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Anti-oxidative effects of silkworm storage protein 1 in HeLa cell

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ABSTRACT

Silkworm hemolymph contains unique proteins that exhibit anti-apoptotic activity in mammalian cells. Among them, 30 K protein, which is one of the major anti-apoptotic molecules in silkworm hemolymph, has been well investigated. However, little is known about the biological functions of storage protein 1 (SP1), another main protein in silkworm hemolymph. In this study, the anti-apoptotic and anti-oxidative activities of SP1 were analyzed. A stable cell line expressing SP1 was constructed, which showed strong anti-apoptotic effect induced by staurosporine treatment. In addition, the cell line exhibited resistance to oxidative stress caused by hydrogen peroxide. For practical applications of SP1, recombinant SP1 was produced in *Escherichia coli*, and the supplementation of recombinant SP1 into culture medium exhibited anti-apoptotic and anti-oxidative activities. In addition, SP1 was found to be a cell-penetrating protein and localized in the cytosol as well as on the plasma membrane. The findings showed that SP1 itself is not an anti-oxidant; rather, it mediates intracellular anti-oxidative activity. In conclusion, the cellular resistance of SP1 to apoptosis and oxidative stress will provide a new strategy that could be utilized in the bio-industry for the production of biologics as well as for the development of anti-aging cosmetics.

1. Introduction

Organisms living in an aerobic environment face continuous threats from exposure to reactive oxygen species (ROS) [1,2]. The increased level of ROS is in turn implicated in triggering intrinsic apoptosis via interactions with proteins related with the mitochondrial permeability transition complex [3,4]. The continuous presence of high levels of ROS causes severe harm to cellular molecules, including DNA, proteins, and lipids [5–7]. Oxidative stress from intracellular or extracellular sources is known to trigger a cellular mechanism that leads to accelerated aging of cells [8]. Thus, identification of a novel anti-oxidant is required in functional cosmetics development. ROS generation during mammalian cell culture of Chinese hamster ovary (CHO), hybridoma, myeloma, and baby hamster kidney (BHK) cells for the production of commercially important proteins was found to cause cell death [9–13]. This results in the reduction of recombinant protein production and has become a critical problem in the bio-industry [13]. The cellular responses to oxidative stress have direct practical implications, and therefore oxidative stress became a serious problem for the pharmaceutical industry to overcome. Therefore, genetic or non-genetic strategies for protecting cells from oxidative stress caused by ROS in mammalian cells are important.

Silkworms produce various proteins, including 30 K proteins and SPs; the anti-apoptotic effects of these proteins have been previously investigated in insect and mammalian cell systems [14–23]. Cell-penetrating and enzyme stabilization effects of 30 K proteins have been demonstrated. There are two types of storage proteins: SP1 and SP2. SP1, a type of hexamerin protein in the insect hemocyanin family, is a female-specific protein and has a high proportion of methionine [24]. SP2 is also a type of hexamerin protein found in insects, especially an arylphorin-type protein [25,26]. It has previously been reported that supplementation of culture medium with SP2 purified from silkworm hemolymph inhibited apoptosis [23]. In addition, SP1 has been shown to inhibit apoptosis of human cells in a stable HeLa cell line expressing this protein [27]. However, in comparison to the 30 K protein, the biological effects of SP1, including its response to oxidative stress, have not been well characterized.

Herein, the anti-oxidative activities of SP1 in human cells were studied for the development of novel and potent material for the bio-industry. First, a stable HeLa cell line expressing SP1 was constructed, and the effects of SP1 on oxidative stress induced by hydrogen peroxide treatment were investigated. Second, to demonstrate the potential applications of SP1, recombinant SP1 was produced and purified for the supplementation of culture medium, and its resistance to reactive oxygen species was determined.

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2. Materials and methods

2.1. Cell culture and treatment

HeLa (human cervical epithelial carcinoma) cells were cultivated in DMEM (Dulbecco's modified Eagle's medium, Biowest, France) containing 10% FBS (fetal bovine serum; Biowest, France) and 1% penicillin/streptomycin (Life Technologies, USA). Cells were sub-cultured in the T-25 flask at 37 °C and under 5% CO_2. The cell line selection medium was prepared with 10% FBS supplemented with 100 or 500 μg/mL of geneticin (G418, Gibco, USA). For the medium containing recombinant SP1, culture medium was supplemented with purified SP1 at different concentrations varying from 6.25 to 50 μg/mL. To test the recombinant SP1 effect, the same concentration of fructose was added to culture medium for the control cells. Staurosporine (STS; Santa Cruz Biotechnology, Inc., USA) was used to induce apoptosis at a final concentration of 1 μM. Hydrogen peroxide (H_2O_2) is a type of reactive oxygen species with free radicals, including hydroxyl radical and superoxide anion. A 0.6 mM concentration of hydrogen peroxide was used for oxidative stress induction. To determine recombinant SP1 effect, cells were seeded in 96-well plates containing growth medium at a density of 1×10^4 cells/cm^2. One day after seeding, the culture medium was replaced with medium containing purified SP1 and further cultured for 1 day. Subsequently, the culture medium containing SP1 was treated with 1 μM STS or 0.6 mM hydrogen peroxide.

2.2. Collection of silkworm hemolymph

Silkworm hemolymph was obtained by clipping the side of an abdominal leg of fifth instar larvae, and subsequently heat-treated at 60 °C or 70 °C for 30 min. Denatured proteins and debris were removed by centrifugation and filtration. SDS-PAGE was carried out using 10% polyacrylamide gels to analyze the existence of SP or 30 K proteins in each silkworm hemolymph. Heat-treated silkworm hemolymph was filtrated using a 0.2 μm syringe and was then added to culture media (5% v/v).

