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 ×g 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, Waltham, MA USA) 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 Kitl(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
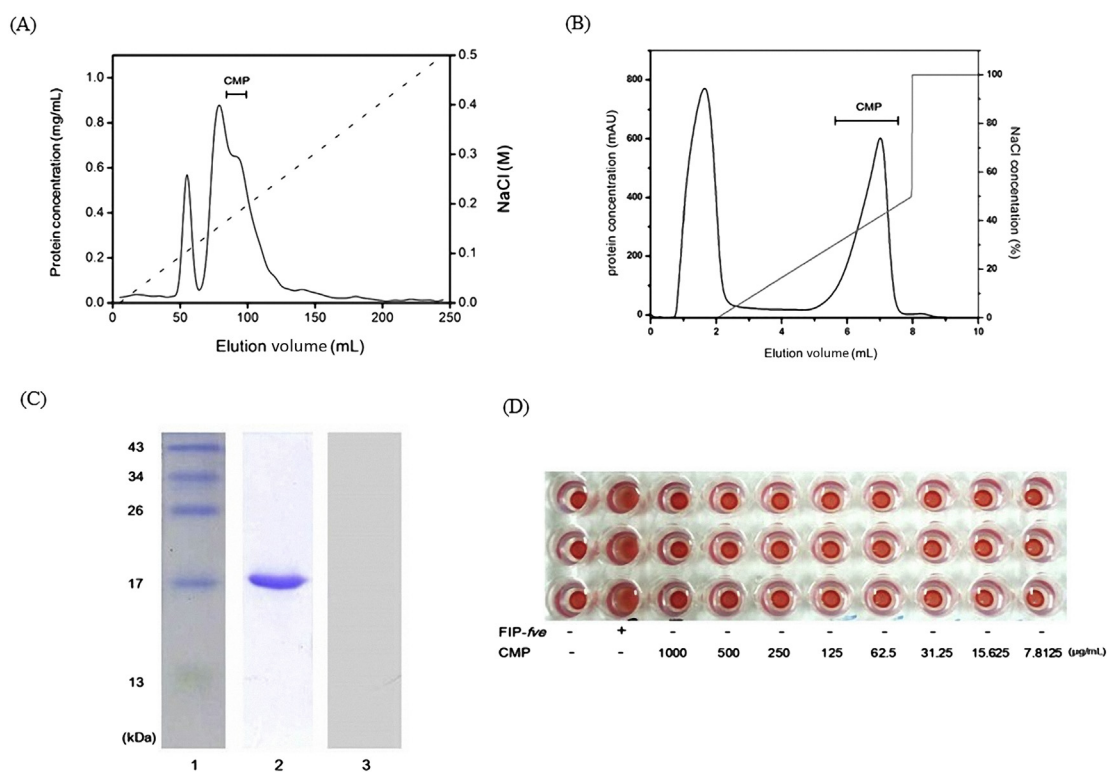
Figure 1 – Purification and biochemical characteristics of CMP. (A) Elution profile of the crude protein of *C. militaris* from a CM-52 cation exchange cellulose column. The distribution of CMP in the elution profile is indicated. (B) Purification of CMP using an FPLC Resource S cation exchange column. The distribution of CMP is indicated. (C) Electrophoretic analysis of CMP. Lane 1: protein marker (Fermentas PageRular Prestained Protein Ladder #SM0671); Lane 2: purified CMP stained with Coomassie brilliant blue R250; and Lane 3: purified CMP stained with periodic acid/Schiff (PAS) reagent. (D) The hemagglutination activity of CMP in the blood of BALB/c mice; 5% (v/v) BALB/c mice blood was coincubated with 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, 0.03125 mg/mL, 0.015625 mg/mL, and 0.0078125 mg/mL CMP18. PBS and 20 μg/mL FIP-F*ve* (funagl immunomodulatory protein-*Flammulina velutipes*) served as negative and positive controls, respectively. CMP = *Cordyceps militaris* protein.

were redialyzed in phosphate buffered saline to ensure that the solution pH was 7.2.

2.6.   CMP N-terminal amino acid sequence analysis

Purified CMP was separated by 15% SDS-PAGE and transferred onto a 0.22-μm polyvinylidene difluoride membrane using the Trans-Blot Cell system (Bio-Rad). The membrane was stained by Coomassie brilliant blue R250 to visualize the protein. The protein band that corresponded to CMP was excised from the membrane and subjected to Edman degradation. Edman degradation and N-terminal amino acid sequence analysis were carried out on an Applied Biosystems Procise Sequencer (Mission Biotech, Taipei, Taiwan).

2.7.   Statistical analysis

All *in vitro* experiments were performed in triplicate ($n = 3$), and representative results from at least two independent experiments are shown as the mean ± standard deviation. One-way analysis of variance and Duncan test for least significant difference were carried out for statistical comparisons, and the differences between the experimental groups were considered to be significant when the obtained p value was < 0.05.

3.   Results

3.1.   Purification of CMP

Dried fruit bodies of *C. militaris* were homogenized and treated with 60% saturation of ammonium sulfate to obtain the crude protein. After dialyzing in 0.01M HEPES buffer (pH 8.0), the dialysate was loaded onto a preequilibrated CM-52 cellulose column and eluted with a linear gradient of 0.0–0.5M NaCl. The proteins were roughly separated into several peaks. The fractions of the third protein peak that contained CMP (Figure 1A) were collected for further purification. The pooled fractions were further applied to a Resource S cation exchange column on an AKTA FPLC system and eluted with a linear gradient of 0.0–0.25M NaCl, and the main fractions that contained CMP were collected for further experiments (Figure 1B).

3.2.   Biochemical characteristics of CMP

To determine the basic biochemical characteristics of CMP, purified CMP was analyzed by SDS-PAGE. A single band of ~18 kDa was visualized after Coomassie brilliant blue R250 staining (Figure 1C), which indicated that the molecular

weight of CMP was ~18 kDa and that CMP was a single unit protein or a homogeneous polymer protein. To determine the carbohydrate content of CMP, we also stained the SDS gel with periodic acid–Schiff reagent. The results suggested that CMP was not a glycoprotein (Figure 1C). To determine whether CMP was a hemagglutinin, series-diluted (1.0–7.8125 μg/mL) CMP was coincubated with 5% (v/v) red blood cells from BALB/c mice. Experimental results showed that CMP was unable to facilitate red cell agglutination, suggesting that CMP was not a hemagglutinin (Figure 1D).

3.3.   N-terminal sequencing of CMP

Purified CMP was analyzed by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and then subjected to Edman degradation for N-terminal sequencing. The resulting sequence, GPSVVVGYRTVSAAQAK, was 76% similar to the *Verticillium albo-atrum* predicted protein (EFY94157.1) sequence and 62% similar to the *Metarhizium anisopliae* hypothetical protein (XP_003002851.1) sequence (Table 1). These percentages of similarity were not particularly high. Moreover, both of these proteins are theoretical proteins. There was no clear and definite evidence that could demonstrate whether both of these theoretical proteins were naturally expressed. These results suggested that CMP may be a novel protein that is different from any known protein.

3.4.   Cytotoxicity of CMP in murine cells

To understand the effects of CMP on mammalian cells, purified CMP was coincubated with mouse primary cells and some cell lines. Compared with the control, cell viability was lower after coincubation with CMP for 72 hours in all five cell types (Figure 2A). Moreover, higher CMP concentrations resulted in lower cell viability, which indicated that this cytotoxic effect was dose dependent.

The murine hepatoma cell line BNL 1MEA.7R.1 was chosen for further testing. BNL 1MEA.7R.1 cells were coincubated with 0.1875–24.0 μg/mL CMP, and cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method at 0, 24 hours, 48 hours, and 72 hours. Without CMP incubation, the BNL 1MEA.7R.1 cell population would normally expend; hence, MTT cell viability was increased. In the presence of CMP, however, BNL 1MEA.7R.1 cell viability was lower than that of the control (Figure 2B). The MTT assay is an indirect assessment of cell viability that detects succinate dehydrogenase activity in the mitochondria.

To determine whether this phenomenon is caused by a cell number reduction or a mitochondrial activity reduction, the cell number was counted after coincubation with CMP for 24–96 hours. The results suggested that CMP decreased the cell number less than the untreated control did (Figure 2C). Furthermore, we determined that lactate dehydrogenase (LDH) was released from BNL 1MEA.7R.1 cells that were coincubated with CMP. LDH is an enzyme that is present in all cells and is rapidly released from cells that are damaged. Cells treated with CMP showed increased LDH cytotoxicity at 24 hours (Figure 2D). Experimental data showed that CMP caused cell damage but did not hinder cell growth.

