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Expression and Characterization of Thiol-Specific Antioxidant Protein, DirA of *Corynebacterium diphtheriae*

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Abstract: A *Corynebacterium diphtheriae* iron-repressible gene *dir*A, that was homologous to TSA of *Saccharomyces cerevisiae* and AhpC subunit of *Salmonella typhimurium* alkyl hydroperoxide reductase, was amplified with PCR and expressed in *E. coli*. The DirA purified from the transformed *E. coli* crude extracts prevented the inactivation of enzyme caused by metal-catalyzed oxidation (MCO) system containing thiols but not by ascorbate/Fe^{3+}/O_2 MCO system. The DirA concentration, which inhibited the inactivation of glutamine synthetase by 50% (IC_{50}) against MCO system, was 0.12 mg/ml. The multimeric forms of DirA were converted to the monomeric form in SDS-PAGE under the thioredoxin system comprised of NADPH, *Saccharomyces cerevisiae* thioredoxin reductase, and thioredoxin. Also, DirA showed thioredoxin dependent peroxidase activity. All of these results were consistent with the characteristics of a thiol specific antioxidant (TSA) protein having two conserved cysteine residues.

Key Words: *Corynebacterium diphtheriae*, DirA, MCO system, TSA, AhpC

INTRODUCTION

In organisms living in aerobic environments, reactive oxygen species (ROS; O_2^-, H_2O_2, ROOH and · OH) were generated by many processes[1]. One of such processes was a nonenzymatic metal-catalyzed oxidation (MCO) system which was comprised of transition metal ion Fe^{3+}(Cu^{2+}), O_2, and thiol or ascorbate[2]. The processes that governed the levels of H_2O_2 and the availability of transition metals may be fundamental to the etiology of various metabolic disorders. The ROS damaged cellular components by oxidizing lipids, proteins, and nucleic acids[3,4], which have been implicated in various diseases, cancers, aging, and apoptosis[4,5]. To be protected from these cytotoxic oxygen species, all aerobic organisms have evolved a number of antioxidant proteins or enzymes which can scavenge ROS as well as repair or remove the damaged cellular components. One of such proteins was a thiol-specific antioxidant protein termed TSA, first found in a yeast *Saccharomyces cerevisiae,* which prevented specifically the inactivation of enzymes caused by a thiol/Fe^{3+}/O_2 MCO system, and did not show any activities of known antioxidant enzymes such as catalase, superoxide dismutases, and glutathione peroxidases[6].

The new antioxidant protein family showing

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homology with TSA was reported to be distributed widely in human pathogenic microbes[7,8]. These pathogenic microbes having antioxidant protein might have a capacity to decompose hydrogen peroxide produced by macrophages or neutrophils and also to scavenge the reactive oxygen species. Such microbes might show even more pathogenic capacity[9]. Recently, *Mycobacterium tuberculosis* mutant (NCI gi: 1002373) resistant to isonizid has shown high expression of the antioxidant protein.

According to the search of GenBank database, *C. diphtheriae* iron-repressible polypeptide known as DirA[10] showed a high degree of homology with TSA of *Saccharomyces cerevisiae*[6] and AhpC subunit of *Salmonella typhimurium* alkyl hydroperoxide reductase[11], which were known to be antioxidant family members. Alignmemt of the amino acid sequences of antioxidant family members revealed highly two conserved cysteine residues, corresponding to Cys-47 and Cys-170 in the yeast TSA. The more N-terminal cysteine was conserved in all family members, whereas the more C-terminal cysteine was present in most but not all members[8]. The antioxidant proteins that contained these two conserved cysteines may be reduced by a mechanism that involved either a single protein, like AhpC, or two proteins, like Trx and TR. It was therefore reasonable to speculate that antioxidant family proteins were also peroxidase, with the conserved N-terminal cysteine being a primary redox catalytic site. This family of peroxidases was named as peroxiredoxin family[9]. The *C. diphtheriae* which was one of human pathogenic bacteria produces maximal toxins to take free irons away from the damaged host tissues when cells were infected with toxigenic phages and grown under iron-limited condition[10,12,13]. The peroxiredoxin proteins were well known as species-specific antigens in *E. histolytica*[14] and *M. avium*[15].