2.3. Plasmids and cell line selection

The SP1 gene (GenBank accession number, NM_001113276) was amplified and inserted into pcDNA3.1 plasmid (Invitrogen, Inc.) at EcoRI and XhoI restriction sites. The primers designed are as follows: forward primer, GAA TTC ATG ACA GCG ATT AGT GGT GGC and reverse primer, GAG CAG CAT GAT GAA CTG ACT CGA G. The pET23a(+) vector was also used with EcoRI and XhoI restriction enzyme sites. The primers designed were as follows: forward primer, GAA TTC ATG ACA GCG ATT AGT GGT GGC and reverse primer, GAT GAT GAG CAG CAT GAT GAA C. After transfecting HeLa cells with the plasmid with or without SP1, cells were incubated for 2–4 weeks in culture media supplemented with G418. Single cells were obtained by limited dilution, and monoclonality was confirmed by microscopic observation. Anti-apoptotic effects in each cell line were screened using the WST-1 assay. Based on the WST-1 assay result, cells with higher viability than the control after STS treatment were used for further selection. After selection of anti-apoptotic cell lines, total RNAs were isolated and converted to cDNAs, and then SP1 expression was determined using reverse transcription PCR for final cell line confirmation.

2.4. Recombinant SP1 production and purification

E. coli BL21 (DE3) was transformed with pET23a(+) plasmid containing the SP1 gene and incubated in 5 mL of LB broth containing ampicillin for 12 h at 37 °C. Four ml of culture was subsequently transferred into 1 L of fresh LB medium and incubated at 37 °C for another 3–4 h until OD_{600} reached 0.6–0.8. Cell concentrations were measured with QUANTOM Tx™ (Logos Biosystems). The cells were treated with 0.5 mM ITPG and subsequently incubated overnight at 30 °C with shaking at 200 rpm, and then cells were harvested by centrifugation at 7000 rpm for 30 min at 4 °C. The cell pellet was resuspended in lysis buffer (10 mM imidazole, 400 mM NaCl, 1% Nonidet-P40, 5% glycerol in 20 mM Tris buffer, pH 8.0), sonicated (on for 5 s and off for 10 s), and centrifuged again at 12,000 rpm for 30 min at 4 °C. The supernatant was filtrated through 0.2 μm filters and then purified using ÄKTA™ start (GE Healthcare). The 6-His-tagged proteins were purified via HisTrap™ HP affinity chromatography (GE Healthcare) with the following buffers: Binding buffer: 20 mM Tris, 0.5 M NaCl, 20 mM imidazole, pH 8.0. Elution buffer: 20 mM Tris, 0.5 M NaCl, 500 mM imidazole, pH 8.0. Purified SP1 was desalted using a HisTrap™ Desalting column with 2 M fructose. Here, fructose was used to avoid the formation of SP1 aggregates as SP1 tended to aggregate when ultrafiltration was used for concentration. Finally, SP1 was concentrated by ultrafiltration and its concentration was measured using the Bradford assay.

2.5. Relative caspase 3 activity

Ac (N-acetyl)-DEVD-AFC (7-amino-4trifluoromethyl coumarin) was used for measuring relative caspase 3 activity. Caspase 3 cleaves the tetrapeptide between aspartate and AFC, thus releasing fluorogenic AFC, which can be quantified by UV spectrofluorometry. Cell lines with or without SP1 were treated with 0.6 mM hydrogen peroxide and harvested at 5, 10, and 15 h after treatment. Cells were washed twice with cold PBS and lysed in 200 μL of lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 10% glycerol (v/v) and 1% NP-40 (v/v)). Twenty microliters of Ac-DEVD-AFC substrate in DMSO was added to 1 mL of 1X HEPES buffer containing DTT and glycerol. After quantification using the BCA assay, cell lysates were diluted to 0.5 μg/μL and 20 μL of each lysate was added into black polystyrene 384 well-plates. After 1 h of incubation at 37 °C, the reactants were measured for caspase activities using a spectrofluorometer with excitation and emission wavelengths of 400 nm and 505 nm, respectively.

2.6. Immunofluorescence of SP1 in cells

After incubation with recombinant SP1, cells were intensively washed with PBS 5 times and then fixed with fresh 4% paraformaldehyde in PBS for 15 min at room temperature. Subsequently, cells were permeabilized with cold 0.2% Triton X-100 in PBS for 5 min. Next, after discarding the remaining Triton X-100 solution, the cells were again washed 5 times and blocked with 5% BSA in PBST (PBS with 0.1% Tween-20) for 30 min at 37 °C. They were then treated with anti-T7 tag primary antibody (Abcam, England) diluted 1:200 in blocking buffer for 1 h at 37 °C. Subsequently, an intensive washing step was performed, goat anti-mouse secondary antibody conjugated with Alexa 594 fluorescence (Invitrogen, Inc.) was added, and the mixture was incubated for 30 min. Subsequently, the nuclei were counterstained with 0.2 μM Hoechst 33342, and SP1 localization was visualized using a fluorescence microscope.

2.7. DPPH radical scavenging activity assay

The DPPH radical scavenging activity of SP1 was tested with Trolox as the positive control. Trolox and SP1 were diluted at 0, 0.01, 0.1, 1, 10, and 100 μg/mL in respective buffers. Forty μl of each solution was added into 96-well plates. Blanks of each solution were prepared after addition of 160 μL of methanol into each solution. Each sample was incubated for 30 min at 37 °C after