JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Production and Characterization of Human Extracellular Superoxide Dismutase in the Methylotrophic Yeast *Pichia pastoris*

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Reactive oxygen species are associated with various diseases including cardiovascular diseases, neurological disorders, and pulmonary diseases. Extracellular superoxide dismutase (ECSOD) is an antioxidant enzyme secreted by cells to prevent overproduction of reactive oxygen species. We expressed an *ECSOD* gene isolated from a human aortic smooth muscle cDNA library in the methylotrophic yeast *Pichia pastoris*. A synthetic secretion cassette was constructed with the inducible promoter of the alcohol oxidase 1 gene (*AOX1*) and the yeast α-mating factor signal peptide. As much as 25% of the total protein was ECSOD in some transformants grown under inducing conditions. After 36 h of methanol induction, ECSOD was exported into the culture medium at a concentration of approximately 440 mg/L with an antioxidative activity of 760 ± 20 U/mg ECSOD. Transformed yeast cells were more resistant to heat shock and H_2O_2 oxidative stress, indicating that the human ECSOD expressed by *P. pastoris* had multiple biological functions. Our data suggest that the methylotrophic yeast inducible system is suitable for large-scale production of enzymatically active human ECSOD.

KEYWORDS: Superoxide dismutase; human extracellular SOD (ECSOD); methylotrophic yeast; *Pichia pastoris*; antioxidative activity

INTRODUCTION

Superoxide dismutase (SOD) is a group of isozymes that function as superoxide radical scavengers. These enzymes protect cells from oxidative stress by catalyzing the dismutation of superoxide anion radicals ($O\bullet^-$) to oxygen (O_2) and hydrogen peroxide (H_2O_2), thereby maintaining a low intracellular concentration of superoxide anions (*1*, *2*). SODs are widespread in aerobic bacteria, eukaryotic cells, tissues, and organs such as the erythrocytes, liver, lung alveoli, heart, and brain (*3*). SODs are metalloenzymes and exist in three different forms in mammals depending on the type of metal ion they bind to and their cellular localization. Copper−zinc SOD (CuZnSOD) is found primarily in the cytoplasm and nucleus of cells (*4*), manganese SOD (MnSOD) is found in mitochondria (*5*), and Cu/Zn-containing extracellular SOD (ECSOD) is found predominantly in the extracellular matrix of tissues (*6*).

Much is known about the physical properties of the CuZn- and MnSODs, and both enzymes have been crystallized (*7*, *8*); however, native ECSOD has not yet been characterized in detail due to difficulties of purifying and crystallizing this enzyme (*9*). ECSOD is reported to be a multimeric glycoprotein composed of at least four identical 30 kD subunits in extracellular fluids with heterogeneous affinity for heparin (*6*). ECSOD exists in high concentrations in both systemic and pulmonary blood vessels and in airways and may be the dominant form of SOD in most arteries (*10*). It is present in both the adventitia and the smooth-muscle cells surrounding blood vessels and airways (*11*). ECSOD may be the most important first line of defense for protecting cell surfaces and extracellular matrix proteins from superoxide-mediated damage.

Oxidative stress, which is usually associated with increased formation of reactive oxygen species (ROS), causes lipid peroxidation and oxidation of thiol groups; these changes are believed to alter membrane permeability and configuration in addition to producing functional modification of various cellular proteins (*12*, *13*). Several studies have proposed that ROS play an essential role in the pathogenesis of myocardial ischemia-reperfusion injury (*14*, *15*). ROS, including hydrogen peroxide (H_2O_2), superoxide radical, hydroxyl radical, and peroxynitrate have been shown to increase upon reperfusion of the heart following ischemia (*16*, *17*). This increase was shown to be prevented by adding SODs to perfusion medium, indicating that antioxidants protected against alternations in the β-adrenore-

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10.1021/jf061379x CCC: $33.50
Published on Web 09/26/2006