3.5.   CMP causes murine cell apoptosis

To further investigate the effect of CMP on BNL 1MEA.7R.1 cells, CMP coincubated cells were fixed with 70% ethanol and stained with PI. The DNA content of the CMP-coincubated BNL 1MEA.7R.1 cells was analyzed using flow cytometry. The results suggested that the DNA content of sub-G1 group of the CMP-coincubated BNL 1MEA.7R.1 cells increased (Figure 3A). BNL 1MEA.7R.1 cells were cocultured with 24 μg/mL CMP for 24 hours, 48 hours, and 72 hours, and the DNA content of sub-G1 group was 5.60%, 13.68%, and 33.35%, respectively, which were all higher than the control (medium) (Figure 3A). These results suggested that the DNA content of cells was <2N, which implied that the cells were undergoing apoptosis. Furthermore, the results of the PI staining cell cycle analysis (Figure 3A) also indicated that the cell number decrease after coincubation with CMP were not due to a cell proliferation decline.

To identify the mechanisms of CMP-triggered cell death, Annexin V–FITC and PI double staining analysis was performed. Annexin-V is a cellular protein with high affinity for phosphatidylserine. We took advantage of this characteristic and easily detected membrane translocation by staining cells with fluorescence-labeled Annexin V. Moreover, during the early stage of apoptosis, although the plasma membrane translocates, the integrity of the cell plasma membrane is maintained. Hence, DNA PI dye could not enter the cell nucleus and combine with DNA. In the presence of 12 μg/mL or 24 μg/mL CMP, the BNL 1MEA.7R.1 cell geometric mean of Annexin V–FITC increased from 9.1 to 15.4 or 18.4 in 72 hours (Figure 3B). This result indicated that CMP may induce BNL 1MEA.7R.1 apoptosis.

The mechanism of apoptosis is mainly divided into two pathways: the mitochondrion-dependent pathway and the

Species	Name	Access no.		Amino acid sequence																	S/E[a]	I[b]
Table 1 – N-terminal amino acid sequences of CMP and the results of protein-protein blast.																							
Cordyceps militaris	CMP18	—	1	G	P	S	V	V	V	G	Y	R	T	V	S	A	A	Q	A	K	17	—	—
Verticilliem albo-atrum	Predicted protein	XP_003002851.1	33		S	V	I	I	G	Y	R	T	V	S	A	E	Q				45	34.1/0.22	10/13 (76%)
Metarhizium anisopliae	Hypothetical protein	EFY94157.1	31	G	Q	N	V	I	I	G	Y	R	T	V	A	E	A	Q	A		46	32.9/0.63	10/16 (62%)

CMP = *Cordyceps militaris* protein.
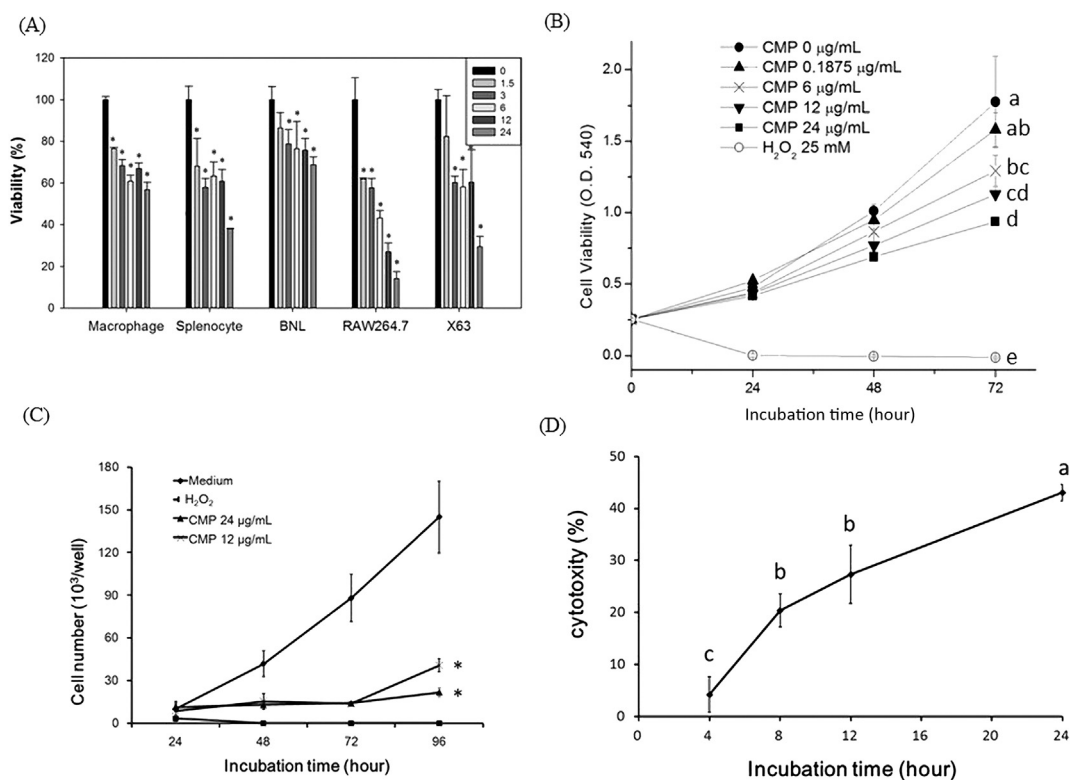[a] Maximum score/E-value.
[b] Identities.

Figure 2 – Cytotoxicity of CMP to murine cells. (A) Mouse primary spleen cells, mouse primary peritoneal macrophages, murine hepatoma cell line BNL 1MEA.7R.1, murine leukemia macrophage cell line RAW264.7, and murine leukemia B cell line X63 were coincubated with 0, 1.5 μg/mL, 3 μg/mL, 6 μg/mL, 12 μg/mL, and 24 μg/mL CMP for 72 hours, and cell viability was analyzed by MTT assay. (B) Murine hepatoma cell line BNL 1MEA.7R.1 was coincubated with 0, 0.1875, 6, 12, and 24 μg/mL CMP for 0, 24, 48, and 72 hours, and 25 μM H_2O_2 was used as a negative control. Cell viability was then analyzed by MTT assay. (C) BNL 1MEA.7R.1 cells were coincubated with 12 μg/mL or 24 μg/mL CMP, 25 μM H_2O_2 (as a negative control) or medium (as a positive control) for 24 hours, 48 hours, and 72 hours. After coincubation, the cells were stained by trypan blue for cell counting. (D) BNL 1MEA.7R.1 cells were coincubated with 24 μg/mL CMP18, medium (a low control) and 1% Triton X-100 (a high control). Medium alone (without BNL 1MEA.7R.1 cells) served as a background control. After coincubation for 4 hours, 8 hours, 12 hours, and 24 hours, cell lactate dehydrogenase release was analyzed. Cytotoxicity = (CMP experimental value − low control)/(high control − low control) × 100%. Data shown are representative of two or three reproducible experiments (Mean ± standard deviation; $n = 3$). *$p < 0.05$ compared with the control group. Different letters indicated that the differences between the groups are significant ($p < 0.05$). CMP = *Cordyceps militaris* protein; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

receptor-dependent pathway. The loss of mitochondrial membrane potential is one of the characteristics of the mitochondrion-dependent pathway, which can be used as an index of mitochondria-dependent apoptosis in the early stage of apoptosis. $DiOC_6$ is a green lipophilic fluorescent dye that binds to mitochondria as a probe of mitochondrial membrane potential loss. As the membrane potential diminishes, the fluorescent intensity diminishes. We found that the $DiOC_6$ florescent intensity geometry means of the CMP-coincubated BNL 1MEA.7R.1 cells were significantly lower than those of the control (Figure 3C). After coincubation with 24 μg/mL CMP for 2 hours, 4 hours, 8 hours, 12 hours, 16 hours, and 24 hours, the $DiOC_6$ florescent geometry means were 366, 364, 302, 265, 190, and 157, respectively (Figure 3C). After coincubation with CMP for 8 hours, the $DiOC_6$ began to decrease. These results suggest that CMP induced cell apoptosis through a mitochondrion-dependent pathway.

