetonitrile gradient. Figure 3b shows a chromatogram of the semi-preparative reverse-phase purification of rEC-SOD. The eluted rEC-SOD was collected and analyzed for protein concentration and rEC-SOD activity. One major peak eluting between 65% and 75% acetonitrile was detected during semi-preparative reverse-phase purification of rEC-SOD.

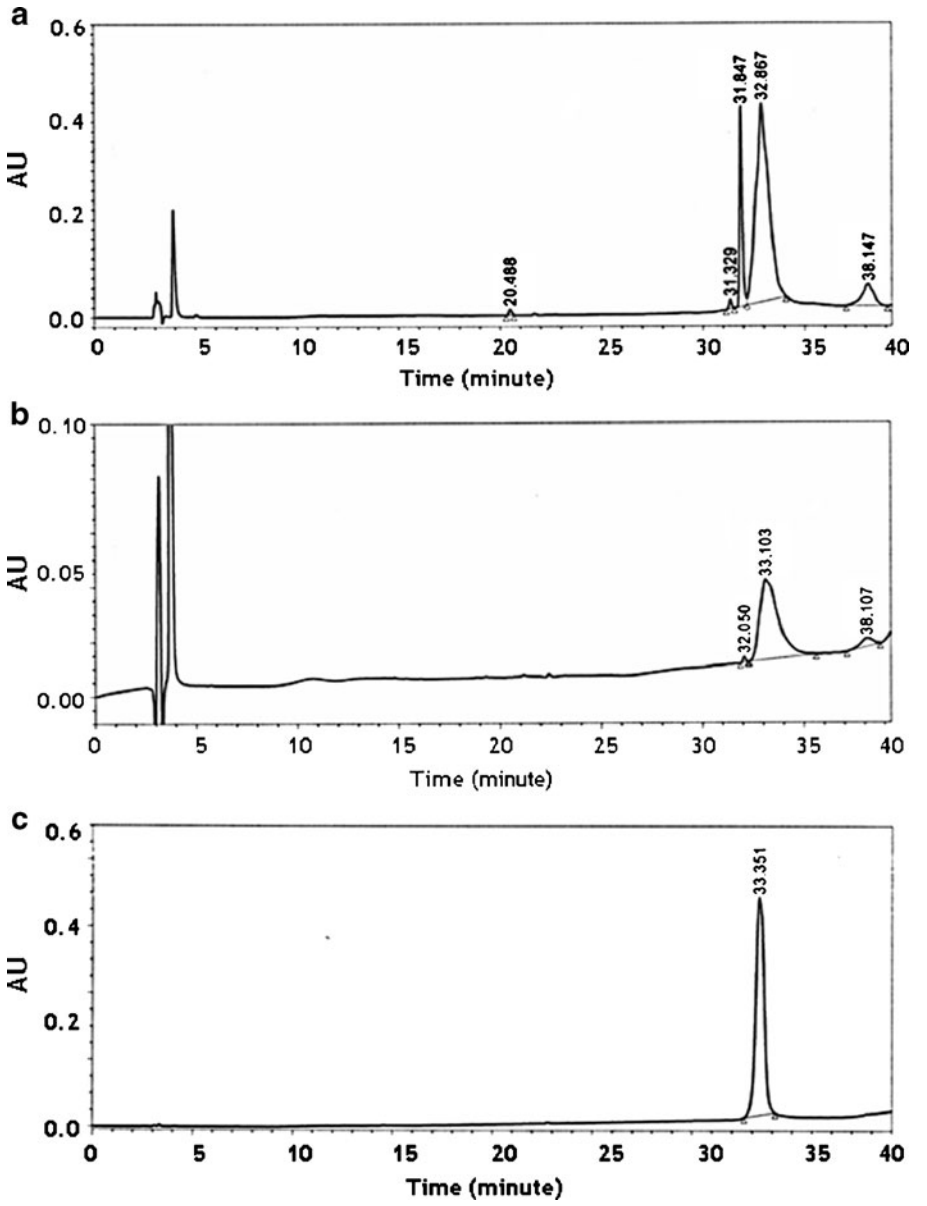


Fig. 4 Analytical reverse-phase chromatogram of rEC-SOD at different stages of purification. **a** The refolded rEC-SOD sample before cation-exchange chromatography. **b** The cation-exchange-purified sample before reverse-phase chromatography. **c** Purified rEC-SOD after reverse-phase chromatography. Analytical chromatography was carried out on an analytical C8 Protein & Peptide column

This peak corresponded to rEC-SOD, which was nearly 100% pure. A subsequent HPLC analysis of the purified rEC-SOD showed a main peak at 33.3 min (Fig. 4c).