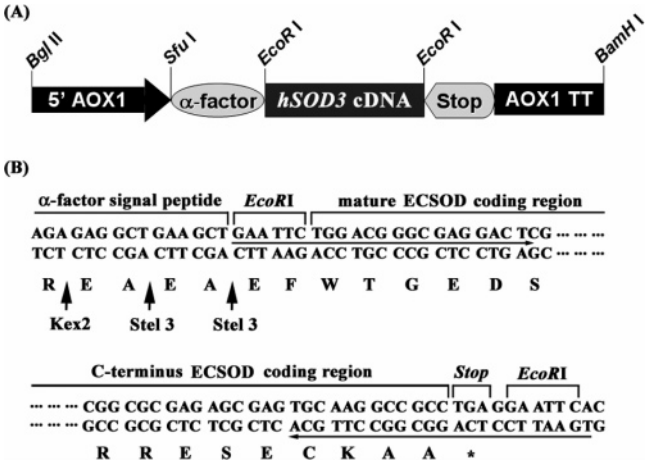

Figure 1. Structure of the *P. pastoris* α-factor/human ECSOD secretion cassette. (**A**) The *hSOD3* cDNA *Eco*RI fragment was amplified by PCR and cloned into pPICZαA flanking the α-factor leader sequence. (**B**) Generation of the ECSOD polypeptide. The sequences of the 5′ and 3′ primers are underlined. The lower line shows the predicted amino acid sequence with α-factor leader sequence and the cleavage sites of proteases *Kex2* and *Ste13* fused to the N-terminus of the mature ECSOD coding region.

ceptor linked signal transduction mechanism following ischemia-reperfusion (*18*). ROS have also been shown to initiate DNA single strand breakage, causing activation of the nuclear enzyme poly (ADP ribose) synthetase (PARS), which eventually leads to severe energy depletion of the cells and necrotic-type cell death. Treatment with SOD antioxidant inhibits the activation of PARS and prevents organ injury associated with acute and chronic inflammation (*19*).

Every biological macromolecule is a potential target for oxygen radicals, so there is interest in the therapeutic potential of ECSOD. A wide range of clinical applications has been suggested, including prevention of oncogenesis and tumor promotion (*20*, *21*), reduction of the cytotoxic and cardiotoxic effects of anticancer drugs, and protection against reperfusion damage of ischemic tissues (*22*, *23*).

The potential demand for ECSOD in human healthcare is great; therefore, large-scale production of biological active ECSOD is necessary. Production of therapeutic proteins by genetically engineered yeasts is a cost-effective alternative to tissue cultures or purification from animal tissues. In this study, we produced and purified human ECSOD with high antioxidative activity in the methylotrophic yeast, *Pichia pastoris*. Furthermore, we verified the bioactivity of overexpressed recombinant ECSOD by testing the transformed yeast culture for its resistance to oxidative stresses such as H_2O_2 and heat shock.

MATERIALS AND METHODS

Construction, Screening, and Selection of ECSOD Expression Plasmid Clones. A full-length *hSOD3* cDNA (GenBank accession number: NM_003102) encoding 240 amino acids of human ECSOD polypeptide (GenBank accession number: NP_003093) was isolated and cloned from a human aortic smooth muscle cDNA library (Uni-ZAP XR system, Stratagene, La Jolla, CA) by a three-round screening process. The recombinant 0.7 kb mature *hSOD3* cDNA fragment with a deleted 21-aa signal peptide was amplified by PCR with the following primers: pSOD3-*Eco*RI (foreword): 5′-GGAATTCTGGACGGGC-GAGGACT-3′ and pSOD3-*Eco*RI (reverse): 5′-TGAATTCCTCAG-GCGGCCTTGCA-3′ (underlined bases indicate the introduced restriction sites; **Figure 1B**). The PCR product was cloned into the pCRII vector by using a T/A Cloning Kit (Invitrogen, Carlsbad, CA), and the sequence was verified by nucleotide sequencing. The resulting pCRII-hSOD3 was digested with *Eco*RI (New England Biolabs, Beverly, MA) at the newly created sites, and the hSOD3 fragment was gel purified. The excised fragment was cloned as an *Eco*RI fragment flanking the α-factor signal peptide in the pPICZαA vector (Invitrogen). This construct was termed pPICZαA-hSOD3 (**Figure 1A**).

The pPICZαA-hSOD3 expression cassette was digested with *Sac*I prior to transformation into *P. pastoris*. For electroporation, a 50 mL culture of *P. pastoris* GS115 (*his4*$^-$) in YPD (10 g/L yeast extract, 20 g/L Bacto peptone, and 20 g/L dextrose) was grown at 30 °C until the $OD_{600} = 1.3-1.5$ ($\sim 5 \times 10^7$ cells/mL). Cells were washed twice and resuspended in 1.5 mL of ice-cold 1 M sorbitol. An aliquot of competent GS115 cells (100 μL) was mixed with 10 μg of linearized transforming DNA and transferred to an ice-cold 2 mm electroporation cuvette. The cells were pulsed according to the parameters for yeast (1500 V, 25 F, and 200 Ω) as previously described (*24*). The cells were incubated for 1 h at 30 °C and then spread on YPDS (YPD + 1 M sorbitol) containing 100 μg/mL Zeocin (Invitrogen). Transformants were visible after 2−3 days at 30 °C. Several hundred Zeocin-resistant transformants were identified during the first round of selection. The Mut (methanol utilization) phenotypes, Mut$^+$ and Mut^S, were confirmed by growth on MDH (minimal dextrose with 40 mg/L histidine) and MMH (minimal methanol with 40 mg/L histidine) selection media, respectively. In the second round, we selected for multi-copy transformants by plating 200 Mut^s transformants on media with 2 mg of Zeocin per mL. Seven transformants resistant to high levels of Zeocin were arbitrarily selected for further analyses.

