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Iron Containing Superoxide Dismutase of *Streptomyces subrutilus* P5 Increases Bacterial Heavy Metal Resistance by Sequestration

Jae-heon Kim*, Kwang yong Han, Ho jin Jung, and Jungnam Lee#

Department of Microbiology, Dankook University, Cheonan 330-714, Republic of Korea

Streptomyces subrutilus P5의 철 함유 Superoxide Dismutase의 중금속 격리에 의한 세균의 중금속 저항성 증가

김재헌* · 한광용 · 정호진 · 이정남#

단국대학교 자연과학대학 미생물학과

#*Present address: Department of Periodontology and Oral Biology, University of Florida, Gainesville, Florida, USA*

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Mitigation of heavy metal toxicity by iron containing superoxide dismutase (FeSOD) of *Streptomyces subrutilus* P5 was investigated. For *E. coli* DH5α, the survival rate in the presence of 0.1 mM lead ions was only 7% after 120 min; however, with the addition of 0.1 μM of purified native FeSOD the survival rate increased to 39%. This detoxification effect was also shown with 0.01 mM copper ions (survival increased from 6% to 50%), and the effect was stronger than with the use of EDTA. *E. coli* M15[pREP4] producing 6xHis-tagged FeSOD was constructed, and this showed an increase in survival rates throughout the incubation time; in the presence of 0.1 mM lead ions,the final increase at 60 min was from 3% to 19%. The FeSOD absorbed about 123 g-atom lead per subunit; therefore, we suggest that FeSOD could sequestrate toxic heavy metals to enhance bacterial survival against heavy metal contamination.

***Keywords*:** *Escherichia coli*, *Streptomyces subrutilus*, heavy metals, resistance, sequestration, superoxide dismutase

Heavy metals are major environmental contaminants and there are reports describing the toxicity of heavy metals with which humans can easily come into contact, e.g., mercury (Hg), cadmium (Cd), lead (Pb), and arsenic (As) (Mamtani *et al*., 2011). They can easily bind to biomolecules with a large number of moieties having high affinity for heavy metal ions, which interferes with protein structure and function as well as DNA structure and leads to their toxicity (Banjerdkij *et al*., 2005; Duruibe *et al*., 2007). In addition, they are known to induce fatty acid peroxidation; thus, impairing membrane function (Howlett and Avery, 1997).

Many species of microorganisms exhibit resistance to heavy metals through a variety of mechanisms. Among them, metallothioneins, identified to be metal-binding proteins, have been attracting much attention for their suitability for bioremediation. Most metallothioneins are eukaryotic cysteine-rich proteins that sequestrate toxic heavy metals by forming complexes with them. Metallothionein-like proteins occur in prokaryotes; for example, the protein SmtA binds zinc and cadmium in *Synechococcus* (Bruins *et al*., 2000). While these proteins are also rich in cysteine residues, the amino acid sequence is not related to the eukaryotic metallothioneins (Turner and Robinson, 1995). In addition, heavy metal-binding proteins with high anionic amino acid content have been identified in prokaryotes, e.g., the cadmium-binding proteins in bacteria (Capasso *et al*., 1996).

Antioxidant enzymes such as superoxide dismutase (SOD) and catalase are directly implicated in the mechanisms of heavy metal resistance in microorganisms because heavy metals cause oxidative stress through intracellular synthesis of free radicals (Eickhoff *et al*., 1995; Geslin *et al*., 2001; Bebien *et al*., 2002; Sumner *et al*., 2005).

In a previous study, *Streptomyces subrutilus* produced extracellular iron containing SOD (FeSOD) that exhibited lead-binding activity (So *et al*., 2001). In this study, we would

*For correspondence. E-mail: jaehkim1@dankook.ac.kr; Tel.: +82-41-551-3452; Fax: +82-41-559-7857

like to verify the heavy metal resistance of *E. coli* by treating with this FeSOD protein in extracellular or intracellular procedure.

Materials and Methods

Strains, plasmids, medium, and culture conditions

Streptomyces subrutilus P5, a lead-resistant strain, was originally isolated by us from the soil sample of a disused gold mine located in Gongju, Korea (Rho and Kim, 2002). The spore solution was prepared from a yeast extract-malt extract agar plate (YEME, ISP No. 2) and stored in 20% (w/v) glycerol solution at -20℃ until used as inoculums. For the extraction of genomic DNA from *S. subrutilus* P5, a liquid YEME containing 34% sucrose was incubated for two days at 30℃, 200 rpm. *E. coli* strains JM109 and M15[pREP4] used for cloning and gene expression were purchased from Promega Korea Ltd. and Qiagen Korea Ltd., respectively and were incubated in LB medium overnight at 37℃, 200 rpm. Ampicillin (100 μg/ml) or kanamycin (25 μg/ml) was added to the LB medium as needed. The pGEM-T easy vector (Promega Korea Ltd., Korea) was used for the cloning of PCR products, and pQE30 (Qiagen Korea Ltd., Korea) containing an N-terminal 6xHis tag was used for the production of recombinant SOD.

Preparation of genomic DNA

Preparation of genomic DNA was performed by the guanidiumthiocyanate extraction method using a cell lysis reagent containing guanidiumthiocyanate, EDTA, and lauroylsarcosine (Pitcher *et al.*, 1989).

Purification of native SOD from culture filtrates

S. subrutilus P5 was incubated at 30℃ for 5 days in YEME liquid medium, and culture filtrate was obtained by centrifugation and filtration using No. 2 filter paper (Advantec MFS, Inc., USA). SOD protein was precipitated by dissolving 0.2 mM lead at 30℃ for 30 min and collected by centrifugation at 12,000 rpm at 4℃ for 20 min. Following a wash with distilled water, the protein precipitates were redissolved in 50 mM Tris-Cl (pH 7.2) containing 0.1 M EDTA. The EDTA-lead complex was subsequently removed by dialysis with triple distilled water at 4℃ for one day (So *et al.*, 2001). The purity of the SOD preparation was verified by the appearance of a single protein band with enzyme activity on 12% native gel (Beauchamp and Fridovich, 1971).

The protein concentration was determined by the Bradford method.

DNA-Amplification, cloning, and expression of SOD

Oligonucleotide primers for PCR of the FeSOD gene were designed based on the homologous base sequences of the upstream and downstream region of the *sodF1* and *sodF2* of *S. coelicolor* A3 (2) and the *sodF* of *S. griseus*. The forward primer, 5′-CTCTTGCTGCTAGCGTGCA GTTG-3′, and the reverse primer, 5′-GGTGAAGGTTGAGAA GACGATCACGAG-3′, were used for amplification of a DNA fragment (750 bp) containing the entire sequence of SOD. All primers were manufactured by Bioneer Co. (Korea). PCR was performed in 20 μl PCR premix (Bioneer Co.) containing each primer (10 pmol) and 1 μl genomic DNA (ca. 95 ng). The reaction followed 30 cycles: initial denaturing at 95℃ for 5 min, denaturing at 95℃ for 1 min, annealing at 68℃ for 1 min, and extension at 72℃ for 1 min; and post-run final extension at 72℃ for 10 min. The base sequence of the amplified DNA fragments (750 bp) confirmed the presence of a SOD gene (657 bp; GenBank KF826799). A new Oligo-Linker Nucleiotides set endowed with the restriction sites *Bam*H1 and *Hin*dIII was used to construct the 6xHis protein expression vector based on pQE30 (Qiagen Korea); the forward Oligo-Linker, 5′-GGATCC GCCATCTACACGCTTC-3′; the reverse Oligo-Linker, 5′-AAGC TTGCCCTTGACGGGGAT-3′. The expression vector harbouring the entire SOD was transformed into *E. coli* M15 [pREP4] cells. The induction and purification of 6xHis-tagged SOD were performed following protocols provided by the manufacturer (Qiagen) and the enzyme purity was confirmed by Laemmli's discontinuous SDS polyacrylamide gel electrophoresis. The activity staining of the 6xHis-tagged SOD was carried out as described earlier.

Determination of heavy metal resistance

E. coli cells in log phase (OD_{600} = ca. 0.6) were harvested and washed with sterile 50 mM MES buffer, pH 6.5, by centrifugation and resuspended into MES buffer. The cell suspensions were treated with separate heavy metal ions to give different final concentrations: lead ($Pb(NO_3)_2$), 1 mM and 0.1 mM; copper ($Cu(NO_3)_2$), 1 mM, 0.1 mM, and 0.01 mM; and iron ($FeSO_4 \cdot 7H_2O$), zinc ($Zn(NO_3)_2$), and cadmium ($CdCl_2$), each 0.1 mM. EDTA and native SOD were added into the cell-heavy metal mixtures as necessary. The cell suspensions containing heavy metals were then incubated for the indicated times with shaking, and then 100 μl of each culture was streaked on LB plate at indicated time intervals for serial dilution and colony counting. Lead and copper resistance was represented as the survival curves and percentage survival rates were determined for other heavy metals.

Measurement of lead-absorbance

Lead ions (87 μM and 130 μM) and the purified 6xHis-tagged SOD protein (3.3×10^{-2} μM) were mixed into 50

mM MES buffer (pH 6.5), incubated at 30℃ for 30 min, and filtrated by centrifugation using centricon YM-10 (Amicon Bioseparations, Millipore, USA) at 4℃, 4,000 × *g* for 40 min. The eluates were assayed for residual lead using an inductively coupled plasma (ICP) mass spectrometer (PQ3, VG Elemental, UK) at the Korea Basic Science Institute (KBSI). The numbers of lead ions per SOD protein were calculated from the difference between the initial and residual concentrations. The measurements were carried out twice and the representative data are shown.

Results

SOD-mediated mitigation of heavy metal toxicity

We suspended *E. coli* DH5α in MES buffer containing either lead or copper ions, and the survival rates were determined at indicated times by residual colony counting (Fig. 1). MES itself exerted no toxicity on *E. coli* DH5α. Lead ions showed no significant toxic effect at 0.01 mM (2 ppm). At 0.1 mM (20 ppm) lead ion, after a short delay of 10 min, survival rates began to decrease and a clear decrease appeared at 20 min (30%). Then there was a relatively slow decrease in the survival rate to 7% by 120 min. At 1.0 mM (200 ppm) lead ion, the toxic effect appeared more clearly, and the bacteria were completely killed after 60 min. Employing the same method, it appeared that copper exerted a stronger toxicity on *E. coli* DH5α than lead. After treatment with 1 mM and 0.1 mM (6.4 ppm) copper ion, all bacteria were killed within 10 min; and, at the lowest concentration of 0.01 mM (0.64 ppm) copper ion, there was a clear decrease to 34% at 10 min with a final survival rate of 6% after 120 min.

Additionally, we analysed the toxic effect of native SOD purified from the culture filtrate of *S. subrutilus* P5 in this experiment. While treatment with 1 μM FeSOD resulted in a survival rate of only 44% after 120 min (data not shown), treatment with 0.1 μM FeSOD did not exhibit any serious toxicity on the strains and this concentration of SOD was chosen to determine its detoxification effect against heavy metal ions. The toxic effect of the higher concentration of purified native FeSOD may be due to excessive synthesis of H_2O_2. This is supported by a report that excessive SOD induces the generation of extremely toxic hydroxyl radicals that destroy cellular macromolecules (Touati, 2000).

To investigate the effect of SOD on the survival of bacteria exposed to heavy metals, we simultaneously treated *E. coli* DH5α with heavy metals and a non-toxic amount of the purified FeSOD (0.1 μM). The addition of native FeSOD increased the survival rate against 0.1 mM lead ion by approximately 2.9 to 5.5 times depending on incubation time; the final survival rate increased from 7% to 39% at 120 min (Fig. 2). This mitigative effect of the native FeSOD was compared with the effect of 0.1 μM EDTA, commonly used as a chelating agent to detoxify metal ions. Although EDTA was also able to reduce the lethal effects of lead ions, the degree of detoxification was lower than that of native FeSOD with a survival rate of 17% at 120 min (Fig. 2). Likewise, the survival rates against 0.01 mM copper ions increased from 6% to 50% at 120 min with FeSOD supplementation (Fig. 3).

In order to ascertain whether the mitigative effect of FeSOD was caused by its intrinsic property and not just a general effect as a protein, we performed the same experiment replacing purified native FeSOD with bovine serum albumin. Bovine serum albumin did not show any influence on the strains' survival rate in the presence of heavy metal ions (data not shown). Through these comparison tests, we could postulate that the mitigative activity of FeSOD occurred through

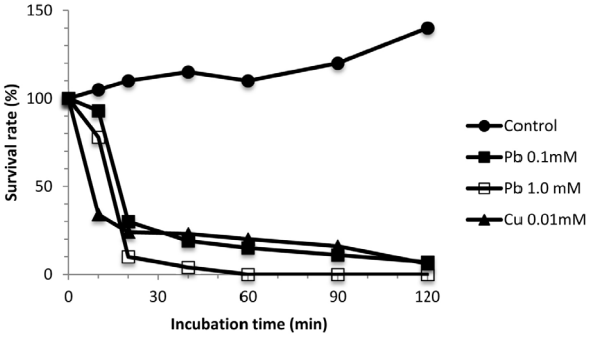

Fig. 1. Survival (%) of *E. coli* DH5α in the presence of Pb^{2+} and Cu^{2+}. The initial number of *E. coli* cells was (2.2 ± 0.2) × 10^6 cells/ml and the proportions of the survived cells at the indicated times were determined by the viable colony counting as described in 'Materials and Methods'.

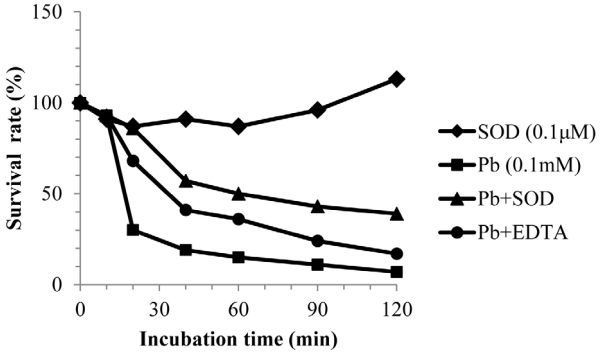

Fig. 2. Mitigation of the lead toxicity by native FeSOD of *S. subrutilus* P5 and EDTA to *E. coli* DH5α. The native FeSOD (0.1 μM) of *S. subrutilus* P5 or EDTA (0.1 μM) was added into the *E. coli* cell suspension containing lead (0.1 mM) and the survival rates (%) were determined at the indicated times. 100% corresponds to (2.2 ± 0.2) × 10^6 cells/ml.

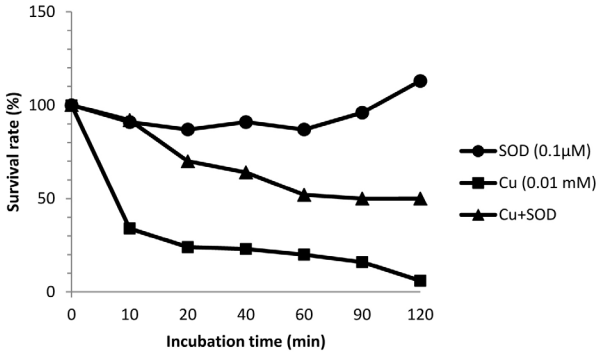

Fig. 3. Mitigation of the copper toxicity by native FeSOD of *S. subrutilus* P5 to *E. coli* DH5α. The native FeSOD (0.1 μM) of *S. subrutilus* P5 was added into the *E. coli* cell suspension containing copper (0.1 mM) and the survival rates (%) were determined at the indicated times. 100% corresponds to $(2.2 \pm 0.2) \times 10^6$ cells/ml.

adsorption of heavy metal ions by the enzyme molecules, which prevented direct contact between *E. coli* DH5α and the heavy metal ions.

Cloning and production of recombinant *S. subrutilus* P5 FeSOD

In order to estimate changes in heavy metal resistance from *E. coli* over-producing FeSOD intracellularly, it was necessary to carry out gene cloning and make a recombinant *E. coli* strain harbouring the SOD gene. We obtained the full FeSOD gene from the genomic DNA of *S. subrutilus* P5 by using PCR amplification. The comparison of base sequences revealed that the FeSOD gene (GenBank KF826799) showed sequence similarities of 82%, 80%, and 85% to the FeSOD I (GenBank AF099014) and FeSOD II (GenBank AF099015) genes of *S. coelicolor* A3(2) and the FeSOD (GenBank AF141866) gene of *S. griseus* respectively. The amino acid sequences (GenBank AHH25151) also showed high similarities of 78% (FeSOD I), 80% (FeSOD II), and 83% (FeSOD) (Fig. 4). Furthermore, the amino acid sequences (GenBank: AHH25151) verified the presence of the characteristic N- and C-terminal domains (pfam00081 and pfam02777 respectively). The gene also contained the SOD signature, D-x-[WF]-E-H-[STA]-[FY] (Priya *et al.*, 2007).

The 6xHis-tagged SOD proteins were produced by inducing the transformed *E. coli* M15. Although the over-expression resulted in the formation of inclusion bodies, we were able to carry out a non-denaturing purification process for SOD proteins to confirm the enzyme activity on native gel, which appeared as a negatively stained band on blue background (Fig. 5). It was obvious that significant amounts of the induced enzyme were soluble and remained intact.

Heavy metal tolerance of *E. coli* producing 6xHis FeSOD

In order to determine if SOD production directly mediates heavy metal tolerance, we used strains that over-express SOD intracellularly instead of supplying the native SOD into the reaction buffer. After transforming FeSOD genes of *S.*

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coelicolorFeSOD1      MSVYTLPELPYDYSALAPVISPEIIELHHDKHHAAYVKGANDTLEQLAEARDKETWGSIN 60
coelicolorFeSOD2      MALYTLPELPYDYSALAPVISPEIIELHHDKHHAAYVKGANDTLEQLAEAREKESWGSIN 60
griseusFeSOD          MATYTLPELPYDYAALEPVINPQIIELHHDKHHAAYVKGANDTLEQLEEARDKEAWGAIN 60
P5SOD                 MAIYTLPELPYDYAALEPVINPQIIELHHDKHHAAYVTGANNTLEQLEEAREKENWGALN 60
                      *: **********:** ***.*:**************.***:***** ***:** **::*

coelicolorFeSOD1      GLEKNLAFHLSGHILHSIYWHNMTG----DGGGEPLDKDGVGELADAIAESFGSFAGFRA 116
coelicolorFeSOD2      GLEKNLAFHLSGHILHSIYWQNMTGPK--DGGGEPLAQDGVGDLADAITESFGSFAGFKA 118
griseusFeSOD          GLQKNLAFHLSGHILHSIYWHNMTG----DGGGEPLAADGVGDLADAITESFGSYAGFKS 116
P5SOD                 GLEKNLAFHLSGHILHSIYWHNMASPKTGEGGGEPTAADGLGDLADAITESFGSFAKFKK 120
                      **:******************:**:.    :*****   **:*:*****:*****:* *:

coelicolorFeSOD1      QLTKAAATTQGSGWGVLAYEPLSGRLIVEQIYDHQGNVGQGSTPILVFDAWEHAFYLQYK 176
coelicolorFeSOD2      QLTKAAATTQGSGWGVLAYEPLSGRLIVEQVYDHQGNVGQGATPILVFDAWEHAFYLQYK 178
griseusFeSOD          QLTKAAATTQGSGWGVLAYEPVSGKLIVEQVYDHQGNVGQGSVPILVFDAWEHAFYLQYK 176
P5SOD                 QLTFASSATQGSGWGVLAYEPVSGRLIVEQVYDHQGNIGVASTPVLVFDAWEHAFYLQYK 180
                      *** *:::*************:**:*****:******:* .:.*:***************

coelicolorFeSOD1      NQKVDFIDAMWAVVNWQDVARRYEAAKSRTNTLLLAP- 213
coelicolorFeSOD2      NQKVDFIEAMWAVVNWQDVAKRHAAAKERGDSLLLKP- 215
griseusFeSOD          NQKVDFIEAMWQVVNWQDVAKRYAAAKERVNVLLLAP- 213
P5SOD                 NQKVDFIEAMWNVVNWQDVAKRYADAKANTPLLIPVKG 218
                      *******:*** ********:*:  ** .   *:
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Fig. 4. Amino acid sequence alignment of FeSOD from *S. subrutilus* P5 with other SODs from *S. coelicolor* A3 (2) and *S. griseus*. P5SOD, *S. subrutilus* P5; coelicolorSOD, *S. coelicolor* A3 (2); griseusSOD, *S. griseus.* Bar, SOD signature; "*", identical; ":", conserved substitutions; ".", semi-conserved substitutions.

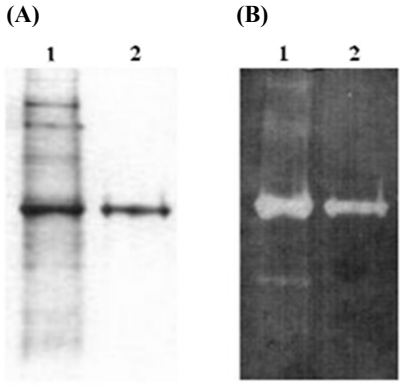

Fig. 5. Purification and SOD activity staining of 6xHis-tagged FeSOD under native condition. (A) Protein staining using coomassie brilliant blue R250, (B) Achromatic SOD activity band on blue background. Lanes: 1, Crude extract of induced 6xHis-tagged SOD in *E. coli* M15; 2, Purified 6xHis-tagged SOD.

subrutilus P5 into *E. coli* M15 strains, we induced SOD expression by treating with 1 mM IPTG for 1 h. Subsequently, we determined the survival rates by calculating the percentage of the colonies that survived the heavy metal treatment in relation to the baseline number of colonies without the heavy metal treatment.

For FeSOD induced *E. coli* culture, the survival rates against lead ion (0.1 mM) increased between 2.5- and 6-times compared with those of non-induced culture (Fig. 6). This mitigative effect was comparable with the same effect shown by the supplementation of native FeSOD. Among other heavy metal ions tested in this study, only iron (II) toxicity could be reduced significantly (1.7 times), while the toxicity of cadmium and zinc ions showed no significant changes (data not shown).

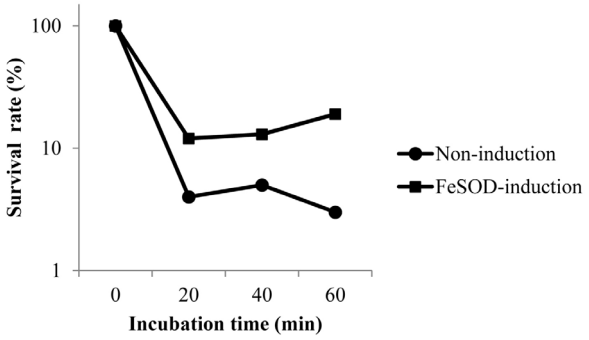

Fig. 6. Survival rates of *E. coli* M15[pREP4] induced and non-induced for FeSOD production in the presence of 0.1 mM Pb^{2+}. The recombinant *E. coli* cells carrying FeSOD gene of *S. subrutilus* P5 were grown to log phase (OD_{600} = ca. 0.6) in LB and induced for FeSOD production by 1.0 mM IPTG. Non-induced cells were also prepared without IPTG treatment. The cells were resuspended into the sterile MES buffer (50 mM, pH 6.5) and the survival rates were determined in the presence of 0.1 mM Pb^{2+} as describe in Fig. 1. 100%: $(2.2 \pm 0.2) \times 10^6$ cells/ml

Table 1. Lead absorption of 6xHis-tagged FeSOD of *S. subrutilus* P5

Pb (μM)	Biosorption of Pb ions (g-atom Pb/mol SOD)
87	80
130	123

The reaction mixture contained 6×His-tagged FeSOD (3.3×10^{-2} μM) and Pb^{2+} in MES buffer (50 mM, pH 6.5). It was incubated for 30 min, 30℃ and the difference between the initial and residual concentrations of lead ions were used for the calculation of the Pb^{2+}-biosorption.

