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The Effect of Translationally Controlled Tumor Protein (TCTP) of the Arctic Copepod *Calanus glacialis* on Protecting *Escherichia coli* Cells against Oxidative Stress

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Translationally controlled tumor protein (TCTP) is one of the most abundant proteins in various eukaryotic organisms. TCTPs play important roles in cell physiological processes in cancer, cell proliferation, gene regulation, and heat shock response. TCTP is also considered an important factor in the resistance to oxidative stress induced by dithiothreitol or hydrogen peroxide (H_2O_2). Arctic calanoid copepods have a variety of antioxidant defense systems to regulate the levels of potentially harmful reactive oxygen species generated by ultraviolet radiation in the Arctic marine ecosystem. However, information on the antioxidant activity of TCTP in the Arctic *Calanus glacialis* is still scarce. To understand the putative antioxidant function of the Arctic copepod *C. glacialis* TCTP (Cg-TCTP), its gene was cloned and sequenced. The Cg-*TCTP* comprised 522 bp and encoded a 174-amino acid putative protein with a calculated molecular weight of ~23 kDa. The recombinant Cg-TCTP (Cg-r TCTP) gene was overexpressed in *Escherichia coli* (BL21), and Cg-rTCTP-transformed cells were grown in the presence or absence of H_2O_2. Cg-rTCTP-transformed *E. coli* showed increased tolerance to high H_2O_2 concentrations. Therefore, TCTP may be an important antioxidant protein related to tolerance of the Arctic copepod *C. glacialis* to oxidative stress in the harsh environment of the Arctic Ocean.

Key words : Antioxidant, arctic copepod, *Calanus glacialis*, recombinant TCTP, TCTP

Introduction

The translationally controlled tumor protein (TCTP) is highly conserved among eukaryotes, and it was originally described as being a growth-related protein in mouse ascites and erythro-leukemic cells [4]. In several organisms, TCTPs are related to diverse cellular processes including apoptosis, microtubule organization, and ion homeostasis, and to interact with many proteins [4].

Oxidative stress is known to drive and support the aging process and the development of various diseases; therefore, there has been a growing interest on identifying genes that can protect cells from oxidative stress damaging effects. Several studies demonstrated that TCTP has important functions in cell growth and anti-apoptotic activity [6, 14, 23, 26]. In particular, upregulation of cellular TCTP levels induced by oxidative stress were found to affect the cellular protection against cell death. Indeed, TCTP upregulation induced by treatment with hydrogen peroxide (H_2O_2) or arsenic trioxide was observed in breast cancer [23]. However, in the case of another tumorigenic cell line (CHO-K1), H_2O_2 treatment did not enhanced TCTP levels [26]. Overall, the underlying mechanism of oxidative stress-promoted TCTP upregulation remains poorly understood.

Reactive oxygen species (ROS), such as H_2O_2, superoxide, and hydroxyl radicals, are produced in cells in the course of normal metabolism, as a result of various oxidative reactions. *In vitro* treatment of cells with exogenous H_2O_2 has been related to intracellular ROS production, with ROS accumulation inside cells triggering DNA strand breaks, the oxidation of lipids, and the decrease of intracellular antioxidants. Moreover, if the oxidative stress is low, cells may surrender to the cytotoxic effects of the accumulated ROS and die by either apoptosis or necrosis, according to the type of cell and microenvironment conditions [13, 25, 29].

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Polar coastal ecosystems have been threatened by increased ultraviolet B (UV-B) radiation due to ozone depletion [24]. UV-B radiation can penetrate significant biological depths in seawater and causes biological damage by altering the DNA in the nuclei of the cells [8]. Direct UV-B exposure has been associated with physiological and biological processes in Arctic amphipods [28], northern temperate zooplankton, and ichthyoplankton [7]. UV-B has been also linked to oxidative stress, as it can induce the generation of ROS in surface waters [1] and to influence the Arctic marine ecosystem [17].

Copepod species of the genus *Calanus* take part in a predominant proportion of zooplankton biomass in the Arctic Ocean, and they have been proposed as useful model organisms for toxicology, genetics, and molecular biology studies [2, 19, 26]. Indeed, copepods have provided interesting insights into stress reactions in gene profiling studies [16, 21, 22]. Interestingly, in order to regulate ROS, the Arctic calanoid copepods have a variety of antioxidant defense systems. However, to date, little is known on the potential antioxidant effect of TCTP in the Arctic *Calanus glacialis*.