Gene Copy Number Detection by Slot Blot Hybridization. Slot blot was prepared using standard protocol as described previously (*25*). Ten micrograms of genomic DNA samples was blotted onto the nitrocellulose membrane Immobilon-NC (Millipore, Bedford, MA) under vacuum sucking of Slot Blot Filtration Manifold (Hoefer Scientific Ins., San Francisco, CA). For DNA cross-linking, the filter was irradiated with UV using a UV-Stratalinker 1800 (Stratagene, La Jolla, CA) with an energy output of 120 mJ. A 0.7 kb fragment of the full-length *hSOD3* cDNA was prepared as a hybridization probe by the random-primed labeling procedure. Hybridization and washing were performed under high stringency conditions (70 °C) as described previously (*26*). After autoradiography, the filter was stripped and rehybridized with a 1.2 kb fragment of yeast glyceraldehyde 3-phosphate dehydrogenase gene (*G3PD*) probe that was used as an internal control. The exogenic *hSOD3* insertion copy was measured by a densitometer with a normalization based on the *G3PD* hybridization signal.

Methanol-Induced Expression of Human ECSOD in *P. pastoris*. A single colony of pPICZαA-hSOD3 transformant cells (clone no. 53) was inoculated in a 250 mL fillister-type flask containing 25 mL of BMGY broth (10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate (pH 6.0), 13.4 g/L yeast nitrogen base (YNB), 0.4 mg/L biotin, and 10 mL/L glycerol) and cultured while shaking (180 rpm) at 30 °C until the OD_{600} was 2−3 (approximately 36−48 h). The cells were harvested and resuspended to an $OD_{600} = 1.0$ in BMMY medium (10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate (pH 6.0), 13.4 g/L YNB, 0.4 mg/L biotin, and 5 mL/L methanol) to induce ECSOD expression. Absolute methanol was added to a final concentration of 0.5% every 24 h to maintain induction. Expression cultures were sampled at 12 h intervals beginning at 0 h and ending 96 h after methanol induction. To differentiate the expressed protein between ECSOD and alcohol oxidase 1 (AOX1), the supernatant was transferred to a new tube, and the cell pellet was subjected to SDS-PAGE and Western blotting for ECSOD protein expression determination (*27*). The supernatant and the cell pellets were stored at −80 °C until assayed.

Immunoblot Analysis of ECSOD Protein in Cell Pellet and Supernatant. Cell pellets were lysed with a lysis buffer (50 mM sodium phosphate (pH 7.4), 1 mM PMSF, 1 mM EDTA, and 50 mL/L glycerol) and acid-washed glass beads (size 0.5 mm; Sigma, St. Louis, MO). For assays of the extracellular secreted human ECSOD, the culture medium was collected, filtered (Centriprep-10, Amicon, Danvers, MA), and resolved on 12% SDS-PAGE. Proteins were electrotransferred from the gel to a nitrocellulose membrane (*28*). ECSOD was detected by a mouse anti-human ECSOD antibody (1:3000 dilution) and a goat anti-

mouse IgG second antibody conjugated with horseradish peroxidase (1:10 000 dilution; Santa Cruz Biotech. Inc., Santa Cruz, CA). The blot was developed with a chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech., Arlington Heights, IL). The amount of secreted ECSOD was determined quantitatively with an enzyme-linked immunosorbent assay (ELISA) as previously described (*29*).

Purification of ECSOD from Culture Medium. *P. pastoris* transformants were cultured in 2 L fillister-type flasks containing 500 mL of BMMY broth at 30 °C while shaking (180 rpm) and 60 h of methanol induction. Three liters of culture medium was concentrated and partially purified by stirred-cell ultrafiltration (YM-10, Amicon, Danvers, MA). The precipitate was resuspended in a 5 mM Tris buffer (pH 7.4) containing 50 mM NaCl and dialyzed against the same buffer. The desalted fractions were passed through a HiPrep 16/10 Heparin FF column and then separated by a fast protein liquid chromatography (FPLC) system (AKTA purifier 10, Amersham Pharmacia Biotech.) for large-scale purification. The fractions containing immunoreactive ECSOD were pooled and concentrated with a Centriplus system (Millipore, Bedford, MA).