3.6. CMP is heat/alkaline intolerant

To understand the stability and the processing tolerance of CMP, CMP was exposed to thermal or acid/alkaline conditions. CMP was heated to 100°C for 5 minutes, 15 minutes, 30 minutes, 60 minutes, 120 minutes, and 180 minutes, and then analyzed by SDS-PAGE. After these treatments, the quantity of the protein declined over time (Figures 4A and 4C). The longer the heat treatment, the less CMP that remained (Figures 4A and 4C). No CMP was found after autoclaving at 121°C for 15 minutes (Figures 4A and 4C). For further investigation, BNL 1MEA.7R.1 cells were coincubated with these heat-treated CMP proteins for analysis of the PI cell cycle. Experimental results demonstrated that the heat-treated protein did not induce cell apoptosis (Figure 5A). This effect was identical to the SDS-PAGE results. These results suggested that CMP was heat intolerant and that the degraded CMP lost the ability to induce apoptosis.
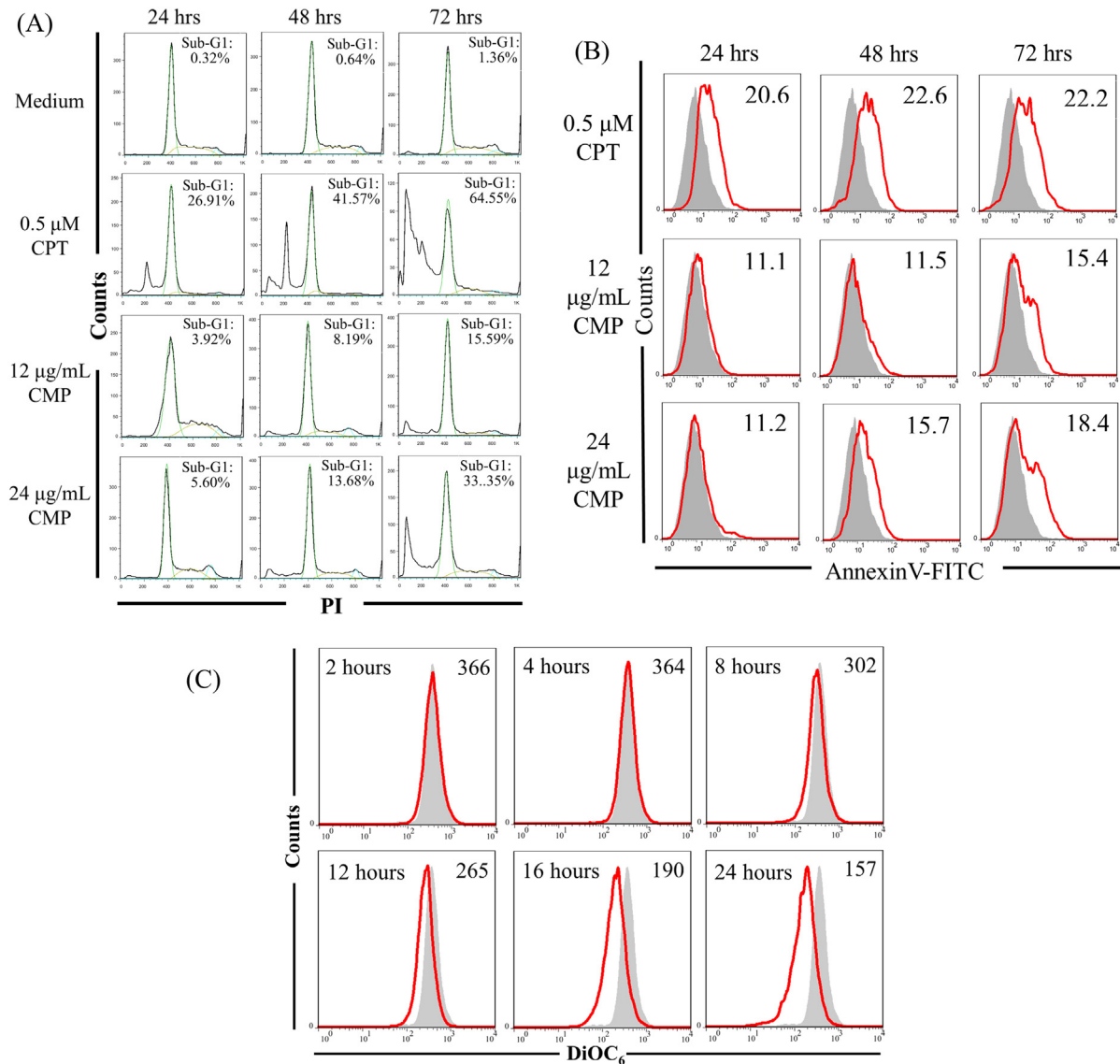
Figure 3 − The analysis of cell apoptosis in the mouse hepatoma cell line BNL 1MEA.7R.1 that was coincubated with CMP. Murine hepatoma cell line BNL 1MEA.7R.1 was coincubated with 12 μg/mL or 24 μg/mL CMP for 24 hours, 48 hours, and 72 hours; medium and 0.5 μM CPT (camptothecin) served as negative and positive controls, respectively. (A) After coincubation, the cells were fixed with 70% ethanol and then stained with PI solution. The percentage of sub-G1 cells was analyzed. (B) After coincubation, the cells were stained with Annexin V−FITC and PI. The PI⁻ cells were gated for Annexin V−FITC analysis. The Annexin V−FITC florescent geometric mean of the control medium (gray background) was 9.1. (C) Murine hepatoma cell line BNL 1MEA.7R.1 was coincubated with 24 μg/mL CMP for 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 16 hours, and 24 hours; medium served as a background control. After coincubation, the cells were stained using DiOC6 for mitochondrial membrane potential analysis. The DiOC6 florescent geometric mean of the control medium (gray background) was 369. All data were analyzed by FLOWJO software. Data shown are representative of two or three reproducible experiments. CMP = *Cordyceps militaris* protein; DiOC6 = 3,3′-dihexyloxacarbocyanine iodide; FITC = fluorescein isothiocyanate; PI = propidium iodide.