In this study, We describe the expression and the purification of DirA with homology to TSA of *Saccharomyces cerevisiae* and AhpC subunit of *Salmonella typhimurium* alkyl hydroperoxide reductase. We also describe the antioxidant activity of DirA against MCO system and the peroxidase activity to reduce H_2O_2 or alkyl hydroperoxide in the presence of thioredoxin system.

MATERIALS AND METHODS

Materials

The Extraction kit for PCR product was purchased from QIAGEN (U.S.A.). Dithiothreitol (DTT) and NADPH were purchased from Sigma (U.S.A.). Glutamine synthetase of *E. coli* strain YMC 10/pGln 6 was purified using a Zn-precipitation method.

Polymerase chain reaction (PCR)

To prepare DirA, forward primer (5'-AAACA-*TATG*TCTATCTTGACTGTTGG-3') containing *Nde*1 site (underlined) and initiation codon (*italic*) and reverse primer (3'-CAAACTCTTT-CCGGAGT*TGA*CTTAAGAAA-5') containing *Eco*R1 site (underlined) and stop codon (*italic*) were constructed. The reaction mixture was made up of 10 mM Tris-HCl (pH 8.3), 40 mM KCl, 0.15 mM $MgCl_2$, 1 mM DTT, 0.5 μg/ml acetylated BSA, 0.25 mM in each dNTP, 100 pmol of each oligonucleotide primer, 0.5 μl of Taq DNA polymerase (5U), and *C. diphtaeriae* colony as template in a 100 μl reaction tube. The reaction was amplified for 2 cycles under 94℃/3min, 45℃/2min, 72℃/30sec, and then for 30 cycles under 94℃/15sec, 55℃/30sec, 72℃/30sec, using Gene Amp PCR System 2400 (Perkin Elmer, USA). PCR products (597bp) were subjected to electrophoresis on a 1% agarose gel, purified by QIA quick gel extraction kit, and then nucleotide sequence was determined.

Overexpression of DirA

The PCR products and expression vector pET17b were cleaved with *Nde*I and *Eco*RI for 1 hour, respectively, separated on a 1%

agarose gel, extracted by QIA quick gel extraction kit, and ligated with T4 DNA ligase for 12 hours at 16℃. The expression vector pET17b containing *dirA* gene was transformed into *E. coli* strain BL21 (DE3). The *E. coli* strain BL21 (DE3) carrying expression vector was cultured overnight, transferred to 1000 ml of fresh LB medium supplemented with ampicillin (100 μg/ml), and cultured further for 3 hours with vigorous shaking at 37℃. Isopropyl-β -thiogalactopyranoside (IPTG) was added to a final concentration 1 mM. After 3 hours of induction, the cells were collected by centrifugation, frozen and stored at -70℃ until use.

Purification of DirA

The cells were suspended in 25 mM Tris-HCl (pH 7.6) and disrupted by sonication. The lysate was centrifuged at 18,000×g for 30 min. The supernatant was loaded onto DEAE-Sephacel column (2.5×20 cm) equilibrated with 25 mM Tris-HCl (pH 7.6). After washing the column with 2 volumes of above buffer, the proteins were eluted with a linear NaCl gradient from 0 to 0.7 M in 600 ml of equilibration buffer (pH 7.6). The fractions containing antioxidant activity were precipitated with 75% saturated ammonium sulfate, dissolved in a minimal volume of 20 mM HEPES (pH 7.6) containing 1 M ammonium sulfate, loaded onto HPLC phenyl column (TSK-Gel, phenyl-5PW, 0.75 ×7.5 cm, Japan), and eluted with a linear ammonium sulfate gradient from 1.0 to 0.0 M for 50 min. The elutes containing activity were concentrated by using a Centricon-30 (Amicon), loaded onto HPLC DEAE column (Bio-Gel, TSK DEAE-5PW, 0.75×7.5 cm, Japan), eluted with a linear NaCl gradient from 0 to 0.7 M for 50 min, and then, the fractions containing antioxidant activity were concentrated with Centricon-30.

Assay of DirA Antioxidant Activity

The DirA antioxidant activity was assayed by monitoring its ability to protect the inactivation of *E. coli* glutamine synthetase caused by a thiol-dependent MCO system as described by Kim *et al.*,[9]. The reaction mixture (25 μl) containing 5 μg glutamine synthetase, 10 mM DTT or ascorbate, 3 μM $FeCl_3$, 50 mM HEPES buffer (pH 7.0) and DirA was incubated at 37℃ for 10 min, then 1 ml of glutamine synthetase assay mixture was added, followed by incubation at 37℃ for 4 min. To determine the remaining glutamine synthetase activities by spectrophotometer at 540 nm, 0.6 ml of the stop solution containing 15.2% $FeCl_3$, 8% trichloroacetic acid and 1 N HCl was added.

Assay of DirA Peroxidase Activity

The DirA activity for reducing hydroperoxide was determined by adding 1 mM H_2O_2 to 500 μl reaction mixture containing 0.2 mM NADPH, 10 μg/ml yeast thioredoxin, 112 μg/ml yeast thioredoxin reductase, 100 mM HEPES buffer (pH 7.0), and 100 μg/ml DirA, and then assayed by monitoring spectrophotometrically the decrease of NADPH absorbance at 340 nm.

Reduction of DirA by Thioredoxin System

Conversion of disulfide bond to sulfhydryl in DirA was performed by adding 5% 2-mercaptoethanol to the reaction mixture (100 μl) containing 0.2 mM NADPH, 10 μg/ml yeast thioredoxin, 112 μg/ml yeast thioredoxin reductase, 100 mM HEPES buffer (pH 7.0), and then electrophoresed on 12.5% polyacrylamide gel by Laemmli method[16].

RESULTS

The expression vector pET17b containing *dirA* gene of *C. diphtheriae* was transformed into *E. coli* strain BL21 (DE3) and then DirA was induced by adding IPTG. The harvested cells were sonicated and centrifuged. The DirA was purified to homogeneity from supernatants by sequencial steps of DEAE-Sephacel chromatography (Fig. 1A), HPLC-phenyl chro-

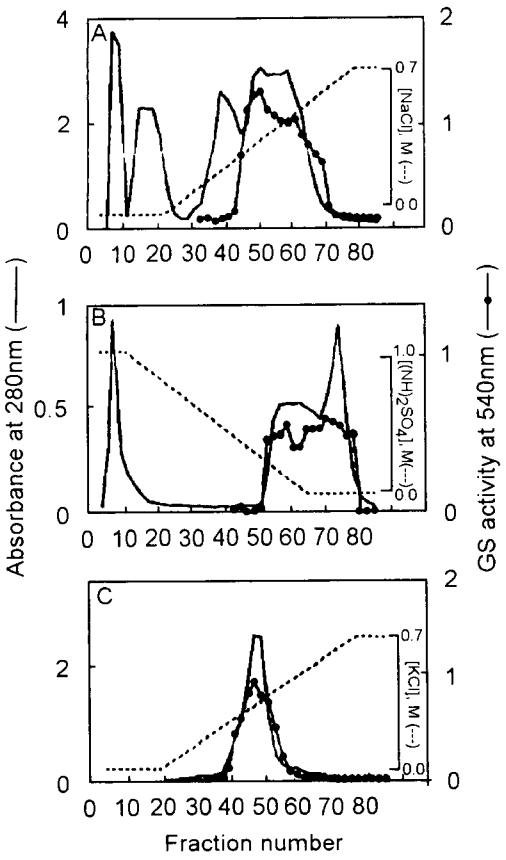

Fig. 1. Purification of DirA. Detailed procedures on column chromatography are described under "Materials and Methods". **A,** DEAE-Sephacel ion exchange chromatography; **B,** HPLC reverse phase chromatography on TSK phenyl-5PW; **C,** HPLC ion exchange chromatography on TSK DEAE-5PW.

matography (Fig. 1B), and HPLC-DEAE chromatography (Fig. 1C). The DirA activity was monitored by assay for the remaining *E. coli* glutamine synthetase activity.

Electrophoresis of the purified DirA was carried out on 12.5% SDS-polyacrylamide gel with 2-mercaptoethanol, stained with Coomassie brilliant blue (Fig. 2A) and subjected to immunoblot analysis (Fig. 2B). In Fig. 2A, lane 2 illustrated that DirA was overexpressed and constituted a major component of proteins in *E. coli* crude extracts. The DirA was about 23 kDa in the reducing condition (lane 3, Fig. 2A). Polyclonal antibodies were prepared using the

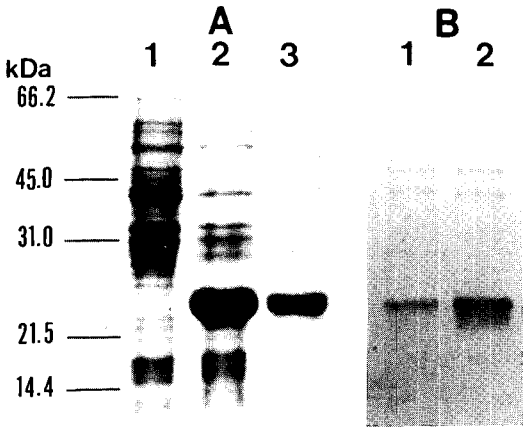

Fig. 2. SDS-PAGE analysis of *E. coli* crude extracts and purified DirA. **A,** Crude extracts (*E. coli* and transformed *E. coli*) and purified DirA were separated by SDS-PAGE on a 12.5% gel in the presence of 2-mercaptoethanol and stained with Coomassie brilliant blue R-250. Lane 1, *E. coli*; lane 2, transformed *E. coli* with expression vector (pET17b), which encodes *dirA*; 3, purified DirA and **B,** crude extracts of *C. diphtheriae* and purified DirA subjected to immunoblot analysis in the reducing condition. Lane 1, crude extracts of *C. diphtheriae*; lane 2, purified DirA.

purified DirA and immunoblot analysis of DirA showed in the lane 2 of Fig. 2B. For the crude extracts of *C. diphtheriae*, the same size protein band was detected by immunoblotting using the polyclonal antibodies against expressed DirA (lane 1, Fig. 2B).

The activity of DirA preventing the oxidative inactivation by MCO system containing either thiol or ascorbate was determined by checking the time-coursed inactivation of *E. coli* glutamine synthetase (Fig. 3). The DirA inhibited the inactivation of glutamine synthetase by thiol-mediated MCO system only (Fig. 3A) but not by the ascorbate MCO system (Fig. 3B). To distinguish the antioxidant specificity of two different MCO system, the MCO system containing both DTT and ascorbate was monitored for its ability preventing the oxidation (Fig. 3C). These results suggested that DirA did not inhibit the inactivation of glutamine synthetase by ascorbate-mediated MCO system and required the thiol compound to in-

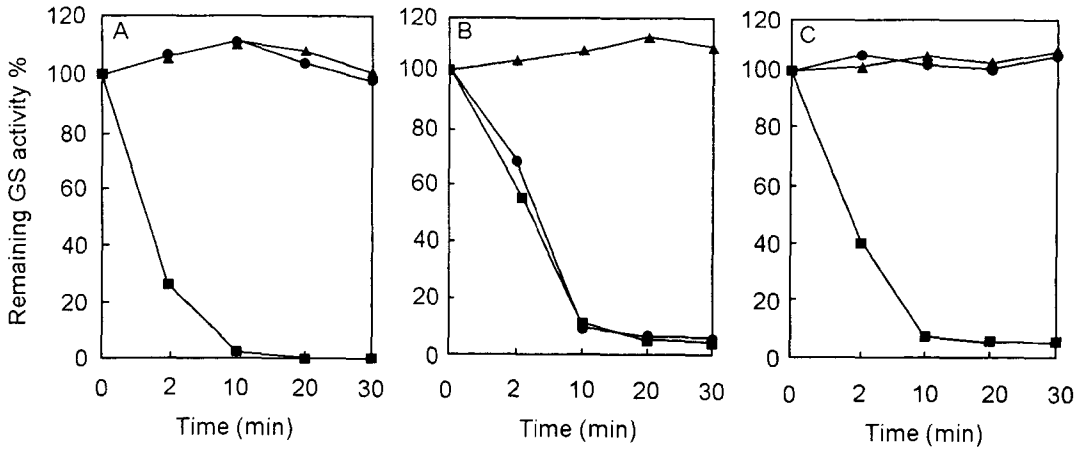

Fig. 3. Protection of glutamine synthetase by DirA. The inactivation mixtures containing 10 μg of gluamine synthetase, 3 μM $FeCl_3$ and 50 mM HEPES, pH 7.0, in a total volume 25 μl were incubated at 37℃. The reaction mixtures were assayed for the remaining activity of *E. coli* glutamine synthetase at various times. Additional reagents added were ■, None; ●, 0.25 mg/ml DirA; ▲, 1 mM EDTA. **A**, DTT/Fe^{3+} MCO system; **B**, Ascorbate/Fe^{3+} MCO system; **C**, DTT/Ascorbate/Fe^{3+} MCO system. 10 mM DTT added additionally in Ascorbate/Fe^{3+} MCO system.

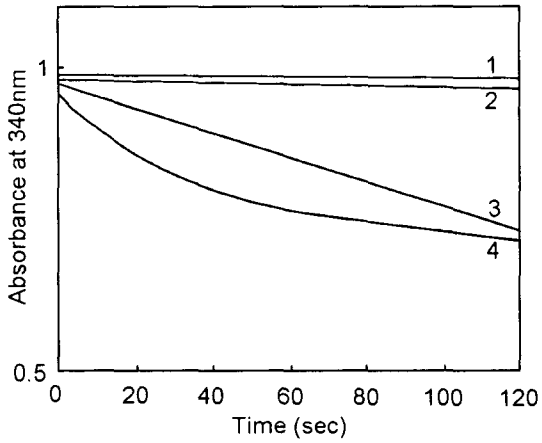

Fig. 4. Oxidation of NADPH by thioredoxin and thioredoxin reductase in the presence of DirA and hydrogen peroxide or cumene hydroperoxide. The reaction mixture contained 10 μg/ml thioredoxin, 112 μg/ml thioredoxin reductase, 0.2 mM NADPH, 100 mM HEPES, pH 7.0 in a total volume of 500 μl. Also added were 1, 1 mM H_2O_2; 2, 1 mM cumene hydroperoxide; 3, 1 mM cumene hydroperoxide + 100 μg/ml DirA; 4, 1 mM H_2O_2 + 100 μg/ml DirA.

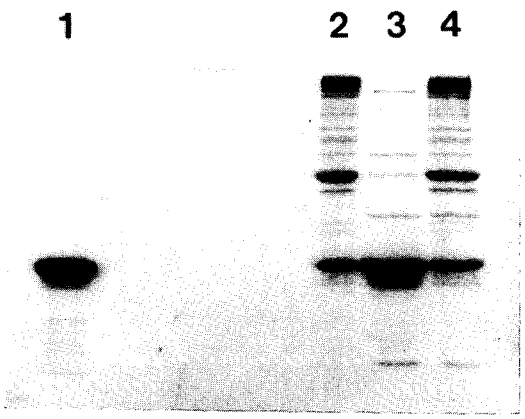

Fig. 5. Conversion of multimeric form of DirA to monomeric form by thioredoxin system Purified *C. diphtheriae* DirA (20 μg) were separated by SDS-PAGE on a 12.5% gel with 2-mercaptoethanol (lane 1) or without 2-mercaptoethanol (lane 2), and NADPH+ thioredoxin/thioredoxin reductase (lane 3) and thioredoxin/thioredoxin reductase (lane 4).

hibit the enzyme by MCO system such as DTT, 2-mercaptoethanol, and gluthatione. The DirA concentration which inhibited the inactivation of glutamine synthetase by 50% against DTT/Fe^{3+} MCO system and ascorbate/Fe^{3+} MCO system with DTT were 0.12 mg/ml, 0.15 mg/ml, respectively (data not shown).

The peroxidase activity of DirA toward H_2O_2 or cumene hydroperoxide in the presence of the thioredoxin system was monitored indirectly by following the decrease in A_{340}, attributable to the oxidation of NADPH (Fig. 4). The rate of

the peroxidase-dependent NADPH oxidation decreased with time. For the same concentration of peroxide, the decrease in rate was more rapid with cumene hydroperoxide than with H_2O_2.

To identify the presence of different forms of the DirA due to reductive activation by the reducing agent such as 2-mercaptoethanol or the thioredoxin system (NADPH, thioredoxin, and thioredoxin reductase), SDS-PAGE was used to analyze the products of such processes (Fig. 5). In the presence of 2-mercaptoethanol which was a reducing agent in the sample buffer, dimer and oligomer of DirA were converted to the monomer form as shown in the lane 1 of Fig. 5. The reduction of DirA disulfide bond by the thioredoxin system was determined by using SDS-PAGE in the nonreducing condition. Complete thioredoxin system also led to cleavage of the disulfide bridge in the DirA (lane 3, Fig. 5). The thioredoxin system without NADPH did not cause conversion of a dimer and oligomer to monomer as shown in the lane 4 of Fig. 5.

DISCUSSION

The *C. diphtheriae* is gram-positive straight or slightly curved rod shape living in the throat. It secreates its toxin into the damaged underlying tissues. For decades, it has been known that toxin production of *C. diphtheriae* is due to the viral genome within the organism (i.e., β-prophage) and the growth under iron-limited conditions and that non-toxic strains may be made toxic by exposing them to β-phage[10,12,13]. When *C. diphtheriae* cells were cultured in the growth medium containing iron chelating agent such as EDDA to remove iron, the cells increased production of proteins known as iron-repressible polypeptides with especially molecular masses of 64, 22, 20, 19, and 18 kDa. The DirA, a major iron-repressible polypeptide (198 amino acid residues, 22.8 kDa), was homologous to AhpC and TSA[10].

The antioxidant activity of DirA was not decreased during in MCO system and the DirA was not degraded by MCO system. Although DirA could not prevent oxidative damage of proteins caused by ascorbate/Fe^{3+} MCO system, it was not impaired by MCO system. Like TSA of *Saccharomyces cerevisiae*[6] and *Candida pseudotropicalis*[17], DirA could not inhibit ascorbate/Fe^{3+} MCO system. However, ascorbate/Fe^{3+} MCO system mediated oxidative damages of protein was completely prevented by DirA in the presence of reduced mercaptans such as DTT, 2-mercaptoethanol. Thus, it can be eliminated that DirA can not inhibit ascorbate/Fe^{3+} MCO system because of reactive species produced such as ascorbate radical in ascorbate system. If DirA required reduced mercaptans to remove reactive oxygen species, ascorbate MCO system can be inhibited by DirA in the presence of reduced mercaptans. The role of reduced mercaptans can be suggested as follow; an oxidized form of DirA produced during reduction of reactive oxygen can be converted to reduced form, catalytically active form, by reduced mercaptans. The protection mechanism of antioxidant protein has not been well described yet. But it was found recently that TSA of *Saccharomyces cerevisiae* has disulfide bond reducible by thiols and removes hydrogen peroxide in the presence of DDT[18].

