의학석사 학위논문

The inhibitory function of recombinant thioredoxin peroxidase of *Clonorchis sinensis* on Fas-mediated apoptosis in Jurkat T cells

간흡충 thioredoxin peroxidase 재조합 단백질의 Jurkat T 세포에서의 Fas-매개 세포자멸사에 대한 억제 효능 관찰

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A thesis of the Degree of Master of Science

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August 2017

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by

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(Directed by Prof. Young Mee Bae)

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ABSTRACT

Clonorchis sinensis is a carcinogenic liver fluke distributed in East Asia including Korea, China, Vietnam, and parts of Russia. Adult worm of *C. sinensis* can dwell in bile duct for over 10 years and its chronic infection promotes persistent oxidative stress such as reactive oxygen species (ROS). To ensure parasite' s long-term survival, anti-oxidant mechanism is important and thioredoxin peroxidase (TPX) is one of the redox enzymes used by parasites. Main function of TPX is neutralizing H_2O_2 and it also has additional biological functions including cell proliferation, intracellular signaling, iron metabolism, immune regulation, and apoptosis. Here, soluble recombinant TPX protein of *C. sinensis* (rCsTPX) was produced in *Eschericia coli* system and investigated its modulation of apoptosis. When rCsTPX was expressed, at first, most of the expressed protein was insoluble form because of low solubility of rCsTPX. To improve its solubility, the expression conditions such as isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, temperature, or duration of induction was changed. Eventually the proper expression condition that can get soluble form of rCsTPX was set at 20℃ for overnight with 100 μM IPTG. With this soluble rCsTPX, its effect of apoptosis was observed in macrophage cell line and T cell line. Only in T cells which is induced apoptosis with anti-Fas antibody, rCsTPX has shown regulatory effect. Caspase cascade was also confirmed by western blot analysis, and rCsTPX had influence on caspase-3 and -9 activation, but not caspase-8. In conclusion, these results suggest the better method of producing soluble rCsTPX and further imply that rCsTPX has

modulatory function of Fas-mediated apoptosis in T cells as a potential candidate for therapeutic application.

Keywords : *Clonorchis sinensis*, Thioredoxin peroxidase, Apoptosis, Anti-apoptotic effect

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LIST OF ABBREVIATIONS

APAF1 : Apoptotic protease activating factor 1

APCs : Antigen presenting cells

BIR : Baculovirus IAP repeat

C. sinensis : *Clonorchis sinensis*

CCA : Cholangiocarcinoma

DCs : Dendritic cells

DIABLO : Direct IAP binding protein with low Pi

DISC : Death-inducing signalling complex

ESP : Excretory-secretory products

FADD : Fas-Associated protein with a death domain

GPx : Glutathione peroxidase

IAPs : Inhibitors of apoptosis proteins

Ig : Immunoglobulin

IL : Interleukin

IPTG : Isopropyl-β-D-1-thiogalactopyranoside

LPS : Lipopolysaccharide

MOMP : Mitochondrial outer membrane permeabilization

NO : Nitiric oxide

Prx : Peroxiredoxin

rCsTPX : Recombinant *C. sinensis* thioredoxin peroxidase protein

RNS : Reactive nitrogen species

ROS : Reactive oxygen species

SMAC : Second mitochondria-derived activator of caspase

SOD : Superoxide dismutase

TGF-β : Transforming growth factor-beta

Th2 : T helper type 2

TMX : Thioredoxin transmembrane related protein

TPX : Thioredoxin peroxidase

TRAIL : TNF-related apoptosis-inducing ligand

Treg cell : regulatory T cell

Trx : Thioredoxin

INTRODUCTION

Clonorchis sinensis is a fish-borne liver fluke predominantly distributed in East Asia including Korea, China, northern Vietnam, and parts of Russia (1). Mammals including humans, dogs, and cats are infected when they consume raw or insufficiently cooked fish containing infective *C. sinensis* metacercariae. Therefore, endemicity of *C. sinensis* is deeply related with cultural habit of consuming raw or undercooked freshwater fish and also a result of the region' s social-ecological custom. When metacercariae of *C. sinensis* enter into the final hosts, they separate from the flesh of fish and excyst in the duodenum until they turn into juvenile stage (2). The juvenile flukes migrate into the host bile ducts and develop into adult flukes. It is estimated that more than 15 - 20 million people are infected, however the exact number are unknown because most of the infected humans have mild symptoms (3). Only heavily infected cases have general complications such as diarrhea, fever, epigastric pain, loss of appetite, and jaundice. Because of the minimal symptoms at the early stage of the infection and often missed diagnosis, adult worm of *C. sinensis* can dwell bile duct for 20 - 25 years (4). Major pathological changes of infected extrahepatic or intrahepatic bile ducts are mucin-secreting metaplasia, ductal dilatation, adenomatous hyperplasia of the biliary epithelium, fibrosis, periductal inflammation, and neoplasia or dysplasia of biliary cells (3). Moreover, there are many epidemiological and experimental evidence strongly indicated that *C. sinensis* is the etiological agents of cholangiocarcinoma (CCA) (5). CCA is the second largest reason to primary liver cancer and a devastating cancer

occurring from bile duct epithelial cells. *C. sinensis* was only classified as a probable carcinogen (group 2A) by contrast with another major liver fluke *Opisthorchis viverrini* by the International Agency for Research on Cancer (1). However, as considerable reports provided additional evidences about the possible correlation between CCA and clonorchiasis, *C. sinensis* was reclassified as a definite carcinogen (group 1) in 2009.

Infection with helminth stereotypically drive T helper type 2 (Th2) immune responses, in which $CD4^+$ T cells especially Th2 cells express the canonical cytokine interleukin (IL)-4, IL-5, IL-13 (6), and also eosinophils, basophils, mast cells and goblet cells are concomitantly involved with the response (7). Additionally responding B cells produce immunoglobulin E (IgE) and also IgG1. Moreover, after discovering new subsets of $CD4^+$ T cells, these new T-helper cells such as Th17 cells and regulatory T (Treg) cells were also described that these cell subsets play important roles in helminth infection (6,8). As shown in many human and experimental animal models, helminths are potential immune modulators which suppress responses that could result in their elimination in order to ensure their long-term survival (9). Because the Th2 response is a main mechanisms of protective immunity to helminths, general feature of helminth immuno-regulation is attenuated Th2 type immune responses including hypo-responsiveness of T cells and regulatory networks that suppress inflammatory responses (10). These networks include anergic T cells, increased portion of Treg cells, and high production of IL-10 and Transforming growth factor-beta (TGF-β) (11-13). As a result of Treg cells and IL-10, B cells also go through changes such as increasing production of specific isotype IgG4, which helps parasites to evade

destruction by host immunity (14). Because both the stimulation and modulation of the immune system is determined by antigen presenting cells (APCs), dendritic cells (DCs) are importantly involved with the modified Th2 immunity as one of the APCs. Helminth antigens are able to modulate maturation and activation of DCs (15,16). In helminth infection, what have down-modulated effect on host immunity is came from live parasites because hypo-responsiveness is alleviated after drug treatment (10). Therefore, excretory-secretory products (ESPs) from live helminths is considered as an substantial immune-modulator (17).

ESPs are complex-metabolic products, which contain different types of proteins, and smaller peptides; also nonprotein factors include glycolipid, glycan, and bio-active lipids (18). These substances are actively released by helminths and secreted depending on the physiological processes like certain life cycle stage of parasite' s body. Effects of ESPs on host immune responses were well-described in many studies. *Trichinella spiralis* muscle larvae ESPs suppressed DCs maturation induced lipopolysaccharide (LPS) from enterobacteria *in vitro*, and also induced the expansion of functional Treg cells (19). Also, adult *Haumonchus contortus* ESPs provoked a partial maturation and weak response of the DCs, and when ESPs were co-stimulated with LPS it reduced some aspects of the Th1/Th2 responses evoked by LPS (20). There are also several studies about protected effect of ESPs for autoimmune diseases. In study using septic mice, *T. spiralis* ESPs improved survival rate, enhanced bacterial clearance, reduced organ injury, and also reduced sepsis-inducing inflammatory cytokines (21). *T. spiralis* ESPs were also shown that prophylactic application of

ESPs ameliorated experimental autoimmune encephalomyelitis (EAE) through shifting to the Th2 type immune response and activating regulatory mechanisms like Treg cells (22). Regulatory effects of ESPs from *C. sinensis* were also reported in many studies. Cells exposed to *C. sinensis* ESPs cause diverse pathological responses such as proliferation (23), migration and invasion (24), apoptosis (25,26), and inflammation (27,28). As mentioned above, ESPs have functional mechanism to regulate host immunity to ensure longer survival of parasite itself. Therefore, there are a lot of approaches to discover defined products from ESPs as a candidate for therapeutic utilization. Also, many studies have used recombinant products of specified part of ESPs to figure out their regulatory functions and discover their features for future application. (Table. 1)

When *C. sinensis* worms reside in the host bile duct, they could be attacked by reactive oxygen species (ROS) such as superoxide radical anion ($O2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$). The ROS are derived from not only aerobic cellular metabolism but also host defense cells like innate immune cells including recruited leukocytes and resident macrophages (29). Produced ROS lead to radical chain reactions following damaging membranes, proteins, and nucleic acids. If these processes are not controlled, it could lead to parasite damage and death. Therefore, to cope with this oxidative stress, parasites need antioxidant mechanisms and count on related redox enzymes. (Fig. 1) Similar with most eukaryotic organisms, in the first line of defense, helminths possess superoxide dismutase (SOD) that decomposes $O2^{\cdot -}$ in the cytosolic and mitochondria (30). After $O2^{\cdot -}$ is transformed to H_2O_2, it can be

converted to oxygen and water by catalases. But parasitic platyhelminthes including Trematode and Cestode have no catalase (29). Instead, neutralization of peroxides in platyhelminthes depends on selenol- and thiol-based peroxidases: selenocysteine (Sec)-containing glutathione peroxidases (GPxs) and 2-Cys peroxiredoxins (Prxs). However, platyhelminthes also have reported that they have low levels of GPxs that are enough to metabolize SOD-generated H_2O_2 (31). Even in *C. sinensis*, it was reported that GPxs expression is restricted within a female-specific sexual organ or cells (32). Accordingly 2-Cys Prxs are considered to be the major enzymes for H_2O_2 removal in platyhelminth such as *Schistosoma japonicum* (33), *O. viverrini* (34), and *Fasciola hepatica* (35).

Thioredoxin peroxidase (TPX) is another term of 2-Cys Prxs and its main function is neutralizing H_2O_2 to water and lipid hydroperoxides to alcohols. Enzymatic activity of TPX is operated through redox cascade involving receiving electrons equivalents from thioredoxin (Trx) (36). In addition to their main function, TPX have various biological functions including cell proliferation, intracellular signaling, iron metabolism, immune regulation, and apoptosis (37-40). Notably, TPX overexpression delayed apoptosis in cells deprived of growth factors or serum, which did not cause an oxidative stress (39). Moreover, an increased TPX expression contributed to the protection against H_2O_2 induced apoptosis caused by Trx (41). According to the previous reports, it suggested that TPX have an essential role in the regulation of apoptosis.

Apoptosis is a genetically controlled, biochemically distinct type of programmed cell death that results during development, maintenance of cellular integrity, immune cell proliferation, and tissue

homeostasis in multicellular organisms (42). Apoptosis has critical role for the survival by getting rid of infected or damaged cells that may prevent normal function (43). Inappropriate apoptosis is associated with a number of parasitic infections along with the cause and development of diseases like aging and cancer (42). The morphologic characteristics of apoptosis are nuclear condensation or segmentation, DNA fragmentation, cytoplasmic shrinkage, and membrane contraction to condensed bodies (apoptotic bodies) (44). The development of membrane-bound apoptotic bodies later undergoes phagocytosis by adjacent cells without associated inflammation.

Apoptosis is initiated by a various stimuli, like the death ligands to their specific surface receptors of the tumor necrosis factor (TNF) receptor (TNFR) family such as Fas, TNF-related apoptosis-inducing ligand (TRAIL) receptors, or TNFR (TNFRI and TNFRII), or by other stimuli such as irradiation, chemotherapeutic agents, growth factor withdrawal, viral infection, or ROS etc. (42, 44). According to these initiation variety, there are two best-understood activation mechanisms: extrinsic pathway (death receptor pathway) and intrinsic pathway (mitochondrial pathway, cause an increase in mitochondrial membrane permeability). (Fig. 2) Recently, besides these two mechanisms, there are additional pathways that are mediated by endoplasmic reticulum (ER) stress or perforin/granzyme (45). However, all these apoptotic pathways converge on the common terminal pathway that is mainly activation of the caspases, a family of cysteine-dependent aspartate proteases (39). Caspases are assorted into initiator caspases like caspase-8 and -9, and effector caspases such as caspase-3, -6, and -7. This classification is based on their position in apoptotic pathway signaling cascades (45). They are inactive

pro-caspases form in healthy cells, and when initiator caspases are activated by auto-cleavage after apoptotic stimuli, downstream proteins including effector caspases become activated following proteolytic cleavage. In accordance with pathway, different initiator caspases become activated: caspase-8 is activated after formation of death receptor-mediated signaling complexes (extrinsic pathway), and caspase-9 become activated following apoptotic stimuli and formed apoptosome that is a multi-protein complex included with several proteins from mitochondria (intrinsic pathway) (39, 45). Proteins formed apoptosome are cytochrome c and other pro-apoptotic molecules, and their release from mitochondria is mediated Bak and Bax, members of the Bcl-2 protein family. Bcl-2 family are involved with creation pores in the outer membrane of mitochondria, therefore, pore formation is controlled by the anti-apoptotic molecules like Bcl-2 and pro-apoptotic members (such as Bim or Bid) of the Bcl-2 protein family respectively (39). Subsequently, the effector caspases cleave essential cellular components to make cell demolished. There are molecules that are able to suppress activated caspases, inhibitors of apoptosis proteins (IAPs) (46). IAPs are molecules that have the baculovirus IAP repeat (BIR) domain and there are eight IAPs in humans, carrying between one and three BIR domains. Proteins emitted from mitochondria include second mitochondria-derived activator of caspase (SMAC)/direct IAP binding protein with low Pi (DIABLO) or apoptosis-related protein in TGF-β signaling pathway (ARTS) and these inhibit IAPs leading apoptosis (47).

There are several studies about antioxidant enzymes of parasite. For example, Trx from *O. viverrini* has been characterized and expressed as a recombinant protein following evaluation of

enzymatic function (48). Also TPX (also termed 2-Cys Prx) of *F. gigantica* has been analyzed about its expression in the worm and antioxidant activity (49). Moreover, there is a report about goat immunization with recombinant Prx of *F. hepatica* (50). In this study, Prx-immunized group showed lower histological damage, reduced infiltration of $CD4^+$, $CD8^+$, IFN-γ^+, TCR^+, $CD2^+$, and IL-4^+ cells in hepatic lesions. Antioxidant enzymes of *C. sinensis* have been also characterized. There is a study that showed histological distribution and expression patterns of two form of 2-Cys Prx (TPX) (32), and the thioredoxin transmembrane related protein (TMX) which is a member of thioredoxin superfamily is identified and immunological characterized (51). Though several antioxidant enzymes were identified and functionally studied, among these enzymes, TPX (2-Cys Prx) has not been studied about its effect on apoptosis. Here, recombinant *C. sinensis* TPX (rCsTPX) was expressed in *Eschericia coli* (*E. coli*) system and observed its effect of apoptosis in macrophage cell line and T cell line. Also the improved recombinant protein expression condition of rCsTPX in a soluble form was discovered to make the protein become more applicable form as a potential therapeutic reagent.

Table. 1. Recombinant products of helminth parasites and their modulatory functions.

Components	Origin	Modulatory outcomes	References
Cystatin with cysteine protease inhibitor activity (rAl-CPI)	*Ascaris lumbricoides*	Reducing inflammation in a mouse model of DSS-induced colitis and modulating the expression of cytokines	(52)
Ferritin heavy chain (*Cs*FHC)	*Clonorchis sinensis*	Triggering free radical-mediated NF-κB signaling which is important factor in the chronic inflammation	(28)
Secreted phospholipase A2 (MBP-CssPLA2)	"	Activation of JNK signaling and accumulation of collagen at hepatic stellate cells	(53)
RNASET2 (r*Cs*RNASET2)	"	Modulation DCs maturation and triggering Th2 immune responses	(54,55)
Severin (r*Cs*severin)	"	Inhibition apoptosis	(25)
Sigma class glutathione transferase (rFhGST-si)	*Fasciola hepatica*	Suppressing Th17 immune responses	(56)
Thioredoxin-1 (recombinant *Ov*-Trx-1)	*Opisthorchis viverrini*	Inhibiting oxidative stress-induced apoptosis of bile duct epithelial cells	(57)
Sj16 (rSj16)	*Schistosoma japonicum*	Inhibiting LPS-induced DCs maturation and cytokine production	(58)

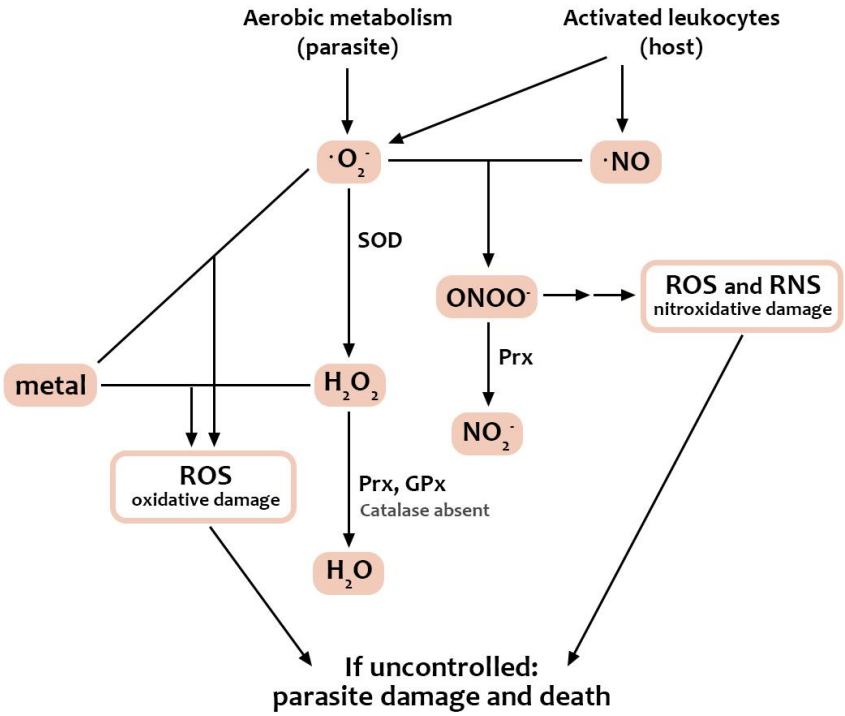

Fig. 1. Simplified map of oxidants and enzyme mechanisms of platyhelminth antioxidant mechanisms. As a consequence of aerobic metabolism, superoxide radical anion ($O_2^{\cdot -}$) is able to give rise to ROS and nitric oxide (·NO) which are generated by activated leukocytes can bring RNS. SOD decomposes $O_2^{\cdot -}$ into H_2O_2 and this is reduced by Prx and GPx, not catalase because catalase is absent in platyhelminth parasites. (Figure adapted from Dowling DJ et al.) (29) ·NO, nitric oxide; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; Prx, peroxiredoxin; GPx, glutathione peroxidase; ROS, reactive oxygen species; RNS, reactive nitrogen species.

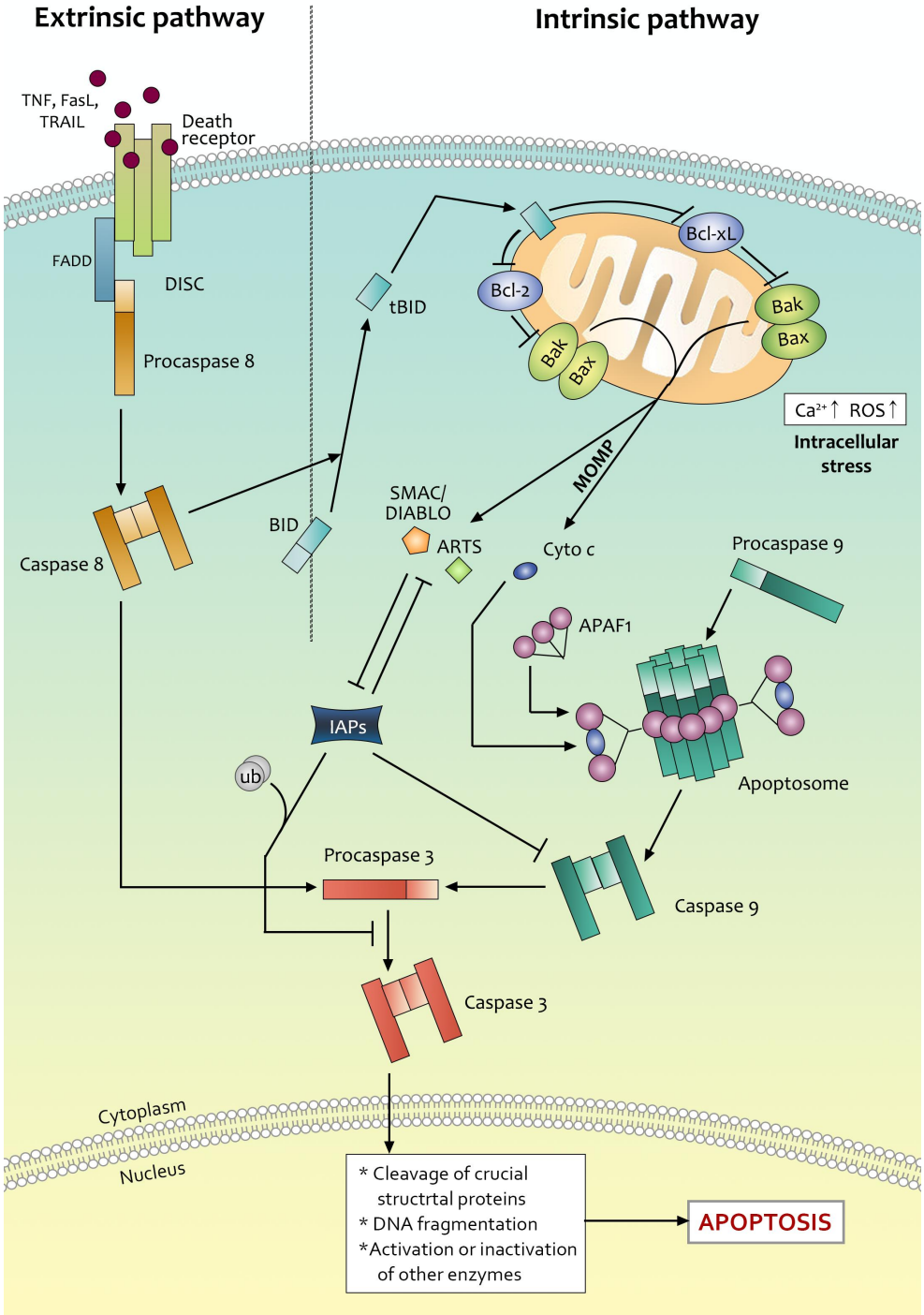
Extrinsic pathway
Intrinsic pathway
TNF, FasL, TRAIL
Death receptor
FADD
DISC
Procaspase 8
Caspase 8
tBID
BID
Bcl-xL
Bcl-2
Bak
Bax
Bak
Bax
Ca2+↑ ROS↑
Intracellular stress
MOMP
SMAC/ DIABLO
ARTS
Cyto c
Procaspase 9
APAF1
IAPs
ub
Apoptosome
Procaspase 3
Caspase 9
Caspase 3
Cytoplasm
Nucleus
* Cleavage of crucial structrtal proteins
* DNA fragmentation
*Activation or inactivation of other enzymes
APOPTOSIS

Fig. 2. The extrinsic (death receptor) pathway and intrinsic (mitochondrial) pathway of apoptosis. There are two activation mechanisms of apoptosis: extrinsic pathway and intrinsic pathway. In both pathways, initiator caspases (caspase 8 and 9) are stimulated within specific molecular platforms and become able to activate effector caspases (caspase 3). The major links between extrinsic and intrinsic pathway is provided by the protein BID, which can inhibit the function of Bcl-2 like proteins following MOMP. (Figure adapted from paper written by Galluzzi L. et al. and added) (59) FADD, Fas-Associated protein with a death domain; DISC, death-inducing signalling complex; tBID, truncated BID; MOMP, mitochondrial outer membrane permeabilization; APAF1, apoptotic protease activating factor 1; SMAC, second mitochondria-derived activator of caspase; DIABLO, direct IAP binding protein with low Pi; ARTS, apoptosis-related protein in TGF-β signaling pathway; IAP, inhibitors of apoptosis proteins.