In the acid/alkaline tolerance experiment, CMP was dialyzed in acid solution (pH 2.0) and alkaline solution (pH 13.0) and further analyzed by SDS-PAGE. After acid treatment at pH 2.0, CMP remained. In contrast, the protein signal decreased after alkaline treatment at pH 13.0 (Figures 4B and 4D). These processed proteins were also coincubated with the BNL 1MEA.7R.1 cells for PI cell cycle analysis. The results suggested that alkaline-treated CMP lost the ability to induce apoptosis (Figure 5B). These results confirmed the degradation of acid/alkaline-treated CMP, as determined by SDS-PAGE analysis. These data showed that CMP is acid tolerant and alkaline intolerant.
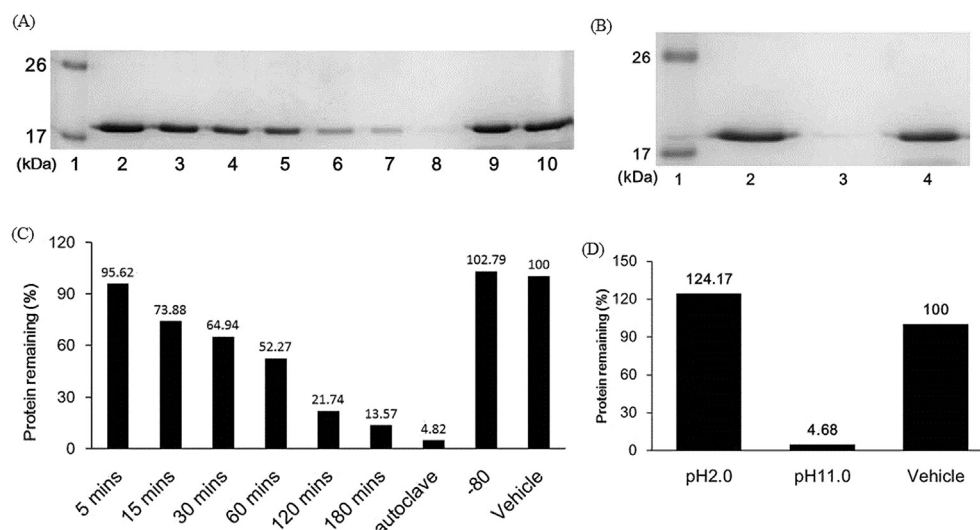
Figure 4 – Resistance of CMP to thermal and acid/alkali treatments. CMP protein was subjected to different thermal treatments (A) and acid or alkaline treatments (B) and then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The results in (A) and (B) were analyzed by GeneTool software to quantify the remaining protein (C, D). The quantity of vehicle was defined as 100%. CMP = Cordyceps militaris protein.

4.   Discussion

In this study, we purified a novel protein, CMP, from *C. militaris*. CMP could be purified through cation exchange chromatography at pH 8.0. We also analyzed CMP using native PAGE with a separating gel at pH 8.8, and the protein band was not visualized in the gel (data not shown). Based on the results of the chromatography and native PAGE, we supposed that the pI of CMP may be >8.0. Further experiments should be performed to confirm this hypothesis. Many fungi/plant toxic proteins are lectins, for example, ricin, which often has hemagglutination activity. As shown in Figures 1C and 1D, we determined that CMP is not a glycoprotein and is unable to agglutinate murine red blood cells, suggesting that CMP may not be a lectin.

