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ARTICLE

A Glycoprotein Extracted from Golden Oyster Mushroom *Pleurotus citrinopileatus* Exhibiting Growth Inhibitory Effect against U937 Leukemia Cells

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Mushrooms have become popular sources of natural antitumor, antiviral, antibacterial, antioxidative, and immunomodulatory agents. Golden oyster mushroom, *Pleurotus citrinopileatus*, is a common mushroom in oriental countries for human consumption. We isolated a functional protein (PCP-3A) from the fresh fruiting body of this mushroom. The isolation procedure included ammonium sulfate fractionation, DEAE-Sepharose CL-6B ion exchange chromatography, and Sephacryl S-300 gel filtration. Electrophoresis demonstrated that PCP-3A is a glycoprotein composed of 10 subunits, each approximately 45.0 kDa in size. In vitro cell study showed that PCP-3A at a concentration about 12.5 μg/mL inhibits the proliferation of human tumor cell line U937, in a time- dependent manner (24, 48, and 72 h). It failed to agglutinate rabbit and human erythrocytes, excluding its possibility from being a lectin. Flow cytometry revealed that it is capable of inhibiting the growth of U937 cells by way of S phase arrest and apoptotic induction. We suggest that PCP-3A is worth further investigating for antitumor use.

KEYWORDS: Mushroom; leukemic; antitumor; glycoprotein

INTRODUCTION

Mushrooms have become popular sources of antitumor, antiviral, antibacterial, antioxidative, and immunomodulatory agents. The bioactive components that have been isolated from mushrooms include terpenoids, steroids, phenols, nucleotides, polysaccharides, and proteins (*1*−*3*). Probably the most important medicinal function of mushrooms is the antitumor activity, which attracts public attention around the world. A number of antitumor components have been isolated from various mushrooms. Lectins (*4*−*6*), FIP-fve (*2*), and FIII-2-b (*7*) are examples.

Pleurotus citrinopileatus, commonly called "golden oyster mushroom", belongs to the family Basidiomycete. This mushroom has recently become a popular delicacy in East Asia, including China, Japan, and Taiwan, for its taste and health value. Studies have found that the extracts of this mushroom are effective in antioxidation (*10*, *11*), cardiovascular disease prevention (*11*), immune regulation (*12*), and the reduction of blood sugar level (*13*). A few bioactive components, including a novel lectin with potent antitumor, mitogenic, and HIV-1 reverse transcriptase inhibitory activities (*4*), have been isolated from the fresh fruiting body. Another protein with immunoregulation effect was also reported (*12*).

Growth inhibitory effects of some crude mushroom proteins on tumor cell lines have been reported from our laboratory (*14*, *15*). Recently, we found a new protein in the extract from *P. citrinopileatus* in a preliminary test. The present study was therefore conducted to investigate the growth inhibