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## Cloning of Active Human Manganese Superoxide Dismutase and its Oxidative Protection in *Escherichia coli*

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**Abstract:** Superoxide radical ( $O_2^{\bullet-}$ ) is a toxic byproduct of oxidative metabolism that extensively damages cellular macromolecules and organelles. Superoxide dismutase (SOD) catalyzes the conversion of superoxide radical to hydrogen peroxide ( $H_2O_2$ ) and molecular oxygen ( $O_2$ ) thus providing a biological defense against oxygen toxicity. The structural gene of human manganese superoxide dismutase (hMnSOD) was successfully cloned into the pET46Ek/LIC by using a Ligation Independent Cloning (LIC) technique. The recombinant human MnSOD was expressed in *E. coli* strain BL21(DE3)pLysS and purified to homogeneity by  $Ni^{2+}$ -NTA. Supplementation of  $Mn^{2+}$  in the bacterial growth media was proven to be crucial for production of enzymatically active hMnSOD. The recombinant enzyme revealed a specific activity up to  $2,857\text{ U mg}^{-1}$  as measured by inhibition of photoreduction of nitroblue tetrazolium (NBT). The molecular weight of each subunit was estimated to be 22 kDa by SDS-PAGE. More interestingly, *E. coli* expressing hMnSOD provides resistance against oxidative stress induced by the herbicide paraquat up to 1.2 mM. These findings gain insights into the biochemical characterization and significant roles of oxidative-protection of the hMnSOD in biological systems.

**Key words:** Human manganese superoxide dismutase (hMnSOD), paraquat, gene cloning, oxidative scavenging enzyme

### INTRODUCTION

Reactive Oxygen Species (ROS) such as superoxide anion ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $OH^{\bullet}$ ) are generated as byproducts during the normal course of aerobic metabolism (Fridovich, 1978). These ROS are accumulated once the organisms have been exposed to various biochemical activation, radiation and stress agents (Halliwell and Gutteridge, 1999). When production of ROS exceeds the rate of their degradation, oxidative stress or oxidative damage will occur to various biological macromolecules especially DNA, RNA, protein and membrane lipids. These oxidative damages, if not repaired, place the organism at risk and consequently lead to several pathological conditions (Sorg, 2004; Opara, 2006).

Superoxide dismutases (SODs) are the first and the most important line of antioxidant enzymes. The SODs can be classified into four types concerning their metal cofactors, copper-zinc type (Cu/ZnSOD), manganese type (MnSOD), iron type (FeSOD) and nickel type (Ni-SOD) (Hassan, 1989; Youn *et al.*, 1996). At present, three

distinct isoforms of SOD have been identified in mammals. Two isoforms contain Cu and Zn in their catalytic center and are localized in the cytoplasmic compartments (Cu/ZnSOD or SOD1) or at the extracellular elements (ECSOD or SOD3). Another isoform of SODs has manganese (Mn) as a cofactor and remains in the mitochondria of eukaryotic cells (MnSOD or SOD2). This active enzyme is a homotetramer composed of 22 kDa subunits each containing one manganese atom (Zelko *et al.*, 2002).

The therapeutic potential of SODs for treatment of oxidative damage has provoked considerable interest. SODs have been proposed as clinically useful for a wide variety of applications e.g., oncogenesis (Oberley, 2001) cardiovascular disease (Besse *et al.*, 2006) ischemia injury (Salvemini and Cuzzocrea, 2002), aging (Levin, 2005), neurodegenerative disorders (Pong, 2003) and inflammatory diseases (Yasui and Baba, 2006). In fact, many research groups are currently evaluating the therapeutic potential of Cu/ZnSOD by several clinical studies (Lakshminrusimha *et al.*, 2006; Nakamura and Ogawa, 2001; Riedl *et al.*, 2005; Yunoki *et al.*, 2003). In

spite of this, a major drawback from the shorter half-life (6-10 min) as compared to that of the MnSOD (5-6 h) may limit its applicability, especially in chronic diseases (Baret *et al.*, 1984; Gorecki *et al.*, 1991). Therefore, many attempts have been geared towards the evolutionary and functional significance of MnSOD as a defense mechanism for oxygen toxicity (Bowler *et al.*, 1990; Purrello *et al.*, 2005; Frealle *et al.*, 2006; Cole *et al.*, 2006). However, explorations on its clinical potential and other molecular characterizations are of limited use due to the unavailability of the enzyme molecule.

Therefore, in the present study, the human manganese superoxide dismutase (hMnSOD) has been cloned and expressed in *E. coli*. Purification and characterization of recombinant protein has been performed. Discovery of the protection effect against oxidative stress of the engineered cells has been carried out. This will provide a greater understanding for future prospects in biotechnological and medical fields.

## MATERIALS AND METHODS

**Materials:** Plasmid pET46Ek/LIC, *E. coli* host strain Novablue (*endA1 hsdR17* ( $r_{K12}^- m_{K12}^+$ ) *supE44 thi-1 recA1 gyrA96 relA1 lac* [ $F'$  *proA*<sup>+</sup>*B*<sup>+</sup> *lacZ*<sup>Δ</sup>*M15::Tn10* (Tc<sup>R</sup>)] and other enzymes required for cloning procedure were supplied as a Ek/Lic Cloning kit (Novagen, EMD Biosciences, Darmstadt, FRG). *E. coli* strain BL21(DE3)pLysS ( $F^- ompT hsdS_B$  ( $r_B^- m_B^-$ ) *gal dcm* (DE3) *pLysS* (Cm<sup>R</sup>)) used for protein expression was also obtained from Novagen. All other chemicals and reagents were of analytical grade.

### PCR amplification and construction of pET46MnSOD plasmid:

Construction of a gene encoding human manganese superoxide dismutase (hMnSOD; NCBI accession No. Y00472) was performed by the aid of a polymerase chain reaction and a ligation independent cloning (LIC) technique (Aslanidis and de Jong, 1990). The forward primer (5'-GACGACGACAAGATGAAGCA CAGCCTCCCCGACC-3') and reverse primer (5'-GAGGAG AAGCCCGGTTTATTACTTTTTCAGCCATGTATC-3') were designed to contain adapters for the LIC vector (shown as underlined text). The mature hMnSOD coding sequence, starting with AAG encoding the NH<sub>2</sub> terminal Lysine and terminating with the TAA stop codon, was amplified. The PCR product was subsequently treated with T<sub>4</sub> DNA polymerase (2.5 U  $\mu$ L<sup>-1</sup>) in the presence of dATP to generate specific single-stranded overhang at both termini. The product was further annealed at 22°C with linearized pET46Ek/LIC vector

containing a compatible site with the treated PCR product, generating the pET46MnSOD. The annealing product was transformed into cloning host (*E. coli* strain Novablue) and plated on LB agar containing ampicillin 100  $\mu$ g mL<sup>-1</sup>.

### Overexpression and purification of recombinant human MnSOD:

For expression analysis, the *E. coli* strain BL21(DE3)pLysS, harbouring the pET46MnSOD, was grown in LB-medium supplemented with 100  $\mu$ g mL<sup>-1</sup> ampicillin and 34  $\mu$ g mL<sup>-1</sup> chloramphenicol, on a rotary shaker (180 rpm) at 37°C. When the OD<sub>600</sub> of the culture reached a value of 0.5, isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) and Mn<sup>2+</sup> were simultaneously added (at a final concentration of 1 mM and 200 ppm, respectively), then, the culture was grown for another 4 h. The culture was harvested by centrifugation at 6000 rpm, washed and resuspended in 50 mM Tris-HCl buffer, pH 7.8, followed by sonication for cell breakage. The supernatant and pellet fractions were separated by centrifugation at 18,000 rpm for 10 min at 4°C and then analysed for protein expression by SDS-PAGE on 12% gels as described by Laemmli (1970). For purification of the recombinant protein, the cell extract was loaded onto Ni-NTA IMAC column (pre-equilibrated with 50 mM Tris-HCl, pH 7.8) and eluted with the same buffer containing 0.5 M imidazole. Fractions possessing the SOD activity were pooled, dialysed overnight against 50 mM potassium phosphate buffer, pH 7.8, containing 200 ppm Mn<sup>2+</sup> and used for further analysis.

**SOD activity assay:** The SOD activity was assayed by the ability of SOD to inhibit riboflavin-mediated photochemical reduction of nitroblue tetrazolium (NBT). Screening of SOD activity was performed by the gel electrophoresis method (Beauchamp and Fridovich, 1971). Total bacterial extract or soluble extract was run onto native PAGE gels. The gel was washed in 2.45 mM nitroblue tetrazolium (NBT) solution for 20 min in the dark and then incubated in a solution containing 28 mM TEMED, 0.028 mM riboflavin and 36 mM potassium phosphate, pH 7.8, for an additional 15 min in the dark with gentle shaking. The gel was subsequently illuminated with fluorescent light and the presence of SOD activity corresponding to achromatic zones in a uniformly deep blue background was detected. The assay for SOD specific activity was slightly modified from that originally described by Beyer and Fridovich (1987). One unit of SOD activity was defined as the amount of enzyme that caused 50% decrease in NBT reduction.

**SOD inhibitor assays:** Crude protein extracts of *E. coli* expressing hMnSOD were loaded onto a native PAGE gel at the final amounts of 20 µg per well and further separated under electric field. The gel was cut into multiple pieces and each was subsequently soaked for 20 min at 25°C in 50 mM potassium phosphate buffer, pH 7.8 containing 3 mM KCN, 5 mM H<sub>2</sub>O<sub>2</sub> or 50 mM NaN<sub>3</sub>. Finally, these gels were subjected to SOD activity assay as described earlier.

**Response of engineered *E. coli* to oxidative stress:** Response against oxidative stress of engineered *E. coli* was investigated by monitoring the survival rate in the presence of a superoxide anion generator. Briefly, overnight cultures of BL21(DE3)pLysS cells transformed either with pET46 control vector or with pET46MnSOD plasmid were grown in fresh LB-medium containing 100 µg mL<sup>-1</sup> ampicillin and 50 µg mL<sup>-1</sup> chloramphenicol under continuous shaking at 37°C. When the OD<sub>600</sub> reached a value of 0.25, the desired concentrations of paraquat (0-1.6 mM) and 1 mM IPTG were simultaneously added. Cultivations were continued for 12 h and the optical density at 600 nm was measured. Growth profiles under oxidative stress were also measured at different time intervals upon addition of 0.6 and 1.0 mM paraquat.

## RESULTS

**Cloning of gene encoding human Mn-superoxide dismutase (hMnSOD):** A gene encoding hMnSOD was successfully constructed by using a polymerase chain reaction in conjunction with ligation independent cloning (LIC). The amplified product (626 bp) carrying a properly designed adaptors was cloned into the pET46Ek/LIC vector where expression of recombinant protein was tightly controlled by an IPTG-regulated promoter. Verification of the MnSOD gene by PCR and restriction endonuclease analysis confirmed the correct orientation in all of the selected transformants (n = 7). Results from DNA sequencing revealed the exact order of nucleotide bases of the recombinant gene (Fig. 1).

**Overexpression and purification of the recombinant hMnSOD:** Expression of recombinant hMnSOD was performed in *E. coli* strain BL21(DE3)pLysS. Induction of the gene took place for 4 h upon addition of IPTG into the LB medium. The overexpressed recombinant enzyme was further identified by electrophoresis on 12% SDS-polyacrylamide gel. Present findings revealed a high level expression of approximately 22 kDa recombinant protein in both the crude supernatant and the pellet

	Hexahistidine										EK cleavage site										
	1	ATG	GCA	CAT	CAC	CAC	CAC	CAT	CAC	GTG	GAT	GAC	GAC	GAC	AAG	ATG					45
	1	Met	Ala	<u>His</u>	<u>His</u>	<u>His</u>	<u>His</u>	<u>His</u>	<u>His</u>	Val	<u>Asp</u>	<u>Asp</u>	<u>Asp</u>	<u>Asp</u>	<u>Lys</u>	Met					15
	46	AAG	CAC	AGC	CTC	CCC	GAC	CTG	CCC	TAC	GAC	TAC	GGC	GCC	CTG	GAA					90
	16	Lys	His	Ser	Leu	Pro	Asp	Leu	Pro	Tyr	Asp	Tyr	Gly	Ala	Leu	Glu					30
Start Mature hMnSOD	91	CCT	CAC	ATC	AAC	GCG	CAG	ATC	ATG	CAG	CTG	CAC	CAC	AGC	AAG	CAC					135
	31	Pro	His	Ile	Asn	Ala	Gln	Ile	Met	Gln	Leu	His	His	Ser	Lys	His					45
	136	CAC	GCG	GCC	TAC	GTG	AAC	AAC	CTG	AAC	GTC	ACC	GAG	GAG	AAG	TAC					180
	46	His	Ala	Ala	Tyr	Val	Asn	Asn	Leu	Asn	Val	Thr	Glu	Glu	Lys	Tyr					60
	181	CAG	GAG	GCG	TTG	GCC	AAG	GGA	GAT	GTT	ACA	GCC	CAG	ATA	GCT	CTT					225
	61	Gln	Glu	Ala	Leu	Ala	Lys	Gly	Asp	Val	Thr	Ala	Gln	Ile	Ala	Leu					75
	226	CAG	CCT	GCA	CTG	AAG	TTC	AAT	GGT	GGT	GAT	ATC	AAT	CAT	AGC						270
	76	Gln	Pro	Ala	Leu	Lys	Phe	Asn	Gly	Gly	Gly	His	Ile	Asn	His	Ser					90
	271	ATT	TTC	TGG	ACA	AAC	CTC	AGC	CCT	AAC	GGT	GGT	GGA	GAA	COC	AAA					315
	91	Ile	Phe	Trp	Thr	Asn	Leu	Ser	Pro	Asn	Gly	Gly	Gly	Glu	Pro	Lys					105
	316	GGG	GAG	TTG	CTG	GAA	GCC	ATC	AAA	CGT	GAC	TTT	GGT	TCC	TTT	GAC					360
	106	Gly	Glu	Leu	Leu	Glu	Ala	Ile	Lys	Arg	Asp	Phe	Gly	Ser	Phe	Asp					120
	361	AAG	TTT	AAG	GAG	AAG	CTG	ACG	GCT	GCA	TCT	GTT	GGT	GTC	CAA	GGC					405
	121	Lys	Phe	Lys	Glu	Lys	Leu	Thr	Ala	Ala	Ser	Val	Gly	Val	Gln	Gly					135
	406	TCA	GGT	TGG	GGT	TGG	CTT	GGT	TTC	AAT	AAG	CAA	CGG	GGA	CAC	TTA					450
	136	Ser	Gly	Trp	Gly	Trp	Leu	Gly	Phe	Asn	Lys	Gln	Arg	Gly	His	Leu					150
	451	CAA	ATT	GCT	GCT	TGT	CCA	AAT	CAG	GAT	CCA	CTG	CAA	GGA	ACA	ACA					495
	151	Gln	Ile	Ala	Ala	Cys	Pro	Asn	Gln	Asp	Pro	Leu	Gln	Gly	Thr	Thr					165
	496	GGC	CTT	ATT	CCA	CTG	CTG	GCG	ATT	GAT	GTG	TGG	GAG	CAC	GCT	TAC					540
	166	Gly	Leu	Ile	Pro	Leu	Leu	Gly	Ile	Asp	Val	Trp	Glu	His	Ala	Tyr					180
	541	TAC	CTT	CAG	TAT	AAA	AAT	GTC	AGG	CCT	GAT	TAT	CTA	AAA	GCT	ATT					585
	181	Tyr	Leu	Gln	Tyr	Lys	Asn	Val	Arg	Pro	Asp	Tyr	Leu	Lys	Ala	Ile					195
	586	TGG	AAT	GTA	ATC	AAC	TGG	GAG	AAT	GTA	ACT	GAA	AGA	TAC	ATG	GCT					630
	196	Trp	Asn	Val	Ile	Asn	Trp	Glu	Asn	Val	Thr	Glu	Arg	Tyr	Met	Ala					210
	631	TGC	AAA	ARG	TAA																642
	211	Cys	Lys	Lys	End																

Fig. 1: A complete sequence of recombinant DNA encoding human Mn-superoxide dismutase (hMnSOD). Amino acid sequence of mature hMnSOD without mitochondrial leading sequence is shown in bold. (Note: sequences of hexahistidine and enterokinase (EK) cleavage site are shown as underlined text)

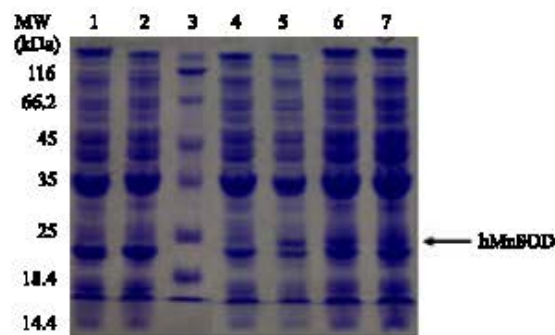


Fig. 2: SDS-PAGE analysis of proteins expressed in *E. coli* BL21(DE3)pLysS containing the pET46 control plasmid or pET46MnSOD plasmid. Lanes 1 and 2 represent supernatant and pellet fractions of pET46 transformed cells. Lane 3 is molecular weight markers. Supernatant and pellet fractions of pET46MnSOD transformed cells grown in the absence (Lanes 4 and 5) and presence (Lanes 6 and 7) of 200 ppm  $Mn^{2+}$  are shown

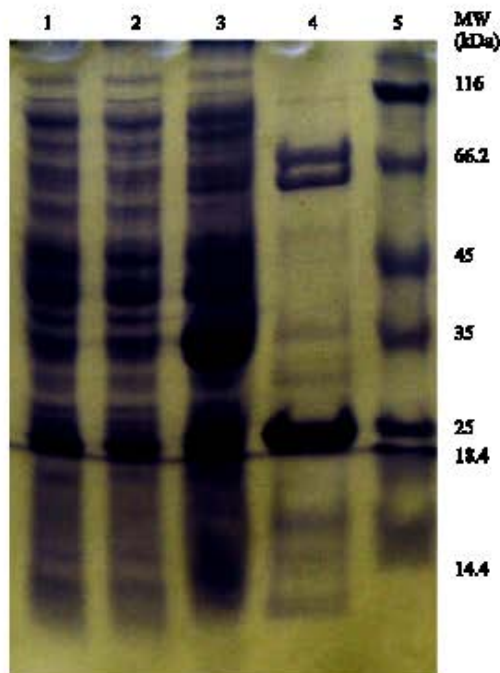


Fig. 3: SDS-PAGE representing the purification profile of the hMnSOD. Lane 1, crude extract; Lane 2, flow through; Lane 3, pellet; Lane 4, purified protein and Lane 5, molecular weight markers

fractions (Fig. 2, lanes 4 and 5). However, it should be noted that the majority of the expressed MnSOD was non-soluble and remained in the cellular pellet.

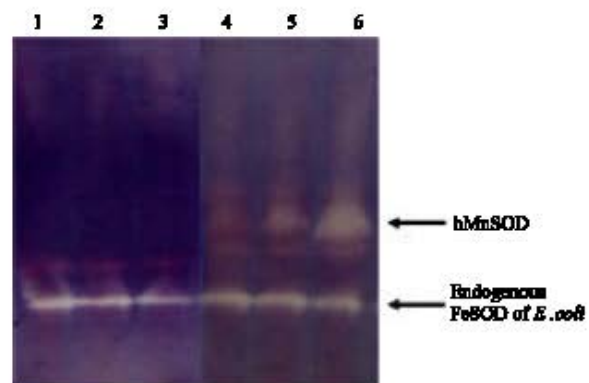


Fig. 4: SOD activity gel: Lanes 1-3, *E. coli* harbouring control plasmid; Lanes 4-6 *E. coli* expressing human MnSOD. Lanes 1 and 4 non induced; Lanes 2 and 5, induced with 1 mM IPTG; Lanes 3 and 6, induced with 1 mM IPTG and supplemented with 200 ppm  $Mn^{2+}$

Supplementation of the culture medium with 200 ppm of  $Mn^{2+}$  resulted in increased solubility of recombinant protein (Fig. 2, lane 6). The overexpressed protein was then purified to homogeneity by Ni-NTA agarose IMAC column as judged by SDS-PAGE analysis (Fig. 3).

**Catalytic activity of the recombinant hMnSOD:** To determine whether recombinant hMnSOD was synthesized in an active form, crude protein extracts were analyzed by using native polyacrylamide gel electrophoresis (PAGE). The recombinant hMnSOD displayed a remarkable high catalytic activity (upper band) and did not co-migrate with the endogenous FeSOD from the *E. coli* cells (lower band) (Fig. 4). Moreover, supplementation with  $Mn^{2+}$  resulted in a high production of hMnSOD (Fig. 4, lane 6), which was consistent with the earlier results (Fig. 2).

To further differentiate the hMnSOD from endogenous bacterial SOD, inhibition assays of SOD activity by various inhibitors were performed. It has been widely established that azide is an inhibitor of MnSOD and FeSOD, while hydrogen peroxide exerts a potent inhibitory effect on the FeSOD and Cu/ZnSOD. In addition, KCN is known to inhibit the activity of Cu/ZnSOD. From our findings, both the hMnSOD and the endogenous FeSOD were not inhibited by KCN (Fig. 5, lane 2). In the presence of hydrogen peroxide, only the hMnSOD could retain its activity (Fig. 5, lane 3). This is in contrast to the results obtained from sodium azide treatment in which none of the hMnSOD and FeSOD activities could be detected (Fig. 5, lane 4). The specific activity of purified recombinant hMnSOD was further determined to be 2,857 units  $mg^{-1}$  protein (Fig. 6).



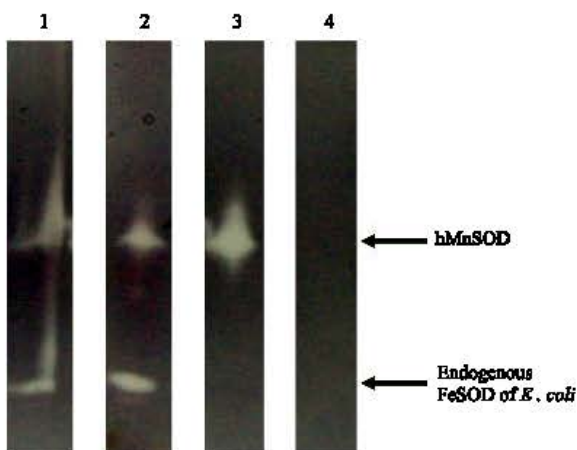


Fig. 5: SOD inhibitor assays: SOD activity gel staining of crude extract (Lane 1), inhibition of SOD activities by 3 mM KCN (Lane 2), 5 mM  $H_2O_2$  (Lane 3) and 50 mM  $NaN_3$  (Lane 4)

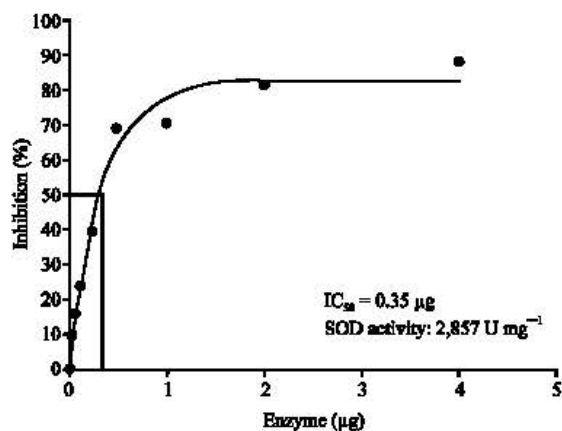


Fig. 6: Specific activity of purified recombinant human MnSOD as determined by inhibition of photoreduction of nitroblue tetrazolium.  $IC_{50}$  and specific activity were calculated to be 0.35  $\mu g$  and 2,857 units  $mg^{-1}$ , respectively

#### Response against oxidative stress of engineered *E. coli*:

To investigate whether the presence of hMnSOD rendered toleration against oxidative stress in *E. coli*, the engineered cells transformed with the pET46Ek/LIC and pET46MnSOD were exposed to various concentrations (0-1.6 mM) of paraquat. A decrease of optical density of control and MnSOD expressing cells was observed upon exposure to 0.4-1.2 mM paraquat. However, notification has to be made that the survival of the MnSOD overexpressing cells was remarkably higher than those of the control cells, particularly at concentrations ranging from 0.6-1.0 mM (Fig. 7). Therefore, the effect of oxidative