An SDS-PAGE analysis of rEC-SOD samples during purification showed that some impurities and high-molecular-weight proteins were removed by cation-exchange

Fig. 5 Analysis of purified rEC-SOD. **a** Reducing SDS-PAGE analysis of purified rEC-SOD. Shown are rEC-SOD samples before cation-exchange chromatography (*lane 1*), major peak 1 from cation-exchange chromatography (*lane 2*), major peak 2 from cation-exchange chromatography (*lane 3*), and rEC-SOD after reverse-phase chromatography (*lane 4*). **b** Analytical gel-filtration chromatography of purified rEC-SOD. Chromatogram showing the final purified rEC-SOD (after reverse-phase chromatography) run on a PROTEIN PAK 300 analytical gel-filtration column. **c** MALDI-TOF mass spectrum of purified rEC-SOD (after reverse-phase chromatography)

chromatography (Fig. 5a). This analysis supported the initial MALDI-TOF results, showing that the first peak in the cation-exchange chromatogram was mainly an impurity with a molecular weight of approximately 14.6 kDa. SDS-PAGE also showed that the second peak in the cation-exchange chromatogram was predominantly rEC-SOD, and confirmed that the peak obtained by reverse-phase chromatography was highly purified rEC-SOD.

The gel-filtration chromatogram and MALDI-TOF MS analysis of purified rEC-SOD showed that both monomeric and dimeric forms of rEC-SOD coexisted in the final purified product (Fig. 5b, c). The amount of monomeric rEC-SOD was somewhat greater than that of the dimer. In the gel-filtration chromatogram, the retention time of monomeric rEC-SOD was 11.83 min and that of the dimer was 11.38 min. Also, MALDI-TOF MS analysis verified that the exact molecular weights of monomeric and dimeric forms of rEC-SOD were 25,255 and 50,514 Da, respectively.

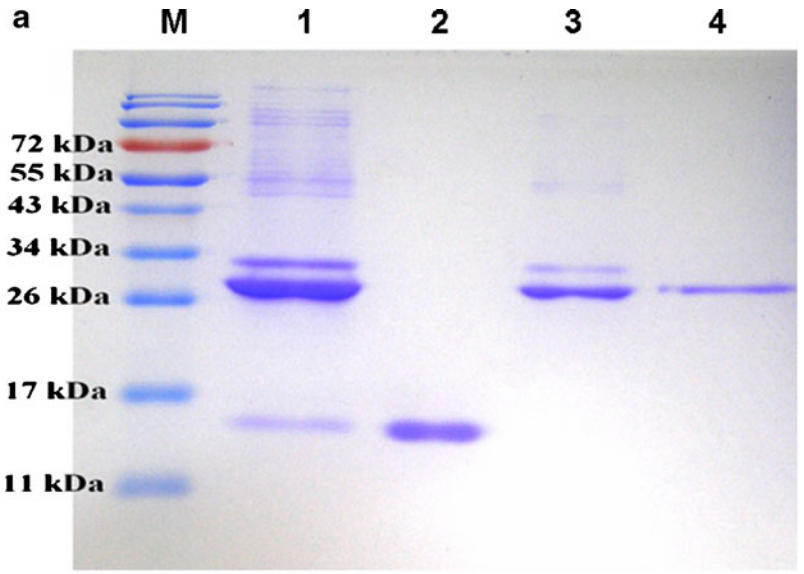
The remaining endotoxin level of purified EC-SOD was 4.3 EU/mg. The solubility of EC-SOD was more than 80% between pH 7 and 10. Especially, the solubility of EC-SOD at pH 7.5, which is similar to human blood, pH 7.4, was 84%. Below pH 7.0, the solubility considerably decreased (Fig. 6).

The recombinant EC-SOD production process was shown as the purification table (Table 1). The volume of cell culture was 2 L and the protein amount of pure inclusion body was 17.14 g. After refolding, we got 2.38 g EC-SOD, whose purity was 78.5%. Finally, 60 mg of EC-SOD (99.9% purity) could be produced through ion-exchange and reverse-phase chromatography, and total activity was 330.24 U.