In this study, the antioxidant activity of TCTP was analyzed in Arctic copepod *C. glacialis* in *Escherichia coli* cells under stress conditions driven by H_2O_2. Such information may provide additional insights on the antioxidant potential of TCTP.

Materials and Methods

Sample collection

C. glacialis was collected in July 2005 from the seawater around the Korea Arctic Research Station, Dasan, in Ny-Ålesund, Svalbard, Norway (79°N, 12°E). Samples were homogenized in TRIzol reagent for RNA extraction. The identification of this species was conducted by partial large subunit ribosomal DNA (LSU rDNA) sequence analysis [18].

Amplification of *TCTP*

Total RNA was extracted using easy-BLUE Total RNA Extraction kit (Intron, Seongnam, Korea), and was dissolved in RNase-free water. The isolated RNA was stored at −80°C until further use. To synthesize complementary DNA (cDNA), 2 μg of total RNA was used, and the cDNA was synthesized using the M-MLV Reverse Transcriptase kit (Enzynomics, Daejeon, Korea) according to the manufacturer's instructions. For recombinant protein expression, the open reading frame (ORF) of *TCTP* (Cg-TCTP) was amplified by polymerase chain reaction (PCR) as per the following conditions: 35 cycles of 94℃ for 30 sec, 46℃ for 30 sec, 72℃ for 50 sec, with a final extension at 72℃ for 10 min. Primers were designed from the expressed sequence tags of *C. glacialis*. The nucleotide sequences of the forward and reverse PCR primers were 5′ATGAAGATCTTCAAGGATGT‐3′ and 5′‐CTAGCACT TCTCCTCTTCAAGAC‐3′, respectively. The amplified fragment was purified using a PCR product purification kit (Intron, Seongnam, Korea).

Expression of and purification of recombinant Cg-TCTP

The PCR product was directly inserted into the pEXP5 TOPO TA vector (Invitrogen, Waltham, Massachysetts, USA), which was used to transform *E. coli* BL21 (DE3) cells. The cloning strategy was designed to induce the expression of TCTP from *C. glacialis* containing an additional C-terminal His6 tag (Cg-rTCTP). The transformants were spread onto Lauria‐Bertani (LB) agar plates containing 50 μg/ml ampicillin and incubated at 37°C, and recombinant cells were cultured on LB medium containing 50 μg/ml ampicillin. Protein expression was induced as the culture optical density (OD) reached 0.7 to 0.8 by the addition of 0.5 and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Optimal expression of Cg-rTCTP was achieved at 0.5 mM IPTG. Cg-rTCTP was directly analyzed on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (Bio-Rad, Hercules, California, USA). The protein samples were boiled for 10 min at 100°C before being loaded onto the gel. Subsequently, the histidine-tagged recombinant proteins were purified using an immobilized metal affinity column chromatography (Clontech, Kusatsu, Japan) according to the manufacturer's recommendations.

Immunoblotting

Upon the gel electrophoresis, the proteins were transferred to a PVDF membrane (Millipore, Cambridge, Massachysetts, USA). Afterwards, the membrane was blocked with 5% skim milk and incubated for 2 hr at room temperature with an Anti-His6 IgG (1:500; Roche, Buonas, Switzerland), followed by a peroxidase-conjugated AffiniPure F(ab′)2 fragment anti-chicken IgG (H+L) (1:20,000; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for additional 1 hr at room temperature. Next, the PVDF membrane was washed, and the SUPEX Western blot detection kit (Neuro-

nex, Providence, Rhode Island, USA) was used to visualize the protein signals. The respective images were detected with a luminescent image analyzer (LAS-3000; Fujifilm, Tokyo, Japan).

H_2O_2 tolerance bioassay

To test the sensitivity of *E. coli* cells to H_2O_2, Cg-rTCTP-transformed bacterial cells were grown until the OD range of the culture reached 0.7-0.8. Then, the cultures were induced with IPTG at a final concentration of 0.5 mM added to the LB medium containing ampicillin. H_2O_2 was serially added to the induced bacterial cells, ranging from 1 to 20 mM and cultured for 12 and 24 hr. A plate culture was used to test the diluted cells (ranging from 1:1 to 1:10), with 10 μl of each dilution being platted on LB agar medium containing 3 and 5 mM H_2O_2. Plates were then incubated at 37°C overnight. Bacterial cells expressing the empty vector were similarly plated as controls. Growth of the bacterial cells after incubation was compared between the control and experimental groups.

Results

Sequence analysis of *C. glacialis* TCTP

To evaluate the TCTP sequence, its ORF was obtained from the full-length *C. glacialis* cDNA. The resulting ORF comprised 522 bp, encoding 173 amino acids. Based on a BLAST search using the inferred amino acid sequence, Cg-TCTP showed high similarity of sequence identity with other eukaryotic *TCTP* genes. Alignment of the predicted Cg-TCTP sequence with the corresponding TCTP from eight different organisms showed a high degree of preservation over long-term evolution. According to the characteristic features of TCTPs, Cg-TCTP also comprised distinctly specified TCTP-1 and TCTP-2 signature, which represent the amino acid positions 45-56 and 129-152, respectively. Alignment of the basic amino acid-rich domain sequences revealed similarities with part of the microtubule-binding domain (MTB) and calcium-binding domain (CaB). Moreover, the Cg-TCTP amino acid sequence showed 71% homology with that of *Tigriopus japonicus*. In addition, alignment of Cg-TCTP with

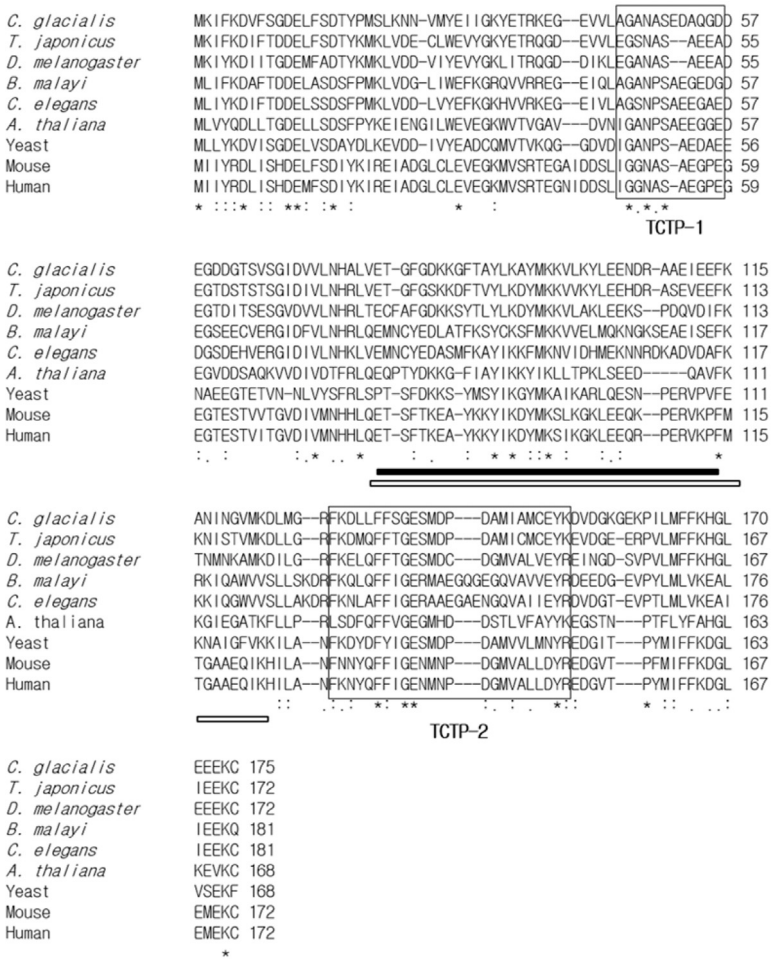

Fig. 1. Multiple sequence alignment of various TCTPs was determined using the ClustalW software. The boxes show the TCTP-1 and TCTP-2 signature regions. Calcium-binding (CaB) and microtubule binding (MTB) regions are indicated by dark and white bars, respectively.

TCTP homologue proteins of *T. japonicus* (accession AAR 88095), *Brugia malay* (XP_001897741), *Caenorhabditis elegans* (Q93573), *Drosophila melanogaster* (Q9VGS2), yeast (NP_594328), *Arabidopsis thaliana* (AAM66134), mouse (P14701), and human (NP_003286) was determined using ClustalW (Fig. 1).

Expression of *C. glacialis* TCTP

The cDNA of *C. glacialis* TCTP was inserted into a eukaryotic expression vector, which was used to induce the expression of a recombinant Cg-TCTP in *E. coli*. Analysis of the obtained Cg-sTCTP revealed a ~23 kDa protein on SDS-PAGE (Fig. 2A, lane 3), which was detected in low amounts in both insoluble and soluble fractions of the cell extracts (Fig. 2A, lane 2). The Cg-rTCTP expression in *E. coli* was detected by immunoblotting using a monoclonal antibody against His-tag (Fig. 2B).

Evaluation of Cg-rTCTP-transformed *E. coli* cells resistance to H_2O_2

To further confirm the antioxidant potential of Cg-rTCTP, *E. coli* cells expressing the recombinant protein or the empty vector alone were cultured in media containing 0-5 mM H_2O_2 for 24 hr. The survival rate of the cells transformed with the empty vector (control) was reduced by approximately 36-40% at 2 and 5 mM H_2O_2 compared with cells in the absence of H_2O_2, whereas Cg-rTCTP-transformed cell survival was largely unaffected by H_2O_2-induced toxicity (Fig. 3). In plate cultures containing 5 mM H_2O_2, which was a critical concentration for inhibiting cell growth, *E. coli* cells harboring *TCTP* and the empty vector did not survived (Fig. 4C). However, Cg-rTCTP-producing cells survived at a density of 1:5 in medium containing 3 mM H_2O_2 (Fig. 4B).

Additional analyses revealed that as soon as *E. coli* cells were exposed to H_2O_2, their logarithmic growth was impaired and the culture reached a stationary-like phase. When the H_2O_2 concentration was 3 mM, which was critical concentration for inhibiting cell growth in plate culture, the survival status of *E. coli* expressing Cg-rTCTP was maintained as compared with the control group (Fig. 5).

Discussion

This study is the first report on the antioxidant activity of TCTP from *C. glacialis* in the Arctic Ocean. Aquatic organ-

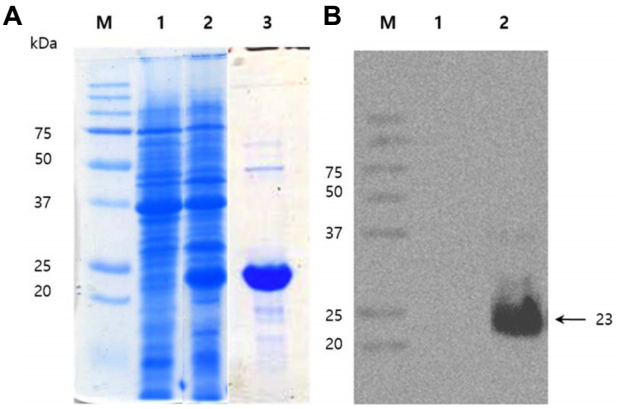

Fig. 2. Chromatographic purification and separation of Cg-TCTP protein. Optimal expression of Cg-rTCTP was achieved at 0.5 mM IPTG. Cg-rTCTP was directly analyzed on a 10% sodium dodecyl sulphate-polyacrylamide gel and stained with Coomassie brilliant blue. The histidine- tagged recombinant proteins were purified using an immobilized metal affinity column chromatography. A. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) results before (lane 1) and after (lane 2) induction. Recombinant Cg-TCTP protein purified on a Co-NTA column (lane 3). B. Immunoblot analysis of the recombinant protein (lane 1; control vector, lane 2; His-tagged Cg-rTCTP). The arrows indicate the TCTP recombinant protein.