Also cells induced for SOD production could survive copper toxicity, although only slightly with a survival rate of about. 0.1% with 0.1 mM copper (II) ions. Throughout the experiments, copper presented stronger toxicity than lead, which is comparable to a study that demonstrated copper exhibited stronger toxicity than cadmium in *S. cerevisiae*. This was explained by the different mode of copper in causing cell death, which is by a combination of a series of reactions, such as direct reaction to nucleic acids, misincorporation into metallothionein, and stronger lipid peroxidation (Howlett and Avery, 1997; Festa and Thiele, 2011).

These experimental results indicate that the recombinant SOD proteins expressed within transformed *E. coli* M15 strains could enhance resistance to various heavy metals. Although the over-expression results in inclusion body formation, considerable amounts of non-denatured protein molecules are likely to be present in the cells that mitigate the effect of toxic heavy metal ions.

In addition, lead absorption by the 6×His-tagged SOD protein was analysed. The purified 6×His-tagged SODs were allowed to react with lead solutions of different concentrations. Although we did not determine the absorption kinetics, lead absorbance increased with increasing lead concentrations to 123 g-atom Pb/mol SOD (Table 1); therefore, the survival of *E. coli* in the presence of heavy metal ions could be due to the sequestration of heavy metals by SOD proteins, both intracellularly and extracellularly.

Discussion

It has been known that some extracellular materials produced by microorganisms confer heavy metal resistance because they can absorb or precipitate heavy metals and avoid direct contact between microbial cells and heavy metal ions. This type of defence mechanism is referred to as extracellular sequestration. Some materials known to exhibit this function are exopolysaccharide, hydrogen sulphide, oxalate, and phosphate (Bruins *et al.*, 2000); however, heavy metal tolerance through the function of extracellular proteins has not yet been reported. The protein investigated in this paper is the Fe

containing SOD of *S. subrutilus* P5, whose lead-binding property has been already reported (So *et al*., 2001).

The toxicity to lead of *E. coli* decreased in a reaction mixture containing purified FeSOD (Fig. 2). This toxicity-reducing effect was found to be stronger than that of EDTA, a chelating agent. Likewise, lead tolerance increased in case a FeSOD gene was expressed in recombinant *E. coli*. These data indicate that this SOD protein contributes to the increased ability of microorganisms to survive in natural environments containing high concentrations of heavy metals.

Metal binding proteins possess characteristic amino acid sequence motives with very high metal affinity. Cystein, glutamic acid, and glycine are the major component of metallotheionneins and phytochelatins (Mejáre and Bülow, 2001). Although the lead-binding activity was determined quantitatively in this report, we could not find any known characteristic amino acid sequence for metal binding in the FeSOD of *S. subrutilus* P5 in this study; therefore, other molecular properties of proteins are likely to be involved in the heavy metal absorbance. In this context, the hollow sphere structure of soy proteins (Liu *et al*., 2013) and porous cage structure of ferritin (Theil, 2003) that can be applied as detoxification agents for various heavy metals (Li *et al*., 2012) are worthy of notice. We should conduct further assays of the absorption isotherm and kinetics to confirm FeSOD as a bio-sorbent for toxic heavy metals.

적 요

Streptomyces subrutilus P5가 생산하는 철 함유 superoxide dismutase (FeSOD)에 의한 중금속 독성의 완화를 조사하였다. 0.1 mM의 납이온이 120분 처리되면 *E. coli* DH5α의 생존율이 7%에 불과 하지만 0.1 μM의 정제된 천연 FeSOD가 첨가되면 생존율이 39%로 높아졌다. 이러한 해독작용은 0.01 mM의 구리 이온에 대해서도 나타나며(생존율이 6%에서 50%로 증가) 그 효과는 EDTA보다 강하였다. 6xHis-tagged FeSOD를 생산하는 재조합 *E. coli* M15[pREP4]는 0.1 mM의 납 이온이 60분 처리된 후의 생존율이 3%에서 19%로 증가하였다. 6xHis-tagged FeSOD는 분자당 123개의 납과 결합하였다. 따라서 FeSOD가 중금속을 세포와의 접촉으로부터 격리함으로써 중금속이 오염된 환경에서 세균의 생존력을 증가시킨 것으로 사료된다.

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