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Original Article

A novel protein from edible fungi *Cordyceps militaris* that induces apoptosis

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ABSTRACT

Cordyceps militaris is a dietary therapeutic fungus that is an important model species in *Cordyceps* research. In this study, we purified a novel protein from the fruit bodies of *C. militaris* and designated it as *Cordyceps militaris* protein (CMP). CMP has a molecular mass of 18.0 kDa and is not glycosylated. Interestingly, CMP inhibited cell viability in murine primary cells and other cell lines in a time- and dose-dependent manner. Using trypan blue staining and a lactate dehydrogenase release assay, we showed that CMP caused cell death in the murine hepatoma cell line BNL 1MEA.7R.1. Furthermore, the frequency of BNL 1MEA.7R.1 cells at the sub-G1 stage was increased by CMP. Apoptosis, as determined by Annexin V and propidium iodide analysis, indicated that CMP could mediate BNL 1MEA.7R.1 apoptosis, but not necrosis. After coincubation with CMP, a decrease in mitochondria potential was detected using 3,3′-dihexyloxacarbocyanine iodide. These results suggest that CMP is a harmful protein that induces apoptosis through a mitochondrion-dependent pathway. Stability experiments demonstrated that heat treatment and alkalization degraded CMP and further destroyed its cell-death-inducing ability, implying that cooking is necessary for food containing *C. militaris*.

1. Introduction

Cordyceps militaris, a fungus that usually parasitizes the pupa of *Lepidoptera* spp., is phylogenetically related to a valuable and rare fungus used in traditional Eastern medicine, *Cordyceps sinensis* (as Dong-Chong Xia-Cao in Chinese). The biochemical components of these two herbal–medical fungi are similar; however, *C. militaris* is less expensive and more easily obtainable than *C. sinensis*. Therefore, *C. militaris* has become a model species in *Cordyceps* research [1–5]. *C. militaris* is known to provide multiple health benefits, including antitumor [6–9], immunomodulatory [10–18], antiinflammatory [19,20], antioxidative [1,4,5], and antibiotic effects [8,21–23]. Some research has indicated that water extraction of *C. militaris* induces apoptosis in cancer cells [6,9,24,25].

Numerous studies have investigated the bioactivity and cytotoxicity of *C. militaris* in the cancer cells. Some of them

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even found there were toxic compounds in edible fungi. However, few studies have paid attention to these effects in normal cells. It is unclear whether there are harmful substances in *C. militaris*. As early as the 1970s, Lin et al [26,27] found the toxin proteins flammutoxin in *Flammulina velutipes* and volvatoxin in *Volvariella volvacea*; both of which are edible mushrooms. In 2006, the toxin protein ostreolysin, was found in an edible oyster mushroom (*Pleurotus ostreatus*) [28]. Most of the studies regarding the bioactivity of *C. militaris* used mixed extractions in their experiments rather than purified compounds. Although *C. militaris* is used as a traditional medicine in many eastern countries, it may contain some toxic substances.

Apoptosis is a type of programmed cell death, and it is different from necrosis or autophagy in terms of both its features and its causes. The main distinctive features of apoptosis include cell membrane shrinkage, chromatin condensation, exposure of phosphatydylserine outside the cell membrane, and formation of apoptotic bodies [29,30]. Apoptosis is a common type of cell death that has been observed in various cell types [30].

In the present study, we purified a new 18-kDa protein from *C. militaris* and designated this protein as *Cordyceps militaris* protein (CMP). We found that this protein could induce cell death in murine primary cells and other cell lines; perhaps through mitochondrion-dependent apoptosis. Furthermore, CMP could be degraded by heat treatment and alkalization, which eliminated its apoptosis-inducing ability.

2. Methods

2.1. CMP extraction and purification

Dried fruit bodies of *C. militaris* were purchased from the Kuen-Shu Chinese herbal medicine shop (Taipei, Taiwan). The dried fruit bodies were soaked in extraction buffer [5% (v/v) acetic acid, 0.1% (v/v) 2-mercaptoethanol, and 0.308M NaCl] overnight and were then homogenized using a Waring Laboratory blender (Torrington, WY, USA). After homogenization, the sample was subjected to sonication using a Sonicator XL2015 (MISONIX, Farmingdale, NY, USA) and subsequently centrifuged at 10,000 rpm at 4°C for 1 hour to remove residuals. To obtain the crude protein, the supernatant was treated with a 60% saturation of ammonium sulfate overnight for protein precipitation. The solution was centrifuged at 10,000 rpm at 4°C for 30 minutes, and the precipitates were collected. After suspension and dialysis in 0.01M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0), the dialysate was loaded into a preequilibrated (10 mM HEPES, pH 8.0) CM-52 cellulose column (2.5 cm × 20 cm; Whatman, Maidstone, UK), which was eluted with a linear gradient of 0.0–0.5M NaCl. The main active fractions containing CMP (Figure 1A) were pooled and dialyzed in 50mM HEPES (pH 8.0) for further purification. These fractions were applied to a Resource S column (GE Healthcare, Little Chalfont, Bucks, UK) on an AKTA FPLC system (GE Healthcare) and eluted with a linear gradient of 0.0–0.25M NaCl; the main fractions containing CMP were subsequently collected (Figure 1B).

2.2. Electrophoresis

Purified CMP was analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a Bio-Rad Mini-protein III system (Bio-Rad, Hercules, CA, USA). Fermentas PageRular Prestained Protein Ladder #SM0671 (Thermo Fisher Scientific) was used to determine molecular weight. After electrophoresis, the gels were stained with Coomassie brilliant blue R250 to visualize the protein. To determine carbohydrate content, the gels were stained with periodic acid–Schiff reagent (Sigma–Aldrich, St Louis, MO, USA).

2.3. Cell culture

The cell lines BNL 1MEA.7R.1 (ATCC number: TIB-75), RAW 264.7 (ATCC number: TIB-71), and P3X63 Ag8 (ATCC number: TIB-9) were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Primary splenocytes and primary peritoneal macrophages were obtained from 6- to 8-week-old female BALB/c mice (National Laboratory Animal Center, Taipei, Taiwan). The animals were maintained under temperature-controlled conditions, and all animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee of the National Taiwan University. All cell lines and primary cells were suspended in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (GIBCO-BRL Life Technologies, New York, USA) and cultured at 37°C with 5% CO_2.

2.4. Fluorescence-activated cell sorting

For cell cycle analysis, 10^6 cells were fixed with 1 mL 70% ethanol, and stained with 400 μL propidium iodide (PI) staining solution (20 μg/mL PI, 0.1% Triton X-100, and 0.2 mg/mL RNase A) at room temperature for 30 minutes. For apoptosis analysis, the cells were stained for fluorescein isothiocyanate (FITC)–Annexin V and PI using an FITC Annexin V Apoptosis Detection KitI(BD Biosciences, San Jose, CA, USA). For mitochondrial membrane potential analysis, the cells were washed and suspended in 0.5 mL phosphate buffered saline with 40nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) at room temperature for 30 minutes. After staining, these cells were acquired by FACScan (Becton Dickinson FACScan, BD Biosciences), and these data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

2.5. Thermal treatment and alkalization for CMP

Purified CMP was heated to 100°C for 5 minutes, 15 minutes, 30 minutes, 60 minutes, 120 minutes, or 180 minutes using a Thermomixer (Eppendorf, Hamburg, Germany) or autoclave sterilized at 121°C for 15 minutes to test protein heat stability. CMP was also stored at −80°C for 1 week to evaluate its frozen storage stability. Pure CMP that was stored at 4°C served as a vehicle control. For the acid or alkaline tolerance tests, purified CMP was dialyzed in 0.6M HCl (pH 2.0) or 5M NaOH (pH 12) at 4°C for 4 hours. Before the cell viability test, protein samples

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