Discussion

Mammalian, insect, and bacterial cell expression systems have been used in an attempt to manufacture human rEC-SOD. The levels of rEC-SOD expression in animal and insect cells were too low to be of commercial value. Although previous studies showed that rEC-SOD could be robustly expressed in *E. coli*, it was expressed in an insoluble form as inclusion bodies; thus, an in vitro refolding step was required to recover EC-SOD activity. In this earlier work, on-column refolding and affinity chromatography steps, such as a His-tagged chromatography [30], were used to purify the rEC-SOD. However, this approach lacks the scalability necessary to economically produce commercial quantities of rEC-SOD.

To maximize the productivity of rEC-SOD, we developed a fed-batch, high-cell-density fermentation process and employed a simple dilution refolding step and commercially viable cation-exchange and reverse-phase chromatography procedures. The overall rEC-SOD production process can be summarized as follows: (1) high-cell-density fermentation of pET-SOD3-1-transformed *E. coli* BL21(DE3) cells, (2) expression of rEC-SOD as an inclusion body after induction with IPTG, (3) homogenization of cells using a high-pressure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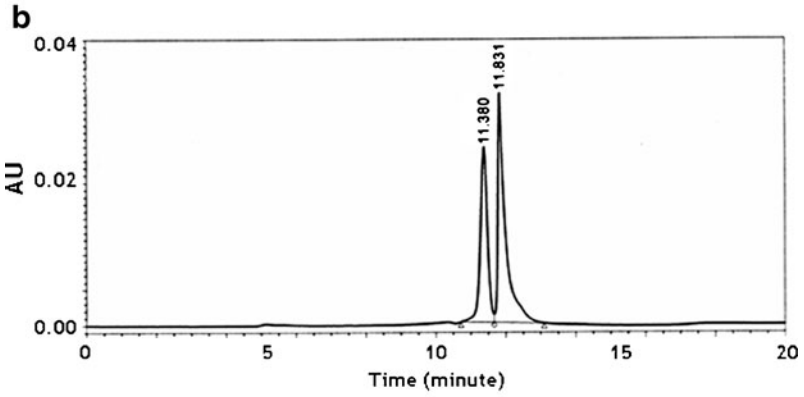
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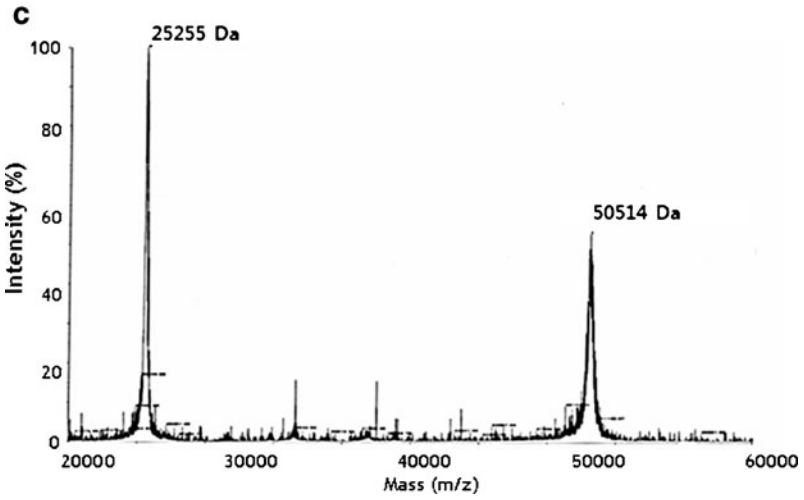
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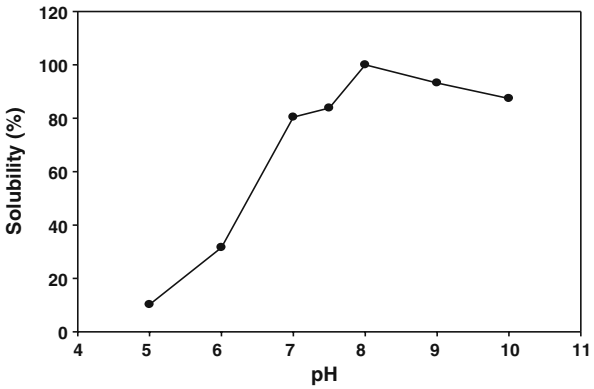