MATERIALS AND METHODS

1. Preparation of recombinant *C. sinensis* TPX protein

1-1. Preparation of competent *E. coli* BL21 cells

To prepare recombinant *C. sinensis* TPX protein (rCsTPX) in *E. coli* system, *E. coli* BL21 cells were prepped as competent cells before transformation of plasmid DNA. The competency was obtained by $CaCl_2$ method. To get large volume of cells, BL21 cell (RBC, RealBio-tech, Taiwan) 1 vial was inoculated into 5 ml Luria-Bertani (LB) media and cultured overnight at 37℃ with shaking. Cultured cells were transferred to 100 ml media and cultured 2 - 2.5 hours at 37℃ with shaking until the $O.D_{550}$ reached to 0.3 - 0.4. After culture, cultured cells were incubated on ice for 5 minutes immediately. Keeping 4℃ of bacterial cells, it was recovered by centrifugation at 2500 rpm for 8 minutes and pellet was re-suspended with 30 ml TFB I solution (100 mM $RbCl_2$, 50 mM $MnCl_2.4H_2O$, 320 mM Potassium acetate, 10 mM $CaCl_2.2H_2O$, and 15% glycerol). Cells were centrifugated again at 2500 rpm for 8 minutes at 4℃ and pellet was re-suspended with 4 ml ice-cold TFBII solution (10 mM MOPS, 10 mM $RbCl_2$, 75 mM $CaCl_2.2H_2O$, and 15% glycerol). Cells were aliquoted in a small volume and freezed at -70℃.

1-2. Transformation of *C. sinensis* TPX cDNA into *E. coli* cells

Previously, in my lab, *C. sinensis* TPX cDNA (CS-TPX cDNA) was cloned into pQE-30 vector (Qiagen, Germany) with restriction

enzyme *Bam*HI and *Hind*III. (Fig. 4A) Using this resulting recombinant plasmid, cloned vector was transformed into *E. coli* BL21 competent cells using heat shock. CS-TPX pDNA 5 ㎕ was put into 50 ㎕ of BL21 competent cells and incubated on ice for 30 minutes. After incubation, it was incubated for 2 minutes at pre-heated 42℃ heating block. To minimize damage of cells, heated cells were incubated for 2 minutes on ice. Cells were inoculated to LB agar plate and cultured overnight at 37℃ incubator.

1-3. CS-TPX pDNA extraction and confirmation of transformation by agarose gel electrophoresis

After culture, CS-TPX inserted colonies were picked from LB agar plate and confirmed by gene using restriction enzyme. Inserted pDNA was extracted using FavorPrep™ Plasmid Extraction Mini kit (Favorgen Biotech Corp, Taiwan). Briefly, grown bacterial culture cells were harvested and re-suspended with buffer containing RNase A in the kit. Cells were lysed and neutralized with solution in the kit and pDNA was extracted using column matrix. Extracted pDNA sample and 6X DNA sample buffer were mixed, and loaded on 1% agarose gel with DNA ladder marker (1Kbp DNA Ladder Marker, Ready-to-use; Elpis Biotech, Korea). Running was performed at 100 V for 30 minutes and image of gel was captured with UV light using MiniLumi (DNR Bio-Imaging systems, Israel). Image was analyzed using the GelCapture software (DNR Bio-Imaging Systems).

1-4. Expression of recombinant CS-TPX in *E. coli* cells and determination of solubility

Picked colonies from LB agar plate were cultured in LB medium containing 100 ㎍/ml ampicillin at 37℃ overnight with shaking. Next day, dilute the non-induced overnight culture 1:20 (e.g., inoculate 10 ml medium with 500 ㎕ overnight culture) with fresh pre-warmed LB medium containing 100 ㎍/ml ampicillin. It was grown at 37℃ with shaking until the $O.D_{600}$ reached to 0.5 - 0.7. By adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), recombinant CS-TPX protein was induced at 37℃ with vigorous shaking for 4 hours. Expression of recombinant protein was checked in 10% SDS-PAGE gel running with coomassie blue staining. To increase the solubility of CS-TPX and efficiency of after purification, induction condition was changed. When protein was induced, 100 ㎛M IPTG was added and induced at 20℃ overnight with vigorous shaking.

To determine of recombinant CS-TPX solubility, the cells were harvested by centrifugation at 13000 rpm for 20 min after induction. Cell pellet was re-suspended by imidazole-containing lysis buffer (10 mM imidazole, 500 mM NaCl, and 50 mM NaH2PO4, pH8.0) with 1 mg/ml of lysozyme and kept in ice for 30 minutes. For complete lysis, additional chemicals were added (0.1% NP-40, 0.1 mM EDTA, and 1 mM PMSF lysed in EtOH). After incubation in ice, lysate was sonicated 5 seconds, 8 times with 30 seconds pauses at 200 – 300 W. Lysate was on ice at all times during sonication. After sonication, lysate was centrifuged at 15000 rpm at 4℃ for 20 minutes. Supernatant (cleared lysate) was collected as soluble protein, and pellet was resuspended in lysis buffer and collected as insoluble

protein. Soluble and insoluble protein were analyzed by SDS-PAGE gel running with coomassie blue staining.

1-5. Purification of rCsTPX

Purification of rCsTPX was performed using Ni-NTA Spin column (Qiagen). Soluble rCsTPX was made by setting specific expression condition, protein was purified under native conditions. Ni-NTA spin column was equilibrated with 600 ㎕ lysis buffer before purification. Cleared lysate 600 ㎕ containing 6x His-tagged rCsTPX was loaded onto the pre-equilibrated column and centrifuged at 1600 rpm for 5 minutes. Flow-through was collected for SDS-PAGE analysis. Ni-NTA column was washed twice with 600 ㎕ washing buffer (20 mM imidazole, 500 mM NaCl, and 50 mM NaH_2PO_4, pH8.0) by centrifugation at 2900 rpm for 2 minutes. After washing, protein was eluted with 200 ㎕ elution buffer (500 mM imidazole, 500 mM NaCl, and 50 mM NaH_2PO_4, pH8.0) by centrifugation at 2900 rpm for 2 minutes. Eluted protein and flow-through were analyzed by SDS-PAGE gel running with coomassie blue staining. Before using eluted protein, it was filtered with 0.22 ㎛ syringe filter (Merk Millipore, USA) and concentration of protein was measured by Pierce BCA Protein assay kit (Thermo Fisher Scientific Inc., USA). Absorbance was read at 540 nm by Tecan infinite M200 pro (Tecan, Switzerland) with Tecan i-control software.

2. Western blotting

Recombinant protein was separated by 10% SDS-PAGE gel, and

transferred to a PVDF membrane (Merk Millipore). Briefly, protein dual color standards marker (Bio-rad, USA) and samples which are mixture of cell lysate and 4X sample buffer were loaded into gel and it was run at 110 V for 1.5 - 2 hours. After gel running, gel was set between 3M paper and sponge with PVDF membrane that is pre-rinsed with methanol for 1 minute. To transfer, gel and membrane were run at 100 V for 1 hour. Before antibody staining, membrane was blocked with 5% skim milk and washed 3 times for 20 minutes with 0.1% Tween 20-containing tris-buffered saline (TBS-T) pH 7.4. Anti-6x His tag antibody (H-3) (Santa Cruz Biotechnology, USA) was used to detect 6x His tag recombinant protein from cell lysate at 1:2000 dilution with 5% skim milk TBS-T under 4℃ for overnight. Anti-*C. sinensis* TPX sera were used to detect rCsTPX from the antigens at 1:2000 dilution with 5% skim milk TBS-T under 4℃ for overnight. After washing 3 times for 20 minutes with TBS-T, anti-mouse-IgG conjugated with horseradish peroxidase (HRP) (Dako, Denmark) were used at 1:2000 dilution with 5% skim milk TBS-T for 1 hour at room temperature.

For caspase analysis, cells were lysed with RIPA buffer (Thermo Fisher Scientific Inc.) containing 1 mM PMSF. Briefly, cultured cells were harvested and washed with PBS twice. After washing, 1.5 times volume of pellet of RIPA buffer with PMSF was added and pellet was re-suspended. Re-suspended pellet was incubated for 20 minutes at -20℃ and centrifugated at 13000 rpm for 1 minute. Supernatant was collected for after protein analysis and concentration was measured with BCA protein assay kit (Thermo). Also loading protein amounts were calculated to be around 20 - 25 ㎍. Samples were loaded on 10% SDS-PAGE gel and then transferred on PVDF

membrane. Antibody to precursor and mature forms of caspase-3, -8 (Santa Cruz Biotechnology), caspase-9 (Cell Signaling Technology, USA) and actin (Bethyl, USA) were used at 1:1000 dilution with 5% skim milk TBS-T under 4℃ for overnight. After washing 3 times for 20 minutes with TBS-T, anti-mouse-IgG conjugated with HRP (Dako) and anti-rabbit-IgG conjugated with HRP (Bethyl) were used at 1:2000 dilution with 5% skim milk TBS-T for 1 hour at room temperature. After antibody staining, membrane was washed 3 times for 20 minutes with TBS-T and visualized by ECL Western blotting detection reagents (Thermo Fisher Scientific Inc.) and LAS-4000 mini (GE health care life sciences, USA) with ImageReader LAS-4000 software.

3. Cell culture

The murine macrophage cell line RAW 264.7 and human T cell line Jurkat were cultured at 37℃, 5% CO_2 in complete Dulbecco Modified Eagle Medium (DMEM) (Welgene, Korea) and Roswell Park Memorial Institute (RPMI) medium (Welgene), respectively, supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotics (Gibco). RAW 264.7 cells with 5 X 10^5 cells per well were cultured within 6-well plate for 24 hours with or without 100 ng/ml LPS and 100 ng/ml rCsTPX for flow cytometry. Jurkat cells with 1 X 10^5 cells/ml media were cultured for 2, 4, or 6 hours with or without 80 ng/ml anti-Fas (CD95) (human) mAb (CH-11) (MBL, Japan) or 1, 2, 4 mM Etoposide (Sigma-Aldrich, USA), and 100 ng/ml rCsTPX for flow cytometry. After proper culture for each study, the cells and culture supernatants were harvested for further analysis.

4. Flow cytometry

Harvested cells were washed with phosphate-buffered saline (PBS) containing 0.05% NaN_3 and then stained with FITC-Annexin V (BD Biosciences, USA) monoclonal antibody and Propidium Iodide (PI) staining solution (BD Biosciences). For staining, Annexin V binding buffer (BD Biosciences) was used and cells were incubated for 15 minutes at room temperature with antibody. After staining, cells were analyzed with a FACSCalibur multicolor flow cytometer (Becton-Dickinson Biosciences, USA) and electric fluorescence intensity was analyzed using the Cell Quest software (Becton-Dickinson Biosciences) as well as Flowjo7.6.2 software (TreeStar, USA).