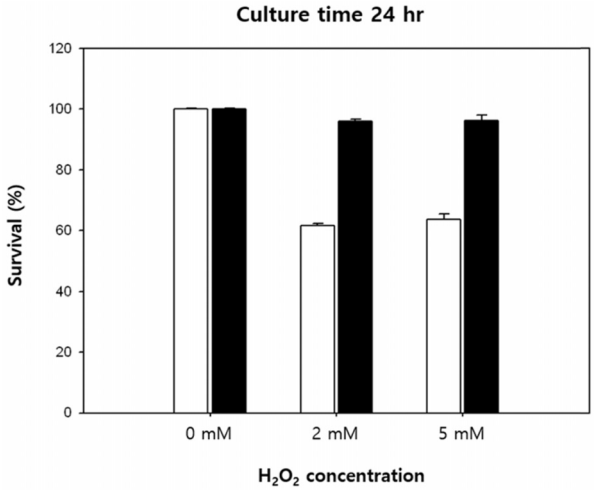

Fig. 3. Comparison of growth resistance to hydrogen peroxide (H_2O_2) between *Escherichia coli* transformed with pEXP5 TOPO TA vector containing *C. glacialis TCTP* and pEXP5 TOPO TA vector alone as control. In order to confirm the antioxidant potential of Cg-rTCTP, *E. coli* cells expressing the recombinant protein or the empty vector alone were cultured in media containing 0-5 mM H_2O_2 for 24 hr. Survival capacity of control *E. coli* (white box) and bacteria expressing the recombinant Cg-TCTP (black box) in the presence of difference concentrations of H_2O_2 for 24 hr.

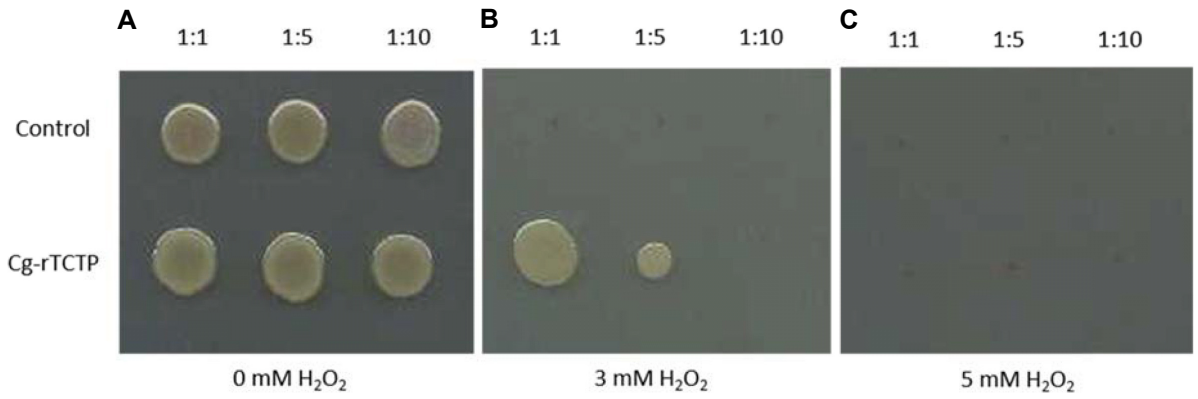

Fig. 4. Hydrogen peroxide (H_2O_2) tolerance of *E. coli* cells transformed with Cg-rTCTP gene. Cells were serially diluted (from 1:1 to 1:10) and were plated on LB agar containing different concentrations (0 mM, 3 mM, or 5 mM) of H_2O_2. After incubation for 12 hr at 37℃, the growth of cells harboring the empty vector was inhibited, whereas cells expressing Cg-rTCTP showed enhanced growth up to a dilution of 1:5 in the presence of 3 mM H_2O_2 (B).