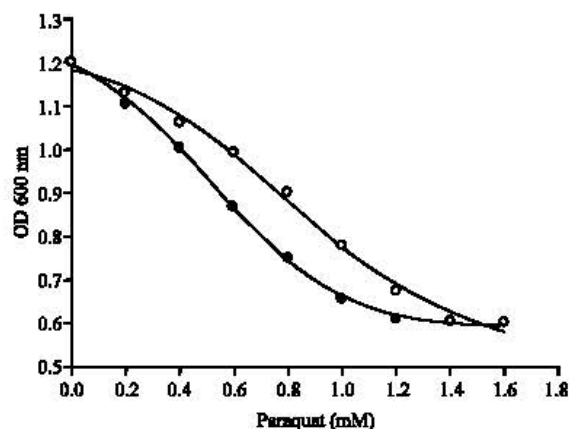


Fig. 7: Effect of paraquat on the survival of SOD expressed *E. coli* in broth culture. The engineered cells expressing hMnSOD (○) and control strain (●) were grown for 12 h in the presence of 0-1.6 mM paraquat. All values shown are the means of three independent determinations

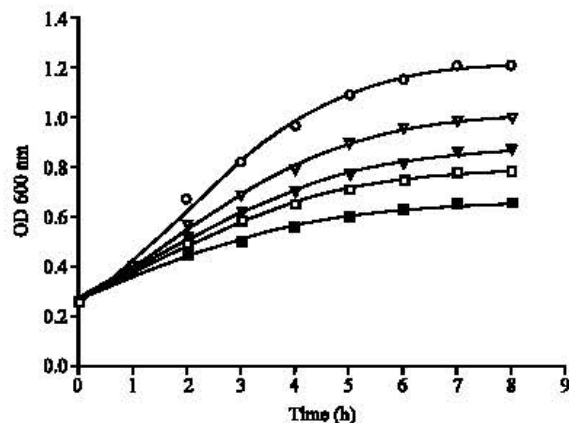


Fig. 8: Effect of paraquat on the growth rate of SOD expressed *E. coli* in broth culture. The growth rate of the bacterial cells was monitored at different time intervals in the presence of varying concentrations of paraquat. The engineered cells expressing hMnSOD and control strains were represented as opened and closed symbols, respectively. Circles denoted cultivation of cells in the absence of paraquat. Triangles and squares represented as cell grown in the presence of 0.6 and 1.0 mM paraquat, respectively

pressure on growth characteristics was evaluated taking into account both the duration of the cell growth and various concentrations of paraquat (Fig. 8). Growth rates of cells expressing hMnSOD were higher than those of the control cells at 0.6 and 1.0 mM paraquat. Taken

together, these data indicate that MnSOD conferred resistance to paraquat and as a consequence, bacterial cultures that express such a protein kept a higher growth rate than the control ones. Nevertheless, bacterial cells could not overcome the deleterious action of paraquat when used in concentrations above 1.2 mM. After exposure to the paraquat at 1.4-1.6 mM, all bacterial cells almost stopped their growth. This finding indicates that such concentrations are not permissible even for a minimal rate of survival, since MnSOD does not equip the cells with an efficient mechanism that completely detoxifies  $O_2^{\bullet-}$  and  $H_2O_2$ .

## DISCUSSION

Regarding the durability and high therapeutic potential of MnSOD (Baret *et al.*, 1984; Gorecki *et al.*, 1991; Epperly *et al.*, 1999), this study explores the successful accomplishments on the cloning and expression of mature human MnSOD in *E. coli*. A Ligation-Independent Cloning (LIC) technique has been applied to provide directional cloning of PCR products into the pET46Ek/LIC vector without the need for restriction enzyme digestion or ligation reactions (Aslanidis and de Jong, 1990). The hMnSOD gene has been placed immediately downstream of a hexahistidine and enterokinase cleavage site (Fig. 1) in order that the fusion tags can potentially be removed after protein purification (Prachayasittikul *et al.*, 2003).