5. Statistical analysis

Statistical analysis was performed using independent samples by t-test. Less than 0.05 of P value was considered as statistically significant and data were represented by mean + standard error using Prism 5.0 software (GraphPad Software, Inc., USA).

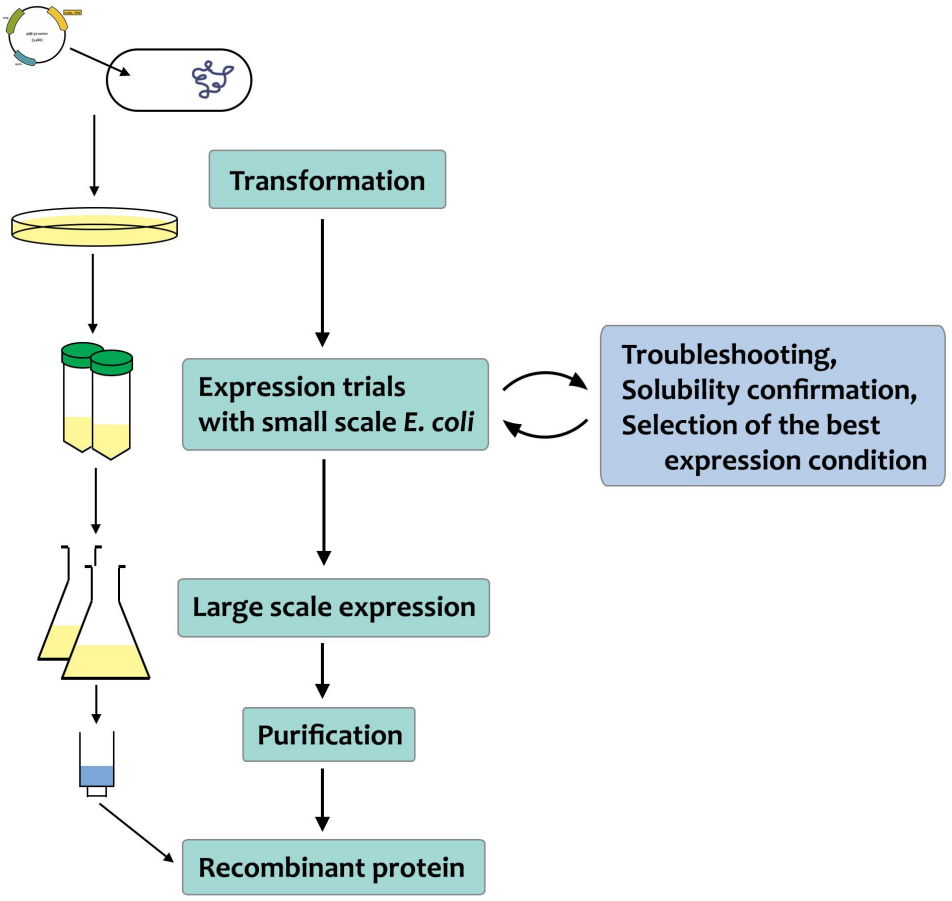

Fig. 3. Simplified workflow chart of recombinant protein expression.

RESULTS

Expression of recombinant *C. sinensis* TPX in *E. coli* system

To express rCsTPX, pQE-30 vector previously cloned with CS-TPX cDNA (Fig. 4A) was transformed into *E. coli* BL21 competent cells by heat shock. Transformation was confirmed by assuring size of pDNA of transformed *E. coli* cells. After induction, pDNA was extracted from cultured cells and incubated with restriction enzyme (*Bam*HI and *Hin*III). After cutting with enzyme, pDNA was analyzed with agarose gel electrophoresis and expected size (585bp) of DNA band was confirmed. (Fig. 4B) Also, extracted pDNA was sequenced to compare with sequence of *C. sinensis* TPX registered at Genbank. (Genbank No. HQ216221) Comparing with registered gene sequence, it was confirmed that CS-TPX gene in extracted pDNA was matched. (Fig. 5)

Transformed BL21 cells were induced to produce recombinant TPX protein with 1 mM IPTG at 37℃ for 4 hours of cultivation. Expression of protein was confirmed by SDS-AGE analysis. As shown in Figure 6A, the emphasized band with the expected size (23kDa) was detected on 10% gel compared to non-induced cell lysate. To assure that this band was recombinant protein resulting from transformation of 6x His tag CS-TPX cloned vector, western blotting with anti-6x His tag antibody and anti-CS-TPX polyclonal antibody was performed and proper size of band was detected. (Fig. 7)

Confirmation of rCsTPX solubility and setting specific induction conditions to improve solubility of rCsTPX

After confirmation of rCsTPX expression, *E. coli* cells were lysed to check the solubility of expressed protein. After induction with 1 mM IPTG at 37℃ for 4 hours, most of the expressed protein was insoluble form as inclusion bodies. (Fig. 6B) To improve its solubility, some conditions of induction were changed and tested. Changed conditions were concentration of IPTG, temperature, and duration of induction. Therefore, with 100 ㎛M IPTG, cells were induced at 4, 20, and 37℃ for 4 hours or overnight. (Fig. 8) First, changed concentration of IPTG was not effective to improve solubility because there was no improved soluble fraction band on SDS-PAGE results of induction with 100 ㎛M IPTG at 37℃. (Fig. 8C) Cultivation at 4℃ was excluded since expression level itself was low. (Fig. 8A) With same concentration of IPTG, expression of recombinant protein as well as soluble form of protein was more at 20℃ than 37℃. Also as duration of induction was longer, the level of expressed soluble protein was augmented. (Fig. 8B) As these results, temperature was critical point to improve solubility and therefore proper condition of induction was set for later experiment; at 20℃ with 100 ㎛M IPTG for overnight.

Purification of recombinant *C. sinensis* TPX

With improved condition of induction, rCsTPX was expressed in large volume and another confirmation was able by comparing with

SDS-PAGE results of cell lysate that cells was transformed with only pQE-30 vector as a control group and went through same induction processes. (Fig. 9) Also this was confirmed again by western blotting with anti-6x His tag antibody and anti-CS-TPX polyclonal antibody. (Fig. 7) Induced soluble rCsTPX was purified using the Ni-NTA spin columns under native conditions. (Fig. 10) Purified protein was filtered with 0.22 μm syringe filter and concentration of protein was measured to treat into cell culture experiments.

The effect of rCsTPX on apoptosis in macrophage cell line and T cell line

With soluble rCsTPX, in order to observe its effect of apoptosis in macrophage cell line and T cell line, cells were treated with or without rCsTPX and different substances that induce apoptosis. Apoptotic cells were measured by flow cytometry with FITC Annexin V/PI staining. First, mouse macrophage cell line RAW264.7 cells were treated LPS 100 ng/ml to induce apoptosis. LPS causes severe immunological reactions, following cell apoptosis (60). Therefore, cells were treated LPS and/or rCsTPX and percentage of apoptotic cells were measured; percentage of Annexin V+/PI- population is early apoptotic cells and Annexin V+/PI+ population is late apoptotic cells. As shown in Fig. 11, rCsTPX did not significantly regulate LPS-induced apoptosis in RAW264.7 cells. Neither early apoptotic cells nor late apoptotic cells were effected by rCsTPX.

Jurkat T cells were also treated with rCsTPX and apoptosis of

these cell were induced by two different stimuli; etoposide and anti-Fas (CD95) Ab. Etoposide is an antitumor reagent which causes apoptosis in normal and tumor cell lines. Anti-Fas Ab is also able to induce apoptosis by binding Fas that is a cell-surface receptor protein and mediates apoptosis. When rCsTPX was treated with etoposide in Jurkat T cells, there was no significant effect on apoptotic cell percentages. (Fig. 12) Etoposide was treated three different concentrations (1, 2, and 4 mM), however all of these group had no regulatory effect. On the other hand, anti-Fas Ab-induced apoptotic cells were reduced when rCsTPX was treated. (Fig. 13) Cells were incubated for 2, 4, and 6 hours after anti-Fas Ab and/or rCsTPX treatment, and all three group showed significant results. Taken together, rCsTPX had regulatory effect on anti-Fas Ab-induced apoptosis in T cells, but not on LPS-induced apoptosis in macrophages and etoposide-induced apoptosis in T cells.

Identification of caspases regulated by rCsTPX in Fas-mediated cell death

In order to confirm of caspase activation, cultured cells were collected and performed western blot analysis with anti-caspase-3, -8 and -9 Ab. (Fig. 14) Jurkat cells were treated with rCsTPX 50, 100, and 200 ng/ml with or without anti-Fas Ab 80 ng/ml. First, after anti-Fas Ab treatment, cleaved capspase-3 expression was increased and this elevated expression was reduced with treatment of rCsTPX. (Fig. 14A, C) Also, caspase-9 was activated and the level of cleaved caspase-9 was influenced by rCsTPX. (Fig. 14A, D) However, increased cleaved

caspase-8 expression after anti-Fas Ab treatment was not restored with rCsTPX. (Fig. 14A, B) In conclusion, these results implicated that rCsTPX has inhibitory effect on anti-Fas Ab-induced apoptosis, and its suppressive effect is involved with activation of caspase-3 and -9, not caspase-8.

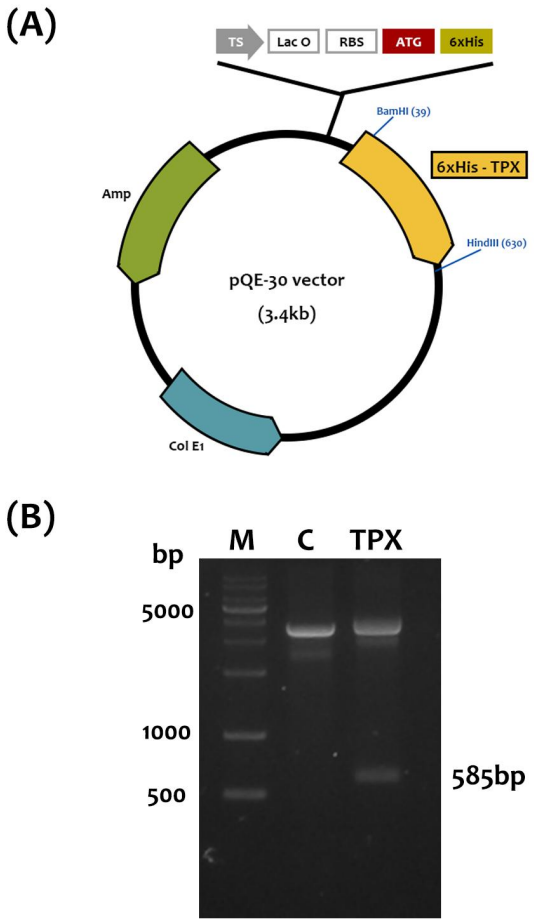

Fig. 4. Diagram for pQE-30 vector composition and confirmation of inserted CS-TPX pDNA. (A) *C. sinensis* TPX cDNA was cloned into pQE-30 vector using restriction enzyme *Bam*HI and *Hind*III. (B) Agarose gel electrophoresis results of pDNA from transformed *E. coli* cells after cutting by restriction enzyme *Bam*HI and *Hind*III. Lane M, DNA markers; lane C, control sample which is pDNA from transformed *E. coli* cells with empty pQE30 vector; lane TPX, pDNA from transformed *E. coli* cells with pQE30 vector containing *C. sinensis* TPX genes.

rCsTPX protien full sequence

1	ATCGCATCACCATCACCATCACGGATCC**ATG**GCTCTCCTGCCGAACCAACCCGCACCGGA	60
61	GTTCTCAGGAATGGCAGTGGTTAATGGCGAGTTCAAGAATATCAGCCTAAAGGATTACCG	120
121	TGGAAAATACGTCATTTTGCTGTCCTACCCACTGGACTTCACGTTCGTCTGTCCAACAGA	180
181	GTTGATTGCTTTCAGCGATGCTGCTGAAGAGTTCAAGTCCAAAAACTGCGTGATTATAGG	240
241	TTGCTCCACGGACTCTGTCTATGCACATCTGCAATGGACCAAAATGGATCGAAAGGTTGG	300
301	TGGCCTGGGAAAGATGAACATCCCGCTTTTGTCGGATAAGAACATGAAGATCTCTCGCGC	360
361	ATACCATGTGCTGGACGAGGAAGAGGGACACGCATTTCGAGGGCAATTTTTGATTGACCC	420
421	GAAGGGTGTTTTGCGTCAGATAACTGTCAATGATCGTCCAGTTGGTCGGTCTGTTGAAGA	540
541	AGCGATCCGCTTGTTGGAAGCCTTTCATTTCCATGAGCAACATGGCGATGTGTGTCCAGC	600
601	GAATTGGAAGCCGAAAGGCAAGACTATGAAGGCAGACCCGGTTGCCGCCCAGGAGTATTT	660
661	CTCGTCCGTTAACAAGCTTAAT**TAG**CTGAGCTTGGACTCCTGTTGATAGATCCAGTAATGA	720

Fig. 5. Full sequence of the recombinant *C. sinensis* TPX protein gene. Start and stop codons are showed as bold letters with underline.