Alignment analysis of the CMP N-terminal sequence suggested that CMP is a novel protein. CMP is similar to two predicted proteins in fungi, the *V. albo-atrum* predicted protein and the *M. anisopliae* hypothetical protein. The similarities are only 76% and 62%, respectively. *M. anisopliae* is a type of insect pathogen and belongs to the same scientific family as *C. militaris*. Both *C. militaris* and *M. anisopliae* are insect-parasitic fungi; hence, the physiological significances of CMP and *M. anisopliae* hypothetical protein in fungi may be similar. However, the functions of CMP or *M. anisopliae* hypothetical protein in these two fungi are not clear, and we cannot infer the functions of CMP in *C. militaris* from previous studies. Further studies should be performed to elucidate the question.

Our results indicated that CMP induced cell death but did not interfere with cell growth or affect the cell cycle. We injected CMP intraperitoneally into healthy Balb/c mice and attempted to immunize these mice for antibody preparation. However, all these mice died within 1 month, which indicated that CMP is harmful to mice. Experimental data indicated that CMP might trigger apoptosis (Figures 2 and 3). Apoptosis is a type of programmed cell death, and it is different from necrosis (which

mainly results from acute cellular injury) or autophagy in terms of both its features and its causes [29]. The main distinctive features of apoptosis include cell membrane shrinkage, chromatin condensation, exposure of phosphatydylserine outside of the cell membrane, and cell membrane blebbing [29]. When apoptosis occurs, phosphatidylserine, which is located in the inner face of the plasma membrane, translocates to the cell surface. As shown in Figures 3A and 3B, our results suggest that CMP induces apoptosis in mammalian cells.

Apoptosis can be induced through two main pathways, a mitochondrion-dependent pathway and a receptor-dependent pathway [30,31]. The loss of mitochondrial membrane potential is one of the characteristics of the mitochondrion-dependent pathway, which can be used as an index of mitochondrion-dependent apoptosis in the early stage of apoptosis. $DiOC_6$ is a green lipophilic fluorescent dye that binds to mitochondria as a probe of mitochondrial membrane potential loss. As the membrane potential diminishes, the fluorescent intensity diminishes. In the mitochondrion-dependent pathway, the Bcl-2 family of proteins mediates mitochondrial outer membrane permeabilization [32]. Proapoptotic Bcl-2 members Bax and Bak are activated, which leads to the loss of mitochondrial outer membrane potential via a conformation change [32]. After mitochondrial outer membrane permeabilization, cytochrome c is released from the mitochondria to the cytosol and binds to APAF-1 (Apoptotic protease activating factor-1) [32]. This binding causes a conformational change in APAF-1 and forms the apoptosome [32], which triggers the caspase cascade and, eventually, apoptosis. In our study, CMP-treated BNL 1MEA.7R.1 cells showed a loss of mitochondrial membrane potential (Figure 3C). This suggests that CMP induces apoptosis through the mitochondrial pathway. Further experiments should be performed to confirm this assumption.