Enzymatic Activity of ECSOD by NBT and WST Assays. ECSOD antioxidative activity was determined by the nitroblue tetrazolium (NBT) method as described previously (*30*). During illumination with fluorescent light, the gel became uniformly blue except at positions containing ECSOD. To quantify ECSOD activity, a microtiter plate assay using a water-soluble tetrazolium (WST-1) system was used (SOD Assay Kit-WST; Dojindo Laboratories, Kumamoto, Japan) with bovine erythrocyte SOD1 (Sigma) as the standard. Aliquots of the solution were immediately pipetted into 96-well flat-bottom microtiter plates containing three empty blanks, a range of concentrations of the SOD standard, and a range of concentrations of each FPLC-purified ECSOD sample. Rates of WST-1 reduction were measured at an OD_{450} with a microplate reader (*31*). Inhibition curves were drawn for each ECSOD solution, and the activity of each was calculated by comparing its 50% inhibition concentration (IC_{50}) to that of the standard SOD. All determinations of ECSOD activity were made in triplicate. The reproducibility of data was all within 10% deviation.

Oxidative Stress and Heat Shock Treatment. Yeast cells transformed with ECSOD were harvested from BMGY cultures before induction and after 36 h of methanol induction; they were then adjusted to a cell density of $OD_{600} = 1.0$ (3.5×10^7 cells/mL) in 100 mM potassium phosphate buffer (pH 7.4) containing a cytotoxic level of H_2O_2 (50 mM). Cell survival was monitored by taking samples at 15 min intervals, diluting them in 100 mM phosphate buffer (pH 7.4), and then plating the samples on YPD plates to obtain a viable count. Heat stress was induced by incubating cultures at 48 °C with samples taken every 15 min to determine cell viability. Wild type *P. pastoris* cells were used as a negative control. Plate counts were made in triplicate, and each experiment was repeated at least three times.

Statistical Analysis. Experimental values are expressed as the mean ± standard deviation (SD). Data were analyzed in the General Linear Models procedures of SAS (SAS Institute, Inc., Cary, NC). A difference between two means was considered statistically significant based on $p < 0.05$.

RESULTS

Cloning and Introduction of a Human *ECSOD* Gene into *P. pastoris*. We used a commercial synthetic secretion cassette with the *P. pastoris AOX1* promoter with an 89-residue prepro α-factor secretion signal sequence fused to the *AOX1* terminator. In this study, a 0.7 kb fragment from the full-length *hsod3* cDNA encoding human ECSOD polypeptide was isolated from a human aortic smooth muscle cDNA library. The *hsod3* cDNA clone was reconstructed by deleting the 21-aa signal peptide from its N-terminus and then cloned as a fragment flanking the α-factor signal peptide and the 3′-*AOX1* terminator sequence (**Figure 1A**). After introducing the *ECSOD* gene into *P. pastoris*, a Zeocin-resistant gene was used as the selectable marker in the screening processes. In this yeast expression system, the 89-aa signal peptide from the α-mating factor was used for secretion of ECSOD, and the cleavage sites of proteases *Kex2* and *Ste13* (**Figure 1B**) were designed to allow proper processing of the heterologously expressed precursor protein and to release the mature ECSOD into the culture medium.

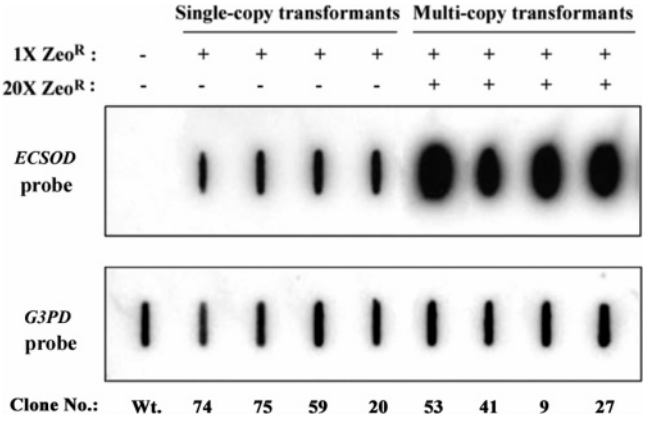

Figure 2. Determination of multi-copy insertion of ECSOD gene in transformants of *P. pastoris* by slot blot hybridization. A 0.7 kb fragment of the full-length hSOD3 cDNA was used as a hybridization probe (upper panel) and a 1.2 kb fragment of yeast glyceraldehyde 3-phosphate dehydrogenase (G3PD) gene was used as an internal control. The amount of input DNA was normalized based on the G3PD hybridization signal. Wt, a wild type yeast clone (GS115) was used as a negative control. The symbol + represents yeast cells resistant to Zeocin selection, and − represents yeast cells sensitive to Zeocin selection. ECSOD transformants 9, 27, 41, and 53 were identified as potential multi-copy transformants based on resistance to high levels (2 mg/mL) of Zeocin. Results are representative of two experiments.