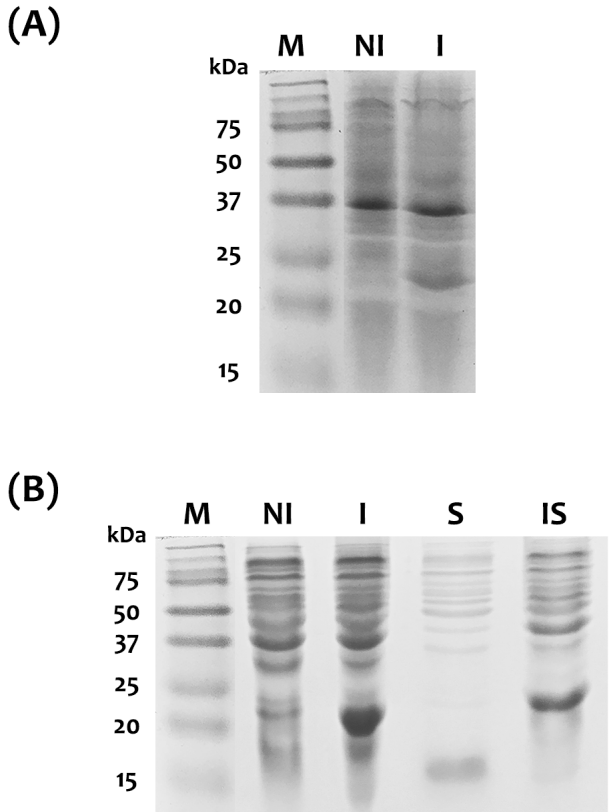

Fig. 6. Rapid confirmation of recombinant protein expression and solubility. Lane M, protein markers; lane NI, non-induced total cell lysate; lane I, induced total cell lysate; lane S, soluble fractions; lane IS, insoluble fractions. (A) After colony pick from culture plate, rapid confirmation of recombinant protein expression by SDS-PAGE and coomasie blue staining. (B) Coomasie blue staining results of *E. coli* lysates to confirm solubility of recombinant protein.

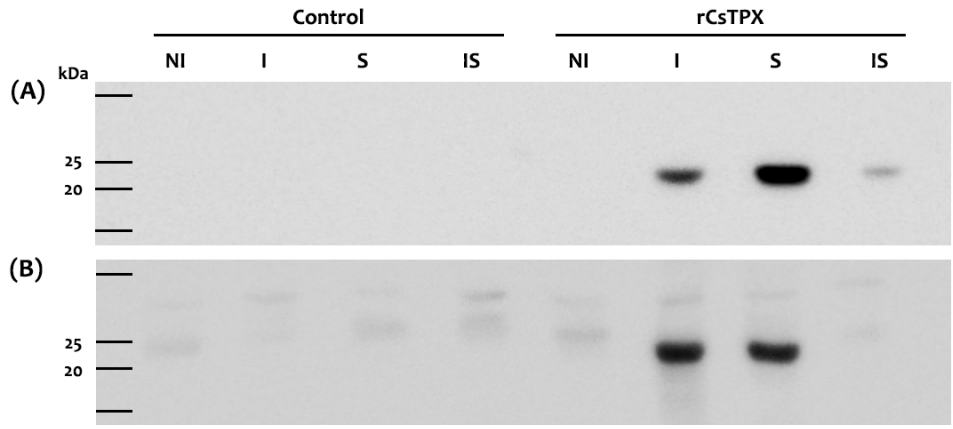

Fig. 7. Western blot analysis of rCsTPX expression with anti-6X-His tag antibody and anti-CS-TPX polyclonal antibody. Lane NI, non-induced total cell lysate; lane I, induced total cell lysate; lane S, soluble fractions; lane IS, insoluble fractions. Control group is samples from transformed *E. coli* cells with empty pQE30 vector. (A) Western blot results of anti-6X-His tag antibody (1:2000 dilution) (B) Western blot results of anti-TPX polyclonal antibody (1:1000 dilution)

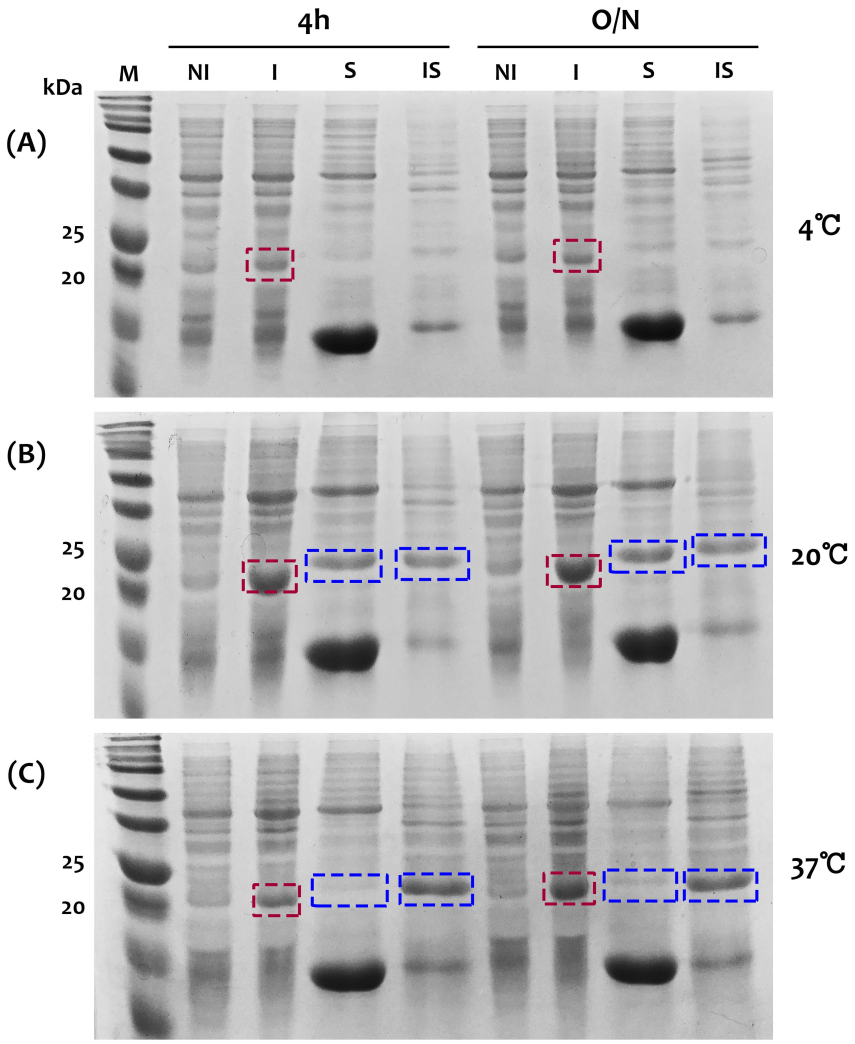

Fig. 8. Results of SDS-PAGE analysis of the solubility of rCsTPX induced at different temperature and duration. Lane M, protein markers; lane NI, non-induced total cell lysate; lane I, induced total cell lysate; lane S, soluble fractions; lane IS, insoluble fractions. Protein was expressed at different induction conditions such as IPTG concentration (100 ㎛M), temperature (4℃ (A), 20℃ (B), or 37℃ (C)), or duration of induction (4 hours or overnight).

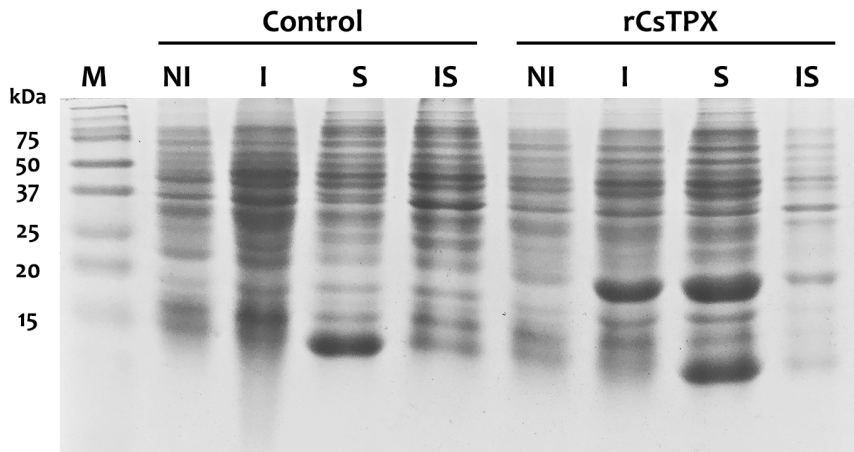

Fig. 9. The expression of rCsTPX in *E. coli* system. Coomasie blue staining results of *E. coli* lysates. Large volume culture was performed at 20℃ overnight with 100 ㎛M IPTG induction. Control group is samples from transformed *E. coli* cells with empty pQE-30 vector. Lane M, protein markers; lane NI, non-induced total cell lysate; lane I, induced total cell lysate; lane S, soluble fractions; lane IS, insoluble fractions.

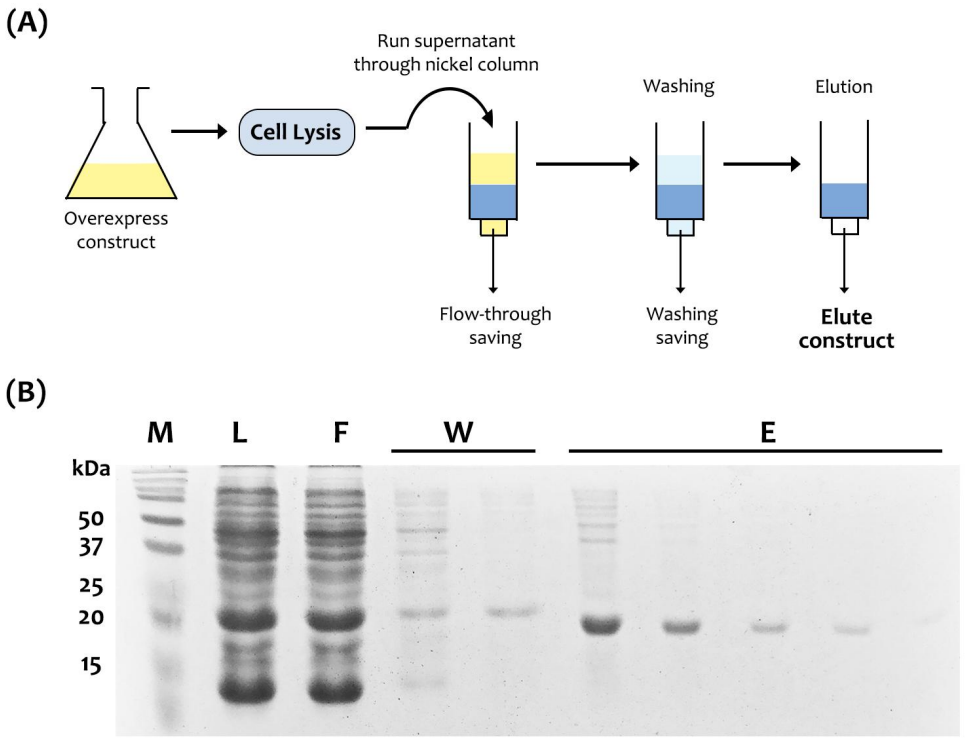

Fig. 10. Elution of recombinant protein and SDS-PAGE analysis of eluted fractions. Protein was purified with Ni-NTA spin column. (A) Simplified workflow chart of recombinant protein elution. (B) SDS-PAGE analysis result of eluted fractions. Lane M, protein markers; lane L, soluble lysate; lane F, flow-through; lane W, washing saving; lane E, elution fractions.