It was known by a search of the GenBank database that DirA had strong homologies with a family of at least 50 antioxidant proteins including AhpC and TSA[9]. They belonged to peroxiredoxin family with thioredoxin peroxidase activity. The AhpC/TSA family had either one or two conserved cysteine residues[9]. Also DirA had four cysteine residues; two of them were coincided highly with conserved cysteine residues of AhpC and TSA[10]. This strong homology between the DirA and AhpC/TSA proteins suggested that DirA may be involved in limiting ROS damage to biological tissues and may have features of thioredoxin peroxidase, which reduced hydroperoxide (H_2O_2, ROOH) with Trx as an im-

mediate hydrogen donor[9]. According to Fig. 4 and Fig. 5, it was suggested that DirA had thioredoxin peroxidase activity and that sequencial redox reactions took place during the flow of electron from NADPH to alkyl hydroperoxide. But thioredoxin peroxidase activity of the DirA had lower than thioredoxin peroxidase activity of *Saccharomyces cerevisiae* because of using TR and Trx of *Saccharomyces cerevisiae*. Therefore, to understand thioredoxin peroxidase function of *C. diphtheriae,* it will be important to identify the physiolgical electron donor that suggests to be mediate the flow of electrons from NADPH to oxidized DirA to reduce H_2O_2 or alkyl hydroperoxide.

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=국문초록=

코리네박테리움 디프테리아 티올 특이성 항산화단백 DirA의 발현 및 특성

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전남대학교 화학과**

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효모의 티올특이성 항산화단백과 아미노산 서열상 상동성을 보이는 50 종류의 단백은 새로운 항산화 단백군을 형성하며 또한 병원성 미생물에도 널리 분포하고 있으나 이들 단백의 생화학적 및 생리적인 기능은 거의 알려져 있지 않은 실정이다. 본 연구는 병원성 미생물의 티올특이성 항산화 단백의 기능에 관한 연구로서 *Saccharomyces cerevisiae*의 TSA 및 *Salmonella typhimurium* alkyl hydroperoxide reductase의 AhpC subunit와 상동성을 나타내는 *Corynebacterium diphtheriae*의 DirA 유전자를 PCR 방법으로 클로닝하고 대장균에 발현시킨 후 정제하여 항산화 특성을 조사하였다. 정제된 DirA는 티올을 함유하는 금속촉매 산화계인 DTT/Fe^{3+}를 선택적으로 억제하였으며 티오레독신 의존성 과산화물 분해활성을 나타내었다. DTT/Fe^{3+} 금속촉매 산화계에 의한 효소의 불활성화를 50% 억제하는 DirA의 농도는 0.12 mg/ml로 효모 TSA 항산화활성의 약1/4 수준이었으며, 효모의 티오레독신계와 반응시켰을 때 과산화물 분해활성은 0.02 unit/mg로서 효모 TSA의 티오레독신 의존성 과산화물 분해활성의 1/20 수준이었다. 정제된 단백질을 이용하여 항체를 제조하였으며 이항체를 이용하여 *Corynebacterium diphtheriae*에서 발현됨을 확인하였다. 이러한 결과를 통하여 *Corynebacterium diphtheriae*의 병원성은 숙주세포의 방어기전인 백혈구에 의하여 생성되는 과산화수소 또는 다른 활성산소종을 제거하는 DirA작용과 연관이 있는 것으로 사료된다.

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†별책요청 저자