Identification of Multi-Copy Insertions of *ECSOD* Expression Cassette in the *P. pastoris* Genome. Among several hundred transformants isolated in primary screening with 1× Zeocin (0.1 mg/mL), 200 colonies were rescreened to identify seven multi-copy transformation events detected as resistant to Zeocin at 2 mg/mL. The presence of multi-copy insertions of the *ECSOD* expression cassette was confirmed by slot blot hybridization with the 0.7 kb *hSOD3* cDNA fragment as a probe. A 1.2 kb fragment from the yeast glyceraldehyde 3-phosphate dehydrogenase (*G3PD*) gene was used as an internal control. Single copy transformants (clone nos.74, 75, 59, and 20, **Figure 2**) survived at low levels of Zeocin (1× Zeo) but not at the high level. A few transformants with multi-copy insertions (>5 copies per cell) of the *ECSOD* expression cassette in their genomes (e.g., transformant nos. 9, 27, 41, and 53 (**Figure 2**)) grew on the 20× Zeo selection plate. Transformant no. 53 had the most copies of the *ECSOD* expression cassette and grew best on media with high levels of Zeocin; therefore, it was selected for further protein expression and functional assays.

Production and Secretion of Human ECSOD Protein in *P. pastoris* Culture Medium. Cells of transformant no. 53 were grown to $OD_{600} = 3$ in BMGY with 10 mL/L glycerol as the carbon source and then shifted to a limited methanol condition (BMMY with 5 mL/L methanol). Aliquots of the culture broth were taken every 12 h for 96 h. The expression level and accumulation of ECSOD protein in the transformed yeast cells were significantly elevated on SDS-PAGE (**Figure 3A**) and Western blot (**Figure 3B**). The highest level of ECSOD expression was reached as much as 25% of the total protein at 36−60 h after methanol induction. The secretion efficiency of the ECSOD protein attributable to the α-factor signal peptide was determined by measuring the secretion of a 70 kD protein in the supernatant of the yeast culture (**Figure 4A**). The amount

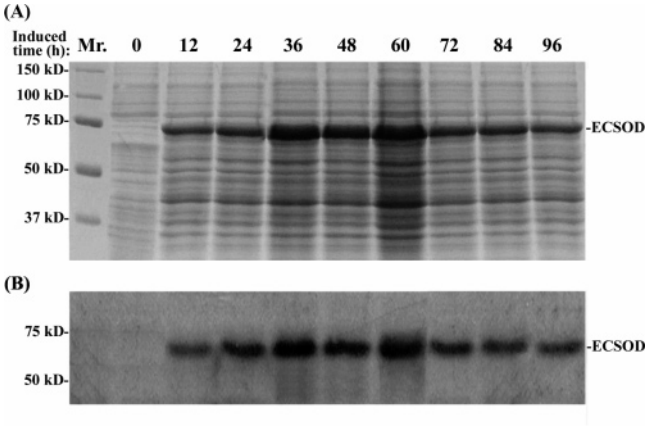

Figure 3. Intracellular ECSOD expression profile of transformant 53. (**A**) SDS-PAGE analysis of cell lysate of cells induced with methanol for the time shown. The protein gel was stained with Coomassie Blue. Dimer formation by the expressed ECSOD monomer occurs after 12 h of methanol induction. (**B**) Western blot analysis of intracellular ECSOD expression with a mouse anti-human ECSOD monoclonal antibody. The highest level of ECSOD expression reached as much as 25% of the total protein at 36–60 h after methanol induction. Results are representative of three experiments.