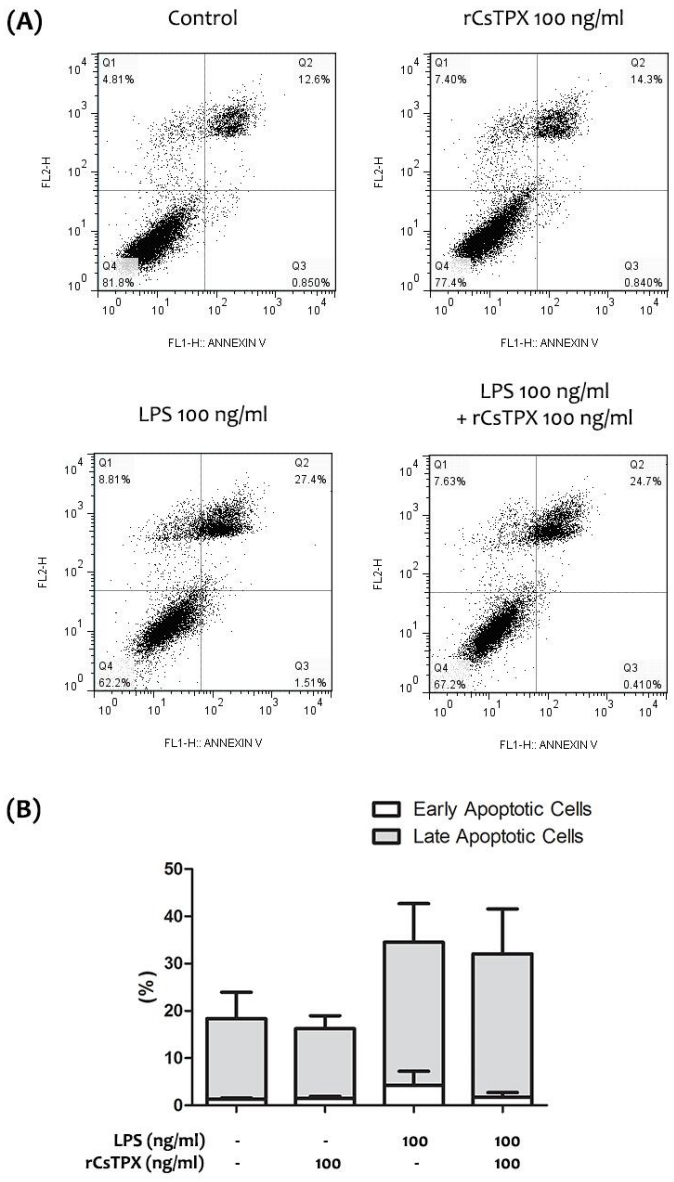
(A)
Control
rCsTPX 100 ng/ml
LPS 100 ng/ml
LPS 100 ng/ml
+ rCsTPX 100 ng/ml
Q1
4.81%
Q2
12.6%
Q4
81.8%
Q3
0.850%
Q1
7.40%
Q2
14.3%
Q4
77.4%
Q3
0.840%
Q1
8.81%
Q2
27.4%
Q4
62.2%
Q3
1.51%
Q1
7.63%
Q2
24.7%
Q4
67.2%
Q3
0.410%
FL2-H
FL1-H:: ANNEXIN V
(B)
Early Apoptotic Cells
Late Apoptotic Cells
(%)
50
40
30
20
10
0
LPS (ng/ml) - - 100 100
rCsTPX (ng/ml) - 100 - 100

Fig. 11. Results of treatment with rCsTPX on LPS-induced apoptosis in RAW264.7 macrophage cells measured by flow cytometry. Cells were untreated (medium alone, contorl group) or treated with rCsTPX 100 ng/ml alone, LPS 100 ng/ml alone, or rCsTPX 100 ng/ml and LPS 100 ng/ml together for 24 hours. After incubation, cells were collected and stained with FITC-Annexin V/PI for flow cytometry. (A) Results of flow cytometry of cells with 24 hours incubation; (B) The histogram of percentages of apoptotic cells population. Values are shown as mean ± SD of four independent experiments.

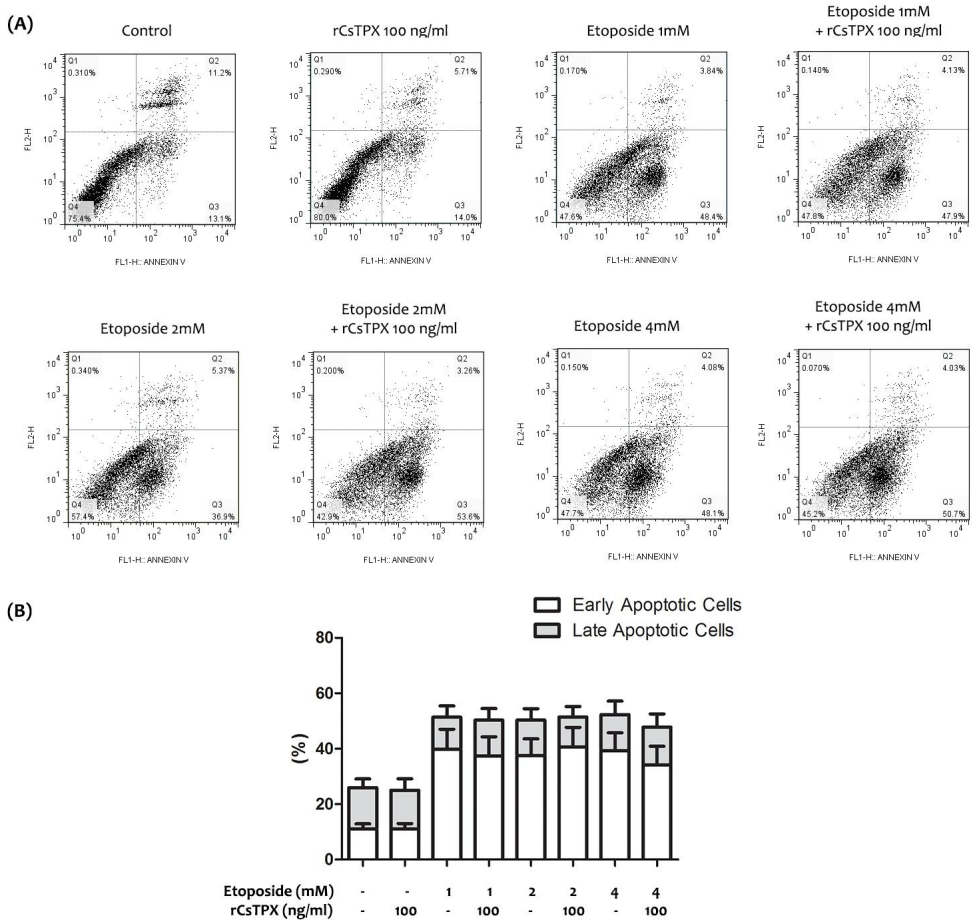

Fig. 12. Results of treatment with rCsTPX on etoposide-induced apoptosis in Jurkat T cells measured by flow cytometry. Cells were untreated (contorl group) or treated with rCsTPX 100 ng/ml alone, etoposide 1, 2, 4 mM alone, or rCsTPX 100 ng/ml and etoposide 1, 2, 4 mM together for 6 hours. After incubation, cells were

collected and stained with FITC-Annexin V/PI for flow cytometry. (A) Results of flow cytometry of cells; (B) The histogram of percentages of apoptotic cells population. Values are shown as mean ± SD of five independent experiments.

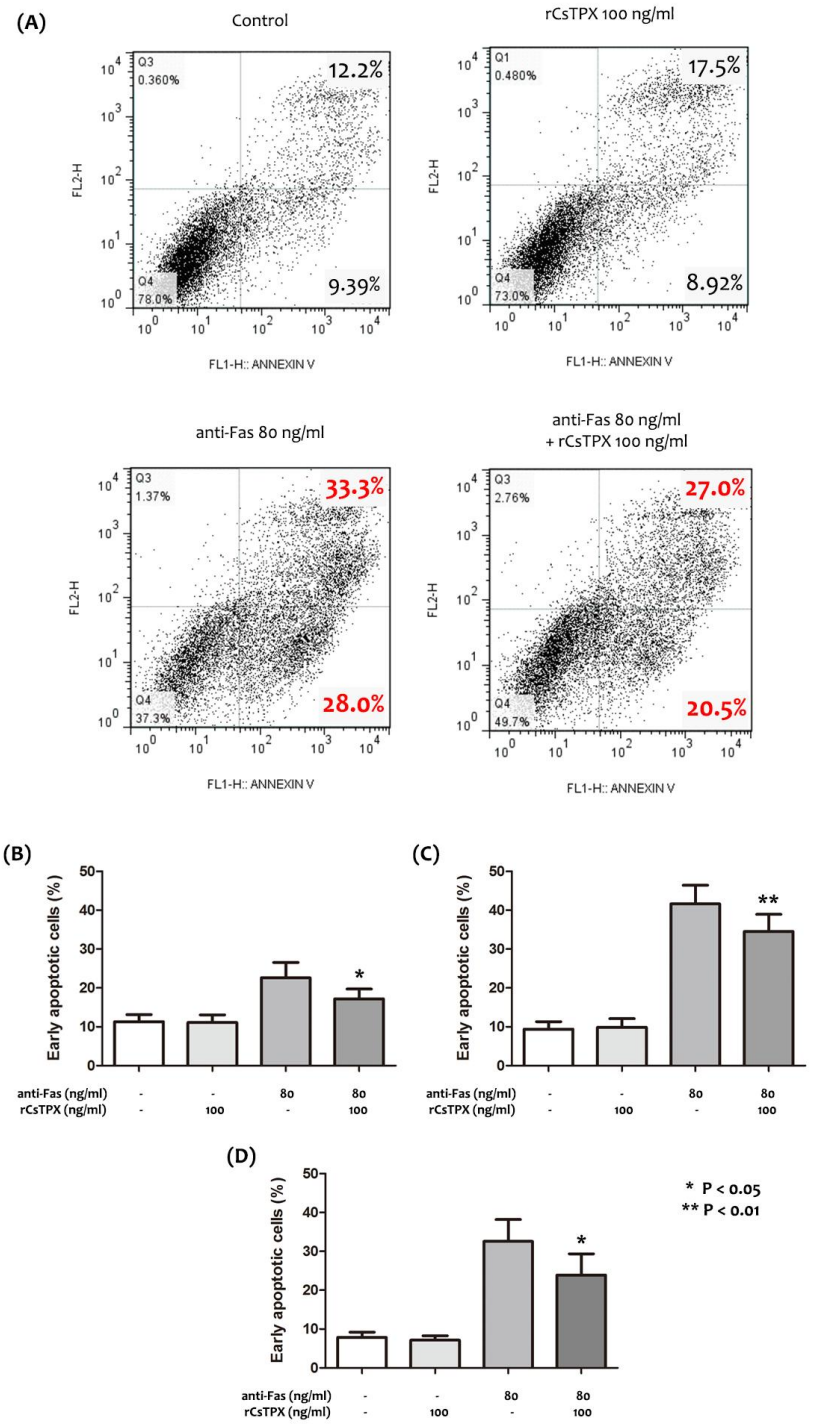
(A)
Control
rCsTPX 100 ng/ml
anti-Fas 80 ng/ml
anti-Fas 80 ng/ml
+ rCsTPX 100 ng/ml
Q3
0.360%
12.2%
9.39%
Q4
78.0%
Q1
0.480%
17.5%
8.92%
Q4
73.0%
Q3
1.37%
33.3%
28.0%
Q4
37.3%
Q3
2.76%
27.0%
20.5%
Q4
49.7%
FL2-H
FL1-H:: ANNEXIN V
(B)
Early apoptotic cells (%)
anti-Fas (ng/ml) - - 80 80
rCsTPX (ng/ml) - 100 - 100
(C)
Early apoptotic cells (%)
anti-Fas (ng/ml) - - 80 80
rCsTPX (ng/ml) - 100 - 100
(D)
Early apoptotic cells (%)
anti-Fas (ng/ml) - - 80 80
rCsTPX (ng/ml) - 100 - 100
* P < 0.05
** P < 0.01

Fig. 13. The inhibitory effect of treatment with rCsTPX on anti-Fas Ab-induced apoptosis in Jurkat T cells measured by flow cytometry. Cells were untreated (medium alone, contorl group) or treated with rCsTPX 100 ng/ml alone, anti-Fas Ab 80 ng/ml alone, or rCsTPX 100 ng/ml and anti-Fas Ab 80 ng/ml together for 2, 4, and 6 hours. After incubation, cells were collected and stained with FITC-Annexin V/PI for flowcytometry. (A) Results of flow cytometry of cells with 6 hours incubation; (B-D) The histogram of results of cells with 2 hours (B), 4 hours (C), and 6 hours (D) incubation, especially percentages of early apoptotic cells population. Values are shown as mean ± SD of four or six independent experiments. $*P < 0.05$ versus anti-Fas Ab alone treated group; $**P < 0.01$ versus anti-Fas Ab alone treated group.

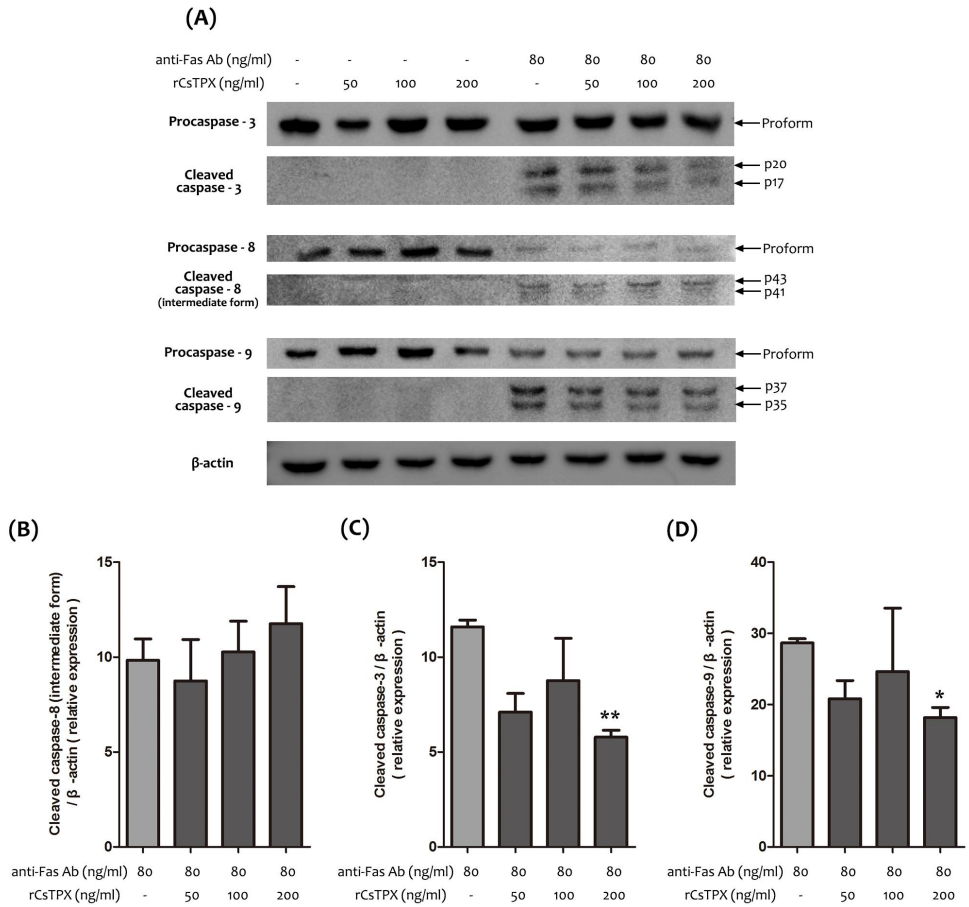

Fig. 14. Western blot analysis of rCsTPX treated Jurkat T cells with anti-Fas Ab to investigate activation of caspase-3, -8, and -9. Cells were untreated or treated with rCsTPX 50, 100, or 200 ng/ml alone, anti-Fas Ab 80 ng/ml alone, or rCsTPX 50, 100, or 200 ng/ml with anti-Fas Ab 80 ng/ml together for 4 hours. After incubation, cells were collected and stained with anti-caspase-3, -8 or -9 Ab. (A) Results of western blotting of anti-caspase-3, -8 and -9 Ab; (B) The histogram

of results of cleaved caspase-3 subunit levels normalized by β-actin. (C) The histogram of results of cleaved caspase-8 (intermediate form) levels normalized by β-actin. (D) The histogram of results of cleaved caspase-9 levels normalized by β-actin. Values are shown as mean ± SD of three independent experiments. $^{*}P < 0.05$ versus anti-Fas Ab alone treated group; $^{**}P < 0.01$ versus anti-Fas Ab alone treated group.

DISCUSSION

When the host is infected with *C. sinensis*, ESPs are continuously released and contribute to interaction between the host and the parasite. As mentioned before, ESPs were evaluated in many studies to validate its effects on host cells or immune responses. In this study, TPX that is one of the components of ESPs is expressed as a recombinant protein and its effect on apoptosis in immune cells, macrophage and T cell line, was demonstrated.

The potential significance of the helminth-induced immunomodulation has been assessed using adult eggs, living adult worms, and ESPs, but more purified molecules is an attractive and promissory field to develop safer therapeutic intervention because of the risk of negative side effect of application (52). There are many trials that studied about the specific component of ESPs, especially as a recombinant product to confirm its modulatory effect. (Table. 1) Anti-oxidant enzymes of parasite have been also identified and expressed to use in many ways. For example, a recombinant thioredoxin-glutatione reductase (TGR) from *F. hepatica* was characterized and expressed in *E. coli* system (61). This recombinant *Fh*TGR was used at rabbit vaccination experiment and it reduced the worm burden significantly following increased IgG response. The omega-class glutathione transferases (GSTs) of *C. sinensis* was also characterized and expressed in *E. coli* system (62). Recombinant CsGSTs was exhibited enzyme activity and showed protection role during worm maturation and the response to oxidative stress. Detoxification in platyhelminthes counts on GPxs and 2-Cys Prxs (TPX)

(30), hence in this study, TPX of *C. sinensis* was a interested component to express as a recombinant protein. Genes of *C. sinensis* TPX was cloned in pQE-30 vector before, therefore using this cloned vector, rCsTPX was expressed in *E. coli* BL21 cells. There is a study that identified two forms of *C. sinensis* TPX (32). These two different sequence of TPX was enrolled in the GenBank database as *C. sinensis* Prx2 and Prx3. By comparing translation sequence of expressed product in this study and two of Prx in the study before, it matched with *C. sinensis* Prx2, not Prx3.

It was difficult to express rCsTPX in water-soluble form as shown at Fig. 6. To improve the solubility of rCsTPX, I tried to express recombinant protein with various induction conditions such as IPTG concentration, temperature, and duration of induction. There is a study that tried to induce *E. coli* cells under different temperatures to optimize better condition of the improved yield of the expressed soluble protein (63). With same *E. coli* cells (BL21 cells) and different vector (pET44 vector) with this study, they expressed NusA-tagged IL-30 proteins at 16, 25, 37℃. In that study, 37℃ was better temperature than others to get more soluble recombinant proteins. However, in the case of rCsTPX, 20℃ was greater condition than other temperature. (Fig. 8) The yield of expressed proteins itself at 4℃ was lower than 20 and 37℃, therefore this condition was excluded to evaluate solubility. In comparison between 20 and 37℃, 20℃ was better condition and induction was continued for overnight to get more proteins. There is other way to improve solubility of recombinant protein. Wu et al. tried to use other vectors carrying genes of protein to solve the problem of recombinant protein solubility (53). To express secreted phospholipase A2 of *C. sinensis*,

they tried several prokaryotic vectors such as pET-26b, pET-30a, pET-32a, pGEX-4T-1, and pMAL-c2X. Only pMAL-c2X vector was expressed as soluble form with good enzymatic activity. pMAL-c2X is a vector with a maltose binding protein (MBP) tag, and MBP is demonstrated as an uncommonly effective tag to promote the solubility of polypeptides (64). Although using other tag protein is also considerable way, changing induction condition is more simple and better way to try, and rCsTPX was able to express as soluble form by changing temperature.

With this soluble rCsTPX, macrophage cell line and T cell line were treated to research its effect on apoptosis in immune cells. TPX has been demonstrated that it is able to regulate apoptosis. There is a study that used thioredoxin reductase (TrxR) inhibitor showing induction of apoptosis in Jurkat T cells (65). TrxR is one of the anti-oxidant enzymes that contribute major role in ubiquitous system connecting with TPX. Therefore, the result that TrxR inhibitor triggers apoptosis suggested the indirect evidence that TPX is involved in regulating apoptosis. There are several studies about apoptosis using antioxidant enzyme of parasite. In the study with the recombinant Trx from *O. viverrini*, it inhibited oxidative stress-induced apoptosis of bile duct epithelial cells by down-regulating pro-apoptotic genes and up-regulating anti-apoptotic genes (57). Also, study with TPX of *Cryptosporidium parvum* (CpTPx) showed that protection effect of apoptosis induced by γ-irradiation (66). CpTPx expressed cells were shown significantly higher survival rate, ROS reduction, and reduced apoptosis amounts. Unlike other helminth parasite, it is hard to find studies about influence of anti-oxidant enzymes of *C. sinensis* on apoptosis. Especially, in the case of *C. sinensis* TPX, there is no study

that investigates its effect of apoptosis. Therefore, here rCsTPX was treated in macrophage and T cell line with apoptosis-inducing reagent.

To examine the effect of rCsTPX, immune cells like macrophage and T cells were chosen because *C. sinensis* TPX is one of the component of ESPs and ESPs influence to host immune responses, and immune cells are one of the critical cells that contact with parasite worm when host was infected. As shown in Fig. 11-12, there was no significant effect of rCsTPX on LPS-induced apoptosis in macrophage and etoposide-induced apoptosis in T cells. However, rCsTPX had inhibitory function on anti-Fas Ab-induced apoptosis in T cells. (Fig. 13) These results suggested that the suppressive effect of rCsTPX is involved with the extrinsic pathway of apoptosis. Activation or apoptosis by LPS is involved with ROS-dependent oxidative stress response, and ROS is one of the stimuli of mitochondrial (intrinsic) pathway of apoptosis (60). Etoposide is a semisynthetic anticancer drug that induces apoptosis via a Bcl-2-dependent pathway, that is mitochondrial (intrinsic) pathway (67). On the other hand, anti-Fas Ab is a monoclonal Ab which binds specific receptor, Fas (also called APO-1 or CD95), and Fas is a death receptor that mediates apoptosis through extrinsic pathway (44). Hence the inhibitory effect of rCsTPX on only anti-Fas Ab-induced apoptosis could be speculated that influence of rCsTPX is associated with death receptor (extrinsic) pathway. These results showed same outcomes with the study that validated the effect of Prx6 (68). In human cervical cancer cell line, Prx6 showed suppressing TRAIL-induced cell death, but not that mediated by intrinsic apoptosis inducers such as etoposide, staurosporine.