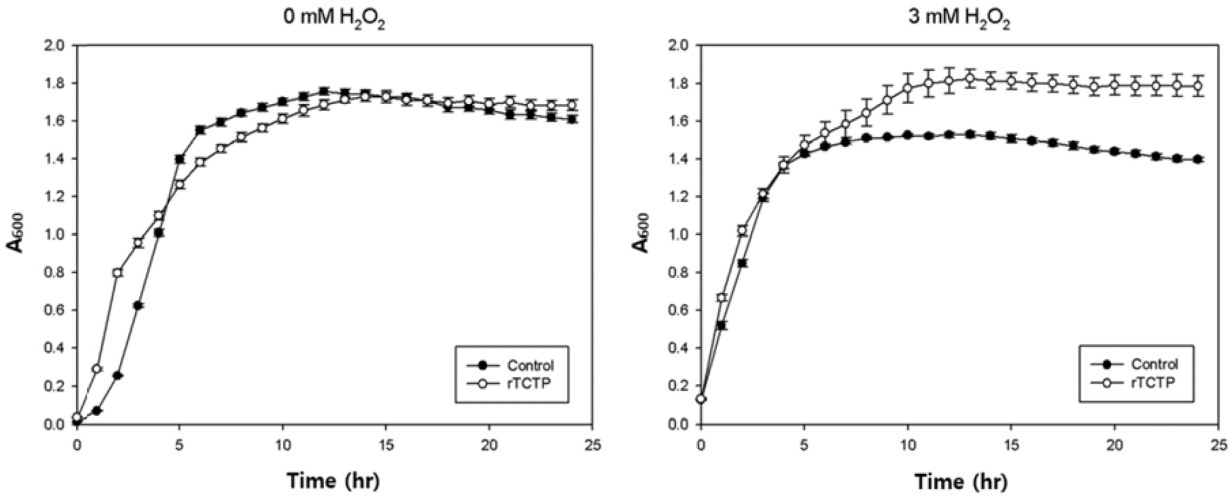

Fig. 5. Growth curves of control (pEXP5 TOPO TA vector control) and transformed (Cg-rTCTP) *E. coli* cells in LB medium containing ampicillin. A plate culture was used to test the diluted cells (ranging from 1:1 to 1:10), with 10 μl of each dilution being platted on LB agar medium containing 3 mM H_2O_2 for 24 hr. Bacterial cells expressing the empty vector were similarly plated as controls. Growth of the bacterial cells after incubation was compared between the control and experimental groups. Growth was monitored spectrophotometrically by following the optical density at A600 nm.

isms are frequently exposed to environmental factors (e.g., cold, heat, and osmotic conditions) and chemical stresses (e.g., endocrine disruptor chemicals and hydrocarbons). Therefore, organisms may respond to these stresses by activating different cellular mechanisms [20], with protective reactions being described in both prokaryotes and eukaryotes [27]. To date, few studies on gene expression and stress response from marine copepods have been published, although they are known to have defense mechanisms [20]. Indeed, previous reports have identified TCTP as an antioxidant enzyme in filarial parasites [15]. TCTP was shown to exhibit an extracellular function as a histamine release factor and to hold anti-apoptotic activity [6]. In addition, the expression of TCTP was upregulated under stress conditions, such as oxidative stress, heat shock, and the presence of metals.

In the present study, a recombinant protein derived from *C. glacialis* TCTP was expressed in *E. coli*. The complete nucleotide sequence of the Cg-*TCTP* was 522 bp in length, and its predicted encoded protein showed high similarity to the *T. japonicus* (71%). TCTP is highly conserved among eukaryotic organisms. Interestingly, Cg-TCTP was found to comprise two signature regions, named TCTP 1 and TCTP 2, which are structurally similar to the Mss4/Dss4 family [30].

Moreover, it also had basic amino acid-rich regions at 45-56 and 129-152 bp, which were very similar to the MTB and CaB domains, respectively. In accordance with this finding, TCTP was previously described as a calcium-binding protein that protects cells from calcium stress-induced apoptosis [15].