Fig. 6 pH solubility of purified rEC-SOD

homogenizer, (4) collection and refolding of rEC-SOD by a simple dilution refolding method, (5) a first step purification of refolded rEC-SOD by cation-exchange chromatography, and (6) a second purification step using reverse phase chromatography. rEC-SOD was successfully expressed at high levels in high-cell-density fermentation cultures of recombinant *E. coli* BL21(DE3)/pET-SOD3-1. High-cell-density fermentation of recombinant protein is a primary prerequisite for a commercially viable production process [35]. Also important in the production of recombinant protein is a simple purification process. For industrial production of rEC-SOD, our simple dilution refolding strategy was clearly superior to on-column refolding methods. Moreover, the ion-exchange and reverse-phase chromatography steps used for rEC-SOD purification are easy to scale up compared to affinity and gel-filtration chromatography. Applying this simple overall purification process yielded highly purified rEC-SOD.

In this report, the expressed rEC-SOD was detected as both a monomer and a dimer (Fig. 5b, c). The dimerization of full-length EC-SOD is caused by formation of a disulfide bond between Cys-219 in two EC-SOD monomers [8, 36]. The ability of rEC-SOD to form a dimer despite the fact that the construct used in this study did not have Cys-219 could reflect the rEC-SOD dimerization by hydrophobic interactions at the N-terminus, which is known to be involved in tetramerization [11]. When the mass to charge ratio in MALDI-TOF analyses was scanned up to 200 kDa, we were unable to detect a peak corresponding to a tetramer (~104 kDa), or any other larger multimeric forms (data not shown).

The C-terminal region can be naturally removed before secretion in a two-step cellular process involving an endoproteinase and a carboxypeptidase [37–39], presumably generating the three subtypes of EC-SOD that differ with respect to heparin-binding affinity: A-type (low affinity), B-type (medium affinity), and C-type (high affinity). The B-

Table 1 Purification of recombinant EC-SOD from *E. coli*.

Purification step	Volume (L)	Total protein (g)	EC-SOD (g)	Total activity (U)	Purity (%)
Fermentation broth	2.00	–	–	–	–
Pure inclusion body	2.00	17.14	–	–	–
Refolding	57.13	3.03	2.38	–	78.5
Ion-exchange chromatography	0.98	0.26	0.23	2,558.06	90.2
Reverse-phase chromatography	0.18	0.06	0.06	330.24	99.9

type is a heteromultimer of A-type and C-type EC-SODs. Since our EC-SOD construct is truncated at the C-terminus, resulting in the deletion of 13 amino acids, the rEC-SOD generated from it can be expected to be the A-type, with minimal heparin-/heparin-sulfate-binding affinity. Despite this difference in affinity, A-type EC-SOD still exhibits a level of enzymatic activity comparable to that of the full-length C-type EC-SOD [12].

Natural EC-SOD is glycosylated at Asn-89, but *E. coli* does not have a glycosylation apparatus. The role of glycosylation in EC-SOD is not clearly known; however, preventing glycosylation by mutating Asn-89 reportedly has no effect on enzymatic activity. The major difference between glycosylated and nonglycosylated forms of EC-SOD appears to be their solubility: the nonglycosylated form is insoluble, but can be made soluble by increasing the pH and ionic strength [40]. We found that the rEC-SOD expressed in *E. coli* was soluble for months in Tris buffer (pH 8.0) after refolding; thus, solubilized rEC-SOD is quite stable and does not tend to aggregate. Even though 6 M urea was used at ion-exchange chromatography, rEC-SOD would not be denatured without adding reducing agents, such as β-mercaptoethanol or dithiothreitol (DTT).

After resolving the freeze-dried rEC-SOD with Tris buffer (pH 8.0), the solubility was more than 80% between pH 7 and 10, making the protein amenable to therapeutic or cosmetic applications. The endotoxin of purified EC-SOD was 4.3 EU/mg. Although there is no guideline for the endotoxin limit of rEC-SOD, the remaining endotoxin level can be considered as not high, comparing to the endotoxin limit of human insulin (10 EU/mg based on USP Guideline).

In summary, we have developed a production process for expressing rEC-SOD in *E. coli* using high-cell-density fermentation, and purifying it using a simple dilution-refolding scheme and commercially viable chromatography steps.

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