C. militaris is a traditional eastern medicine, and people typically use it as food in diet therapy. Because CMP appears to
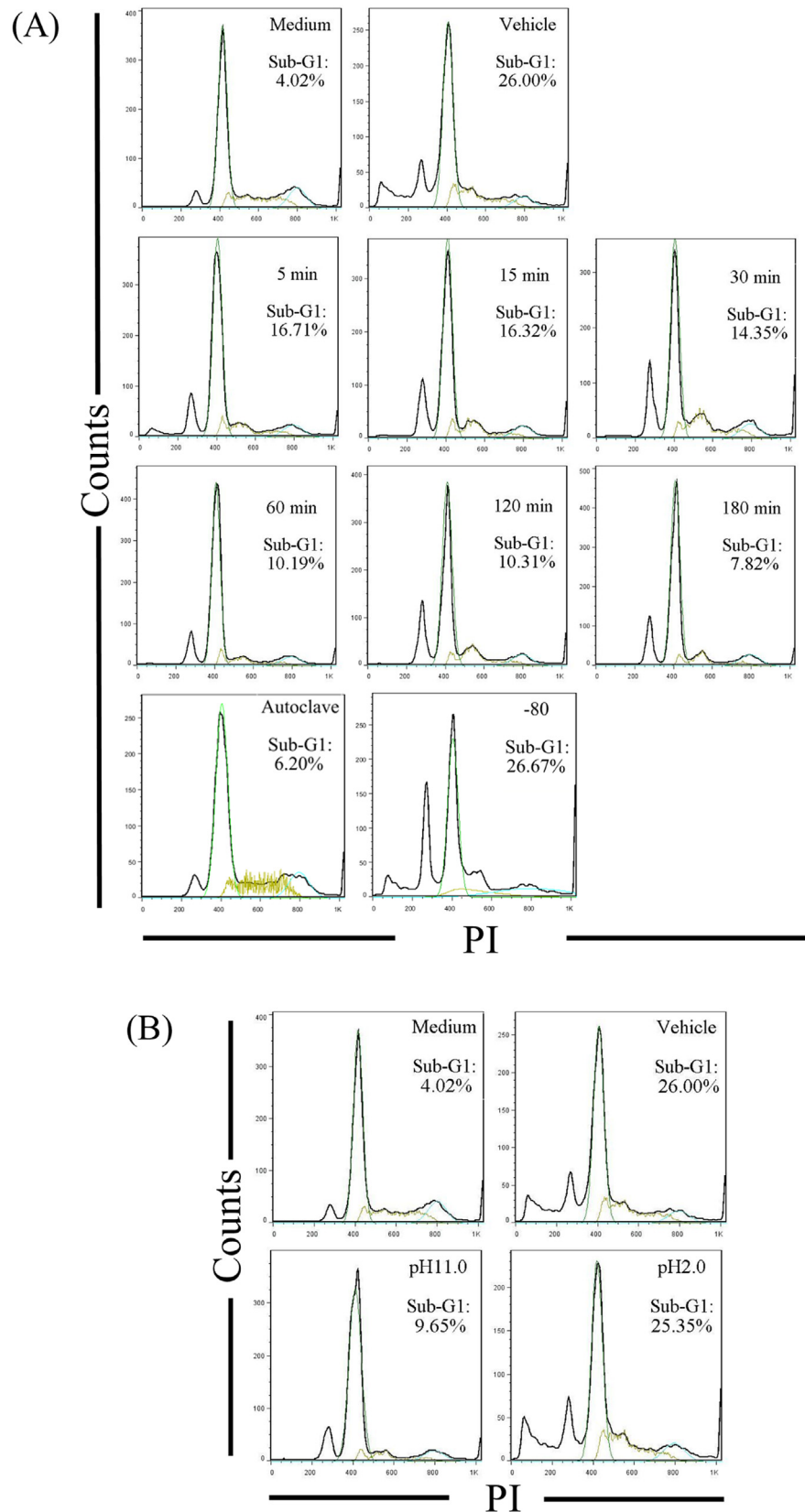
Figure 5 − The effects of heat treatment and acid/alkali treatment on CMP cytotoxicity in BNL 1MEA.7R.1 cells. The murine hepatoma cell line BNL 1MEA.7R.1 was coincubated with 24 μg/mL (A) heat-processed and (B) acid/alkali-processed CMP for 72 hours; medium served as a negative control. After coincubation, cells were fixed with 70% ethanol and then stained using propidium iodide solution. The sub-G1 percentage was analyzed by FlowJo software. CMP = Cordyceps militaris protein.

be harmful to cells, the safety of consuming *C. militaris* also warranted investigation. Thus, our study investigated the processing tolerance of CMP. Our results suggested that CMP was a heat/alkaline intolerant protein. After heating or alkaline treatment, both the quantity and the apoptosis-induced ability of CMP declined. In eastern countries, Chinese herbal plant medicines, including *C. militaris,* are usually boiled to extract their beneficial ingredients. In traditional diet therapy, *C. militaris* is also boiled in soup. For understanding whether it is safe after boiling, and how much cooking time could make *C. militaris* safety to consume, we analyzed CMP residue using SDS-PAGE and the toxin remains using PI cell cycle analysis. Because CMP showed thermal intolerance, the common cooking methods of *C. militaris* should make CMP safe to eat. These results regarding the thermal/alkaline intolerance of CMP are also valuable for the industrial food processing of *C. militaris*. We suggested that *C. militaris* should be boiled for >3 hours (based on Figures 4 and 5) or treated with an alkaline solution to reduce toxicity before consumption.

In conclusion, CMP isolated from *C. militaris* is an 18-kDa protein that lacks glycosylation. Moreover, CMP induces murine cell death, which may occur through a mitochondrion-dependent apoptosis pathway. Notably, CMP was heat and alkaline intolerant after being heated for two hours, which eliminated its apoptosis-inducing activity. Thus, our study provides valuable information regarding the potential risk of consuming *C. militaris* and offers a solution for its safe consumption.

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