Gnanasekar and Ramaswamy (2007) confirmed that presence of three cysteines located in the central portion of the protein in filarial TCTPs and suggested that these cysteine residues in recombinant TCTP from *Brugia malayi* were critical for its antioxidant function. However, only two cysteines were identified at 149 and 175 position of the Cg-TCTP sequence [14]. Despite of this difference, the findings herein described clearly suggest that Cg-TCTP may hold antioxidant activity. Notably, Cg-rTCTP-transformed *E. coli* cells survived under oxidative stress conditions. In the broth culture, Cg-rTCTP expression enabled *E. coli* cells to survive in the presence of 5 mM H_2O_2 for 24 hr (Fig. 3). However, cell growth on a plate containing 5 mM H_2O_2 was completely inhibited for 12 hr. When the H_2O_2 level was increased to 10 and 20 mM, the growth of transformed and control cells was strongly inhibited (data not shown). It has been reported that recombinant TCTP homologs survive at a maximum concentration of 1.2 mM H_2O_2 [14]. However, in this study, it was confirmed that Cg-rTCTP-producing cells were capable of sustaining growth at high concentrations (3 mM) of H_2O_2. Control cells were incubated with purified TCTP at various concentrations (0, 0.007, 0.07, and 0.7 mg), which revealed that high concentration of TCTP (0.7 mg) enhanced bacterial growth, whereas in the absence of TCTP it was inhibited (data not shown). As shown for the control condition in Fig. 5, as soon as the induced cells were exposed to H_2O_2, growth entered a stationary-like phase. In contrast, the growth of Cg-rTCTP-transformed cells was maintained regardless of H_2O_2 presence, which was similar to the growth profile of control cells not exposed to H_2O_2. Altogether, these results suggest that the TCTP of *C. glacialis* may have a protective function against H_2O_2-induced damage. Nevertheless, additional studies are still necessary to explore the antioxidative protective mechanisms of *C. glacialis* TCTP.

Over the past few years, several studies have explored the biologically relevant functions of TCTP. For example, TCTP is a known target for artemisinin and has protective functions against heat stress [3, 25]. Artemisinin is a highly valuable drug used to treat malaria [11, 31] and is also known to exhibit anticancer and anti-inflammatory activities [9, 10, 32, 33]. Additionally, Eichhorn *et al*. (2013) suggested that the antimalarial activity of artemisinin may be related to molecular interaction with TCTP [12]. Furthermore, some researchers have suggested that *TCTP* mRNA is down-regulated in yeast cells exposed to heat shock [5], and that *TCTP* expression is modulated by stresses such as starvation and heat stress [6]. Contrasting to these findings, evaluation of the impact of artemisinin treatment and exposure to heat shock on the growth of Cg-rTCTP-transformed cells failed to show any significant effects (data not shown).

To confirm the antioxidant effect of Cg-TCTP, Cg-rTCTP-transformed *E. coli* cells were exposed to oxidative conditions using H_2O_2. Overall, the transformed cells showed increased tolerance against oxidative stress, indicating that overexpression of Cg-rTCTPs protect bacterial cells from oxidative damage caused by H_2O_2 exposure. Altogether, this study suggests that Cg-TCTP may have an important function as an antioxidant protein, and its antioxidant effect can be related to improved oxidative stress tolerance of *C. glacialis* in the harsh environment of the Arctic Ocean. Additional studies are still warranted to provide more information on function of TCTP in *C. glacialis* in the Antarctic and Arctic Oceans.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 북극 동물플랑크톤 *Calanus glacialis* TCTP (Translationally Controlled Tumor Protein)가 산화적 스트레스 상태에서 *E. coli* 세포의 저항성에 미치는 효과

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TCTP는 다양한 진핵생물에서 풍부하게 존재하는 단백질 중에 하나이며, 암, 세포 증식, 유전자 조절 등과 관련된 세포의 생리학적 기작에서 중요한 역할을 담당하는 것으로 알려져 왔다. 더구나, TCTP는 dithiothreitol (DTT)나 hydrogen peroxide (H_2O_2)에 의해 유도되는 산화적 스트레스에 대한 저항성에 관여하는 중요한 단백질로 주목받아 왔다. 한편, 극지역 서식 생물들은 강한 자외선에 의해 발생한 활성산소를 조절하기 위한 다양한 항산화 방어체계를 가지고 있다. 본 연구에서는 북극 동물플랑크톤 *Calanus glacialis*에서 분리된 TCTP가 산화적 스트레스 하에서 *E. coli* 세포의 저항성에 미치는 효과를 관찰하였다. *C. glacialis*에서 분리된 TCTP 유전자(ORF 522 bp) 서열을 분석하였고, 약 23 kDa의 재조합 단백질을 제작하였다. 관찰 결과, TCTP 재조합 단백질이 *E. coli* 세포에서 과발현되었을 때, 세포들은 H_2O_2에 의해 유도된 산화적 스트레스에 대한 저항성이 증가하는 것을 확인하였다. 본 관찰을 통해, 북극 *C. glacialis* TCTP 단백질의 항산화 조절자로서의 역할에 대한 가능성을 처음으로 제시하였다.