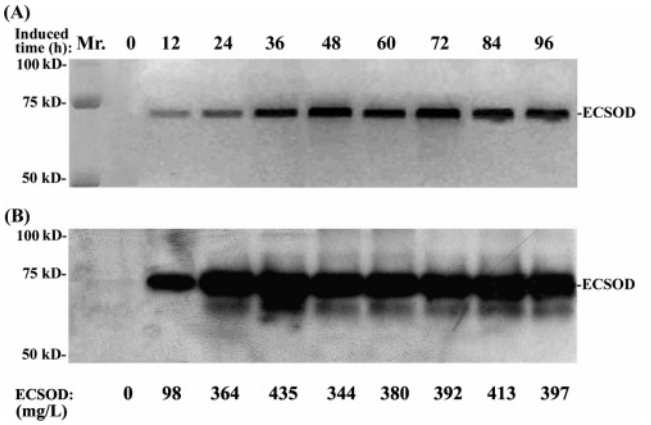

Figure 4. ECSOD secretion profile of transformant 53 in culture medium. (**A**) SDS-PAGE analysis of concentrated supernatant of the yeast culture induced by methanol for the time shown. The secreted ECSOD protein appears as a major band after 24 h of methanol induction. (**B**) Western blot analysis of secreted ECSOD with a mouse anti-human ECSOD monoclonal antibody. The amount of ECSOD secreted from the transformant cells was determined quantitatively by ELISA. Results are representative of three experiments.

of secreted dimeric ECSOD protein (70 kD) was further quantified by Western blot and ELISA 12 h after methanol induction; by 36 h post-induction, it had reached 435 mg/L in culture medium (**Figure 4B**). ECSOD secretion remained constant through days three and four, so the yeast cultures were harvested at 36 h post-methanol induction for analysis of optimal ECSOD yield.

Antioxidative Activity of Human ECSOD from *P. pastoris* Supernatant. Supernatants from cultures of yeast transformants were concentrated, separated on a nondenaturing polyacrylamide gel, and assayed by NBT staining for SOD activity. Serial dilutions of bovine SOD1 were used as a positive control (**Figure 5**). A significant level of SOD activity was detected in culture media in which *P. pastoris* transformants carrying the *ECSOD* gene had been grown (transformant nos. 41, 53, and 59) but not in media in which either wild type or a strain

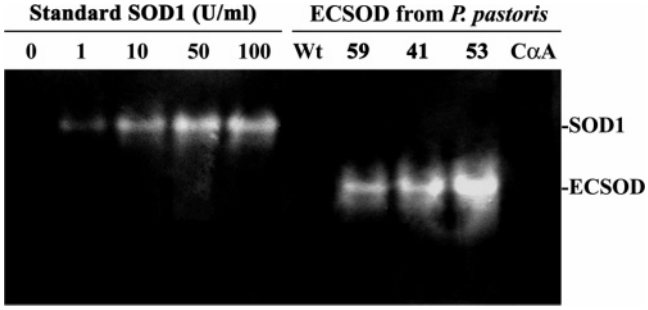

Figure 5. SOD activity was measured by gel electrophoresis of secreted ECSOD. Protein extracts were resolved on a 10% nondenaturing polyacrylamide gel. The first five lanes contain different levels of a commercial bovine SOD1 protein. The last five lanes contain proteins from culture media of wild type (WT), three ECSOD transformants, and an empty vector transformed yeast (CαA).

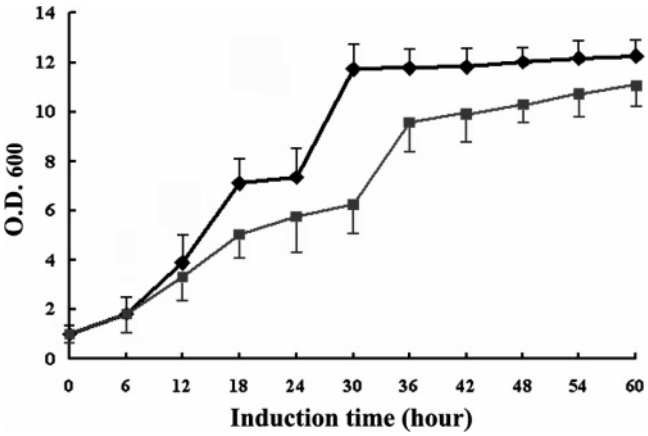

Figure 6. Cell growth of wild type and transformant 53 during late logarithmic phase in BMMY after methanol induction and expression of ECSOD. ◆: Transformant 53 and ■: wild type. The assays were performed in triplicate.

transformed with just the vector (CαA) had been grown. The highest antioxidative activity was detected in lane 9, which contained transformant no. 53 and that was sampled 36 h after methanol induction.

ECSOD activity units were determined through a WST-1 microtiter quantitative assay. ECSOD derived from transformant no. 53 was purified through a HiPrep heparin affinity column by FPLC. The standard inhibition curve was plotted by measuring serial concentrations of bovine SOD1 in a total reaction volume of 200 μL. The IC_{50} of the purified ECSOD was 0.15 μg/mL in the assay solution, with an antioxidative activity of 760 $\pm$ 20 U/mg protein in extracellular culture medium.