To attain a high level and tightly-controlled of protein expression, the recombinant plasmid has been transformed into *E. coli* strain BL21(DE3)pLysS, a host bearing the T7 RNA polymerase gene. This is imperative since *E. coli* RNA polymerase normally does not recognize the T7 promoter presented in the pET series. Regulating of protein expression has also been precisely controlled by the presence of pLysS plasmid (encoding T7 lysozyme, a natural inhibitor of T7 RNA polymerase), which can minimize the ability to transcribe target genes in uninduced condition. From present findings, maximum expression of hMnSOD can be obtained after induction with IPTG for 4 h (data not shown). However, most of the expressed enzyme is non-soluble and is in an inactive form. Therefore, addition of  $Mn^{2+}$  is required for production of soluble and enzymatically active hMnSOD (Fig. 2 and 4). A similar occurrence has recently been investigated upon expression of MnSOD in the cytoplasm of *Saccharomyces cerevisiae* (Luk *et al.*, 2005). In all known cases of eukaryotic MnSOD, the enzyme is initially synthesized as a precursor polypeptide containing, at its N-terminus, a pre-sequence for mitochondrial targeting that is subsequently cleaved out in the mitochondria.

Luk *et al.* (2005) have engineered a mutant form of *S. cerevisiae* MnSOD that lacks such a mitochondrial pre-sequence. The modified MnSOD polypeptide accumulates in the cytosol rather than in the mitochondria and is enzymatically inactive. Lack of activity reflects manganese deficiency in the enzyme molecule, because activity can be restored upon culturing yeast cells in a medium containing high manganese ions. The study demonstrates that MnSOD requires a mitochondrial localization to efficiently acquire manganese. However, the same is not true for endogenous expression of *E. coli* MnSOD since it is normally expressed in the cytoplasm but remains enzymatically active (Gregory *et al.*, 1973). This may be enlightened that under normal physiological conditions, the bioavailability of manganese in *E. coli* cytoplasm is sufficient for its own enzyme but when heterologous enzyme is overexpressed the manganese appears too low to activate the large amount of synthesized MnSOD. Another possibility for enzyme inactivity may be attributable to the fusion sequence at the N-terminal of the expressed hMnSOD. Expression of yeast MnSOD without removing the mitochondrial targeting sequence was demonstrated to confer enzymatic inactivity (Schrack *et al.*, 1988), thus in our case, removing fifteen amino acids at the N-terminal may confer better enzymatic activity and protein folding. Detection of enzymatic activity by gel staining showed that an induced culture containing pET46MnSOD supplemented with  $Mn^{2+}$  has much higher activity than those of the control (Fig. 4). Furthermore, the purified hMnSOD is composed of identical polypeptides (22 kDa/subunit) and demonstrates the specific activity close to that of the authentic human liver enzyme (McCord *et al.*, 1977).

Expression of hMnSOD has been shown to protect *E. coli* from paraquat-generated oxidative stress. Paraquat is a redox cycling agent widely used as a source of  $O_2^{\bullet-}$  in a variety of experimental systems (Hassan and Fridovich, 1978). However, protection is observed only upon cultivation of bacteria in the absence of  $Mn^{2+}$ . On the contrary, supplementation with  $Mn^{2+}$  renders bacterial cells more susceptible to paraquat killing (data not shown). This may be explained by the synergistic effect between paraquat and manganese ions on the remarkable induction of MnSOD activity via metal-dioxygen dependent mechanism (Pugh *et al.*, 1984; Pugh and Fridovich, 1985). As a consequence, rapid dismutation of  $O_2^{\bullet-}$  may lead to an intracellular accumulation of  $H_2O_2$  at a toxic level. Other supportive evidences can also be drawn that a 11 fold increase in production of FeSOD does not confer protection against the effects of paraquat in *E. coli* (Scott *et al.*, 1987), whereas protection is obtained in transformants expressing a 4.8 fold increase in

superoxide dismutase activity (Bhattacharya *et al.*, 2004). Therefore, effective protection against oxidative stress may require balanced production of superoxide dismutase in parallel with other antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GPX).

The SOD has been proposed as clinically useful for the treatment of oxidative damage in many circumstances. Therefore, production of active human MnSOD herein will gain insights not only into the physical and biochemical characterizations, but also take into consideration the high protective performance against oxidative damages at the cellular level. Further investigations, e.g., construction of chimeric antioxidant enzymes (SOD-CAT, SOD-GPX and SOD-Bacterial hemoglobin) providing sequential catalytic sequestrations of reactive oxygen species; expression of antioxidant enzymes in mammalian and tumor cell lines and comparison of the efficacy to detoxify superoxide anion among the recombinant enzymes and SOD mimetics, are underway as ongoing research in our laboratory.

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