To investigate this modulatory effect of rCsTPX, protein was treated in cell line and the consequence of treatment means the influence of extracellular TPX. As mentioned before, rCsTPX has same translation sequence with Prx of *C. sinensis* known as Prx2. Prx2 is known as cytosolic enzyme but this is also produced by stromal cells and might be secreted like other soluble molecules (69). The key molecules of biological functions of this extracellular Prxs could be TLRs. Especially TLR4 is known as an important host factor in both infection and self-defense, and many extracellular Prxs are known to activate TLR4-mediated signaling when they regulate intracellular signaling. Therefore, it could be speculated that effect of rCsTPX is also involved with TLR4 signaling cascade when protein was treated in cell line.

To figure out the mechanism of the effect of rCsTPX, caspase-3, -8, and -9 activation was confirmed by western blot analysis. When Fas ligand binds with its specific receptor Fas, Fas recruits the adaptor molecule Fas-associated protein with a death domain (FADD). (Fig. 2) FADD provide a docking surface for caspase-8 (44). Fas, FADD, and caspase-8 form a complex called the death inducing signaling complex (DISC). After forming DISC, if cells express and recruit enough amounts of pro-caspase-8 to the DISC (Type I cells), cells are proceeded apoptosis through direct activation of pro-caspase-3. In contrast, if low level of caspase-8 is activated at the DISC, cells cannot undergo through direct mechanism and process pathway depend on the mitochondria (Type II cells). In those cells, signaling depends on the Bid, leading to the inhibition of anti-apoptotic molecules like Bcl-2 and release of cytochrome c and other molecules from the mitochondria. However, type I versus type II

discrimination is not a black or white categorization, rather stand for the extremes of a spectrum of reaction about Fas occupied by different cell types. Jurkat T cells used in this study were not specific type of cell, therefore both caspase-8 and -9 were activated. (Fig. 14) However, they were differently regulated by rCsTPX. Expression of cleavage caspase-3 and -9 was reduced after treatment with anti-Fas Ab and rCsTPX together, but caspase-8 activation was not influenced. These results implied that effective point of rCsTPX is on mitochondria pathway of apoptosis induced by Fas. After inducing apoptosis by Fas receptor, the first factor that is involved with mitochondrial pathway is Bcl-2. However, as it was reported that Bcl-2 activation is not influenced by many antioxidant enzymes including peroxiredoxin (70). Bcl-2 might not be a critical molecule for modulatory function of rCsTPX.

Thus considering other factors that could be involved with rCsTPX effect, the possible factor of caspase-3 and -9 inhibition could be IAPs. The members of IAP family are intrinsic cellular regulators of apoptosis. Of all IAPs, XIAP is the only IAP that can inhibit caspase directly in a strict biochemical sense (46). Other IAPs, like cIAP1, cIAP2, DIAP1, and DIAP2 are inefficiently inhibit caspases *in vitro* (46). XIAP has three BIR domains that bind and inhibit caspase-3, -7, and -9. Residues between the BIR1 and BIR2 domain bind to the active site of caspase-3 and -7, and BIR3 domain binds to the surface of caspase-9 (71). The XIAP' s binding affinity to the caspase-3 and -7 is very strong, whereas binding to the caspase-9 is relatively weak and binding to the caspase-8 is undetectable (72). XIAP is known that expressed ubiquitously in most fetal and adult tissues. One of the

regulatory factors of negative control of XIAP is IAP-antagonist such as SMAC/DIABLO and ARTS, which is released from mitochondria when apoptosis signaling is induced through mitochondria. (Fig. 2) As the activation of caspase-3 and -9 were influenced by rCsTPX, but not caspase-8, (Fig. 14) it could be understood that direct suppressive factor of caspase, XIAP, is a possible major factor of rCsTPX effect.

Also, the possible mechanism of modulatory function of rCsTPX could be involved with apoptosome formation. When apoptosis signaling is process through mitochondria, cytochrome c is released and form apoptosome with APAF1 and several pieces of procaspase-9. (Fig. 2) There is a study that pharmacological inhibition of APAF1 prevented procaspase-9 activation and delayed mitochondrial damage (73). Also cell viability was relatively increased and caspase-3 activity was reduced by inhibiting APAF1. There is another study used cytochrome c knock-in mice having a mutation in gene locus of cytochrome c (74). This mutation downregulates the ability of cytochrome c to form apoptosome and motor neuron of this mutant mice displayed enhanced neuron survival. In addition, cIAP1 which is one of the members of IAPs was demonstrated that it blocks the cytochrome c-dependent apoptosome (75). Not only cIAP1 also XIAP have no inhibitory ability of procaspase-3 or -9, they can inhibit only active form of caspases. However, cIAP1 was showed that this interaction with oligomerized active caspase-9 formed apoptosome and inhibited procaspase-3 activation (75). Like these results of studies, there are several possible factors and points included with apoptosome that could be the critical mechanism of rCsTPX effect.

In addition to the specific pro- or anti-apoptotic factors, the ROS controlling function of TPX is also considerable in other cell

types. Prx2 is known as the abundant enzyme in mammalian neurons and described that it has a protective function under oxidative stress in dopaminergic neurons via inhibition of apoptosis signal-regulating kinase1 (ASK1) activation (76). In other words, Prx2 suppresses ASK1 involved mechanism by ROS through its peroxidase function keeping a reduced state of Trx in neurons. After ASK1 activation, not only caspase-independent cell death but also mitochondrial apoptosis pathway through c-Jun N-terminal kinases (JNK) is induced (77). According to these reasons, peroxidase activity of TPX controlling ROS is also a possible factor involved with inhibitory effect of rCsTPX in apoptosis and, for further study, whether ASK1 activation is influenced by rCsTPX would be interesting.

In the case of etoposide- and LPS-induced apoptosis, there were no effect by treatment of rCsTPX even they have been known that their apoptosis is involved with mitochondria pathway. About this results, further studies could be needed to figure out if rCsPTX only has effect on mitochondria pathway induced by Fas or death receptor. Also whether there is another bypass factor involved in anti-apoptotic function of rCsTPX could be a good subject for future studies. Through additional investigation, more details about anti-apoptotic function of rCsTPX need to be validated. Moreover, discovering more precise fraction of rCsTPX that has its crucial function is also needed to increase potentiality of utilization for future application. Additionally, *in vivo* experiment can be performed to figure out variable characteristics of rCsTPX. Most of *in vivo* studies used the ways that using TPX overexpressing or knock-out mice. However there was a study that inoculated recombinant Prx5 to investigate its

neuroprotective effects against neonatal excitotoxic trial (78). Although recombinant Prx5 in that study was mammalian enzyme protein, rCsTPX also could be used at *in vivo* experiment to explore its functional role in many disease model involved with inappropriate apoptosis like experimental models of autoimmune disorders such as autoimmune thyroid disease, type I diabetes, lupus and others. Excessive apoptosis and destruction of cells in each disease models, especially involved with Fas-mediated signaling, could be anticipated to be reduced by rCsTPX.

In conclusion, rCsTPX has regulatory function on Fas-mediated apoptosis in T cells and this effect are involved with activation of caspase-3 and -9, assuming mitochondrial pathway. Inhibition function of apoptosis in T cell line is useful ability for many areas of several diseases. Jurkat T cell line is T cells already activated, therefore it can be thought that rCsTPX is able to modulate signaling in activated T cells although clear mechanism is not yet elucidated. Furthermore, as mentioned before, this function could be used for treatment of diseases like type I diabetes that inappropriate T cell is a critical problem. Also rCsTPX was showed that it has a regulatory effect on Fas-mediated apoptosis. There are several kinds of cells that have many Fas on their cell membrane such as hepatocyte. Thinking that rCsTPX is a protein of liver fluke *C. sinensis*, its application to cells involved with main location of *C. sinenis* worm infection such as liver or biliary tract could be anticipated positive results. There was a study that indicated inhibitory effect of *O. viverrini* Trx on apoptosis of bile duct epithelial cells (57). *C. sinensis* and *O. viverrini* are liver-flukes that are related closely each other and many proteins of them are

shown similar than any other parasites (79). Thus alike influences are assumed comparing with protective effect of *O. viverrini* Trx on apoptosis, and also Matchimakul et al., authors of that study, mentioned drugs that inhibit Trx/TrxR signaling mechanisms and similar approaches can be tried for opisthorchiasis-induced bile duct cancer. In the same vein, based on the features of rCsTPX, inhibiting TPX signaling system may be a good strategy for intervention of clonorchiasis-mediated cholangiocarcinoma.

Although it has not defined all features of rCsTPX and its regulatory effect on apoptosis, rCsTPX has enough potential for further studies but also as a substrate for more than experiment. Like *Trichuris suis* therapy in Crohn' s disease (80, 81), rCsTPX can be applied in many diseases with the modulatory ability of Fas-mediated apoptosis in T cells. Especially diseases that deregulated apoptosis of cells are critical features and Fas is important factor such as type I diabetes and autoimmune thyroid disease are promising area of application. As one of the components of *C. sinensis* ESPs, with more studies about its effect on apoptosis to elucidate details clearly, it could be a future focus of developmental therapeutic reagent.

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국문초록

간흡충은 한국, 중국, 베트남, 러시아 일부분을 포함한 동아시아에 주로 분포하는 발암성의 인체기생충이다. 간흡충의 성체는 감염 후 담관 내에 10년 이상 기생할 수 있으며 이러한 만성 감염으로 인해 감염된 체내에서의 활성산소와 같은 지속적인 산화 스트레스를 유발하게 된다. 이 때, 기생충의 장기적인 생존을 위해 항산화 기전이 중요한 역할을 하게 되며, 이를 위해 기생충에 의해 사용되는 산화 환원 효소 중 하나로 thioredoxin peroxidase (TPX)가 있다. TPX의 주된 기능은 H_2O_2를 중화하는 것이며, 이는 또한 세포 분열, 세포 내 신호전달, 철 대사, 면역 조절, 세포자멸사와 같은 다른 생물학적 기능에도 관여한다. 이 연구에서 가용성의 재조합 간흡충 TPX 단백질 (rCsTPX)을 대장균을 이용하여 발현시켰으며, 발현시킨 단백질의 세포자멸사에 대한 조절 여부를 확인하였다. 초반 rCsTPX를 발현시켰을 때에 단백질의 낮은 용해도로 인해 거의 대부분의 발현 단백질이 불용성의 형태를 띠고 있었다. 낮은 용해도를 높이기 위하여 isopropyl β-D-1-thiogalactopyranoside (IPTG) 농도, 발현 시 온도, 발현 시간과 같이 여러 발현 조건들을 바꿔보았고, 결국 100 μM IPTG를 넣고 20℃에서 하룻밤 동안 발현시키는 최적의 조건을 잡게 되었다. 이렇게 발현시킨 가용성의 rCsTPX로 마크로파지 세포와 T 세포에서의 세포자멸사에 대한 영향을 확인하였다. 오직 T 세포에서 anti-Fas 항체로 유도한 세포자멸사에서만 rCsTPX의 조절 기능을 확인할 수 있었다. Caspase cascade 또한 western blot 분석을 통해 알아보았고, caspase-8의 활성화 같은 경우 rCsTPX에 영향을 받지 않은 반면 caspase-3와 -9의 활성화는 변화를 보였다. 결론적으로, 이 논문에서는 rCsTPX의 가용적 발현을 위한 개선된 방법을 제시하고, 이 단백질이 Fas-매개 세포자멸사에서 조절 능력을 갖고 있음을 보이고 있다. 더불어 rCsTPX와 같은 경우, 추가적인 실험을 통해 더 자세한 연구가 이루어 질 때 좋은 주제가 될 수 있을 것이며, 추후 치료적 적용면에도 충분한 잠재적 가능성을 갖고 있을 것이다.

주요어 : 간흡충, Thioredoxin peroxidase, 세포 자멸, 항-세포자멸 효과

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