Growth Rate in ECSOD-Expressing Transformant and Wild Type *P. pastoris*. Wild type and transformed *P. pastoris* cultures were transferred to methanol induction medium for ECSOD expression and cultured continuously for 3 days to evaluate cell growth performance in the late exponential and stationary phases. Growth curves were constructed from data obtained by measuring growth of wild type and transformed yeast cells every 6 h (**Figure 6**). By 18 h, the OD_{600} of the cells expressing ECSOD exceeded that of the wild type strains ($P < 0.05$). The growth rate during the late logarithmic phase accelerated, and the maximal cell concentration for the transformant ($OD_{600} = 12$) was reached 30 h after methanol induction. The wild type culture had lower logarithmic phase growth and reached the stationary phase ($OD_{600} = 10$) at 36 h post induction.

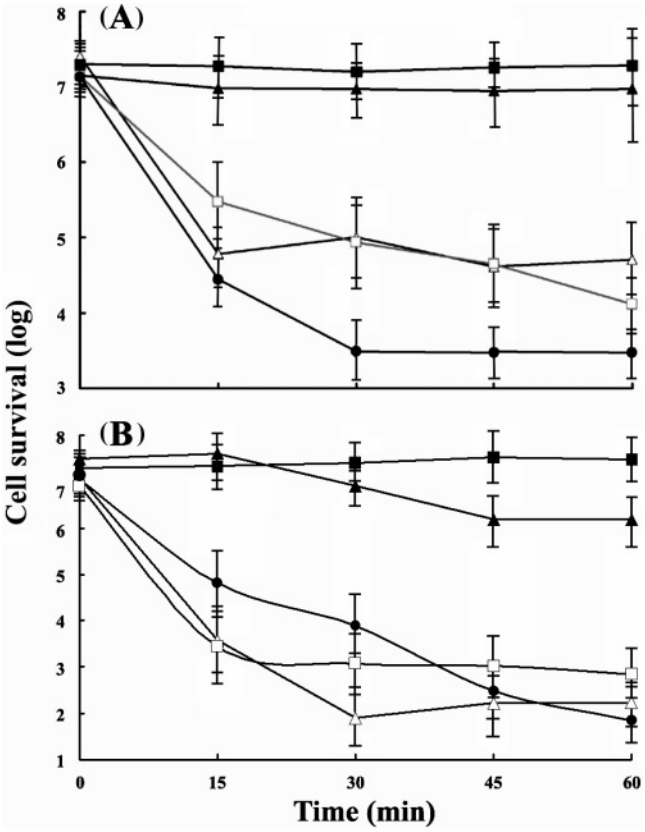

Figure 7. Cell viability as a measure of resistance to oxidative stress in transformed yeast cells before and after ECSOD expression. Cells were treated with (**A**) 50 mM H_2O_2 or (**B**) heat shock (48 °C). ▲: Transformant 41/BMMY; Δ: transformant 41/BMGY; ■: transformant 53/BMMY; □: transformant 53/BMGY; and ●: wild type/BMMY. The assays were performed in triplicate.

ECSOD Expression and Resistance to Heat Shock and Oxidative Stress. Early exponential phase cultures, OD_{600} = 1.0, were very sensitive to H_2O_2 concentrations >20 mM and to temperatures >45 °C. In the oxidative stress tolerance study (**Figure 7**), a cytotoxic level of 50 mM H_2O_2 was used to test the viability of transformants 41 and 53 before and after ECSOD expression. The number of viable cells dropped at least 2−3 orders of magnitude during the first 30 min in the wild type yeast and in both of the uninduced transformants ($P < 0.05$). The protective effect of expressed ECSOD was observed in both of the transformants after 36 h of methanol induction (**Figure 7A**). The ECSOD-secreting transformants also maintained a steady-state survival rate during the entire heat shock treatment period when comparing with wild type and uninduced transformants ($P < 0.05$), indicating that these transformants were highly resistant to heat stress (**Figure 7B**).

DISCUSSION

P. pastoris is uniquely suited for expressing transgenic proteins due to its ability to grow rapidly on simple media. Production of heterologous proteins can be regulated by using the *AOX1* promoter from *P. pastoris* and methanol as an inducer (*24*, *32*). The heterologous protein may constitute as much as one-third of the total soluble protein (*33*). Addition of an 89-aa signal peptide from α-mating factor with *Kex2* and *Ste13* protease cleavage sites (**Figure 1**) also can be used to increase the secretion and release of the heteroloous protein into the culture medium. Screening for increased heterologous protein expression by using the extent of Zeocin resistance also is an efficient technology for identifying strains with the potential to express even higher levels of the exogenous protein.

This is the first paper to express human ECSOD in *P. pastoris* yeast culture system. The most significant aspect of this ECSOD production system is the high level of ECSOD expression in cell pellets (**Figure 3**) and in culture media (**Figure 4**). These high levels of expression were attained in shake flask cultures and also in fermenters in our following study. The dominant form of ECSOD produced by *P. pastoris* was a dimer with a molecular weight of 70 kD (**Figure 4**), as has been previously described for native and heterologously produced enzyme (*6*). The mammalian CuZn-containing ECSOD dimer is an unusually stable protein. It is more thermostabile than any other characterized globular protein, retains activity in 8 M urea, and is not dissociated by 4% SDS, and its electrophoretic pattern is not altered by boiling for 30 min (*6*, *34*), indicating that a covalent interaction is present.

The metabolic generation of ROS can affect cellular life span; for example, in fungi, mutants defective in ROS detoxification have limited viability during the stationary phase (*35*). We found that *P. pastoris* transformants expressing ECSOD were resistant to heat shock stress. The relationship between oxidative stress and heat shock in eukaryotic systems is currently under intense investigation (*36*, *37*). Most studies of the correlation between oxidative stress and heat shock have focused on the expression of genes coding for antioxidant defenses and cellular signaling mechanisms (*38*). Induction of ECSOD may be very important because higher ECSOD levels in the cell and in the culture medium may protect cells from heat shock and oxidative stress.

Transformed cells overexpressing ECSOD also were resistant to oxidative stress induced by 50 mM H_2O_2 (**Figure 7**). Other H_2O_2 metabolizing enzymes, such as glutathione peroxidase and catalase, are located intracellularly, so ECSOD may be critically important in decomposing H_2O_2 in the extracellular space. This property could be important in the highly oxidative environments of yeast culture and the mammalian vascular system. Several previous reports have shown that expression of ECSOD in the human vascular system is highly regulated in pathophysiological and physiological conditions, including atherosclerosis (*39*, *40*), angiotensin II-induced hypertension (*41*), and exercise training (*42*). We speculate that under those conditions, increased ECSOD expression not only reduces interstitial $O_2^{\bullet -}$ but also minimizes interstitial H_2O_2 levels.

Although there are many nonenzymatic anti-oxidants (e.g., α-tocopherol, ascorbate, and glutathione), the most efficient way to eliminate ROS is by catalysis with antioxidant enzymes (*41*). The metalloenzyme superoxide dismutases (SODs) are the primary enzymes involved in cellular ROS clearance, and interstitial ECSOD may be an important first line of defense that protects cell surfaces and the extracellular matrix proteins from superoxide-mediated damage.

In conclusion, we have successfully expressed and secreted human ECSOD in *P. pastoris* cells in a methanol-induced system. The antioxidative activity of 760 ± 20 U/mg ECSOD from culture supernatant and the effects of ECSOD on reducing heat shock and oxidative stresses indicate that human ECSOD produced by *P. pastoris* has multiple biological functions, including antioxidative ability against reactive oxygen species, enhancing high-density cell growth, and protecting cells from oxidative damage. Further investigation of ECSOD produced by *P. pastoris* is required to determine if this enzyme has potential clinical, healthy food, or feedstuff additive uses.

ABBREVIATIONS USED

AOX1, alcohol oxidase 1 gene; BMGY, buffered complex medium-containing glycerol; BMMY, buffered complex medium-containing methanol; ECSOD, extracellular superoxide dismutase; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; *G3PD*, glyceraldehyde 3-phosphate dehydrogenase gene; H_2O_2, hydrogen peroxide; Mut, methanol utilization phenotype; PARS, poly (ADP ribose) synthetase; ROS, reactive oxygen species; YNB, yeast nitrogen base; WST-1, water-soluble tetrazolium.

ACKNOWLEDGMENT

We thank Prof. James D. Crapo of the Department of Medicine, National Jewish Medical and Research Center, Denver, CO, for providing us with a mouse anti-human ECSOD monoclonal antibody and Dr. Jeffrey Conrad for critically reading the manuscript. We also thank Mr. Chung-Yu Lin for his assistance in gene construction.

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Received for review May 16, 2006. Revised manuscript received August 13, 2006. Accepted August 21, 2006. This research was supported in part by Grants NSC-92-2313-B-212-011 and NSC-93-2313-B-005-023 from the National Science Council, Republic of China, and Grant COA-93-4.1.3-U1(3) from the Council of Agriculture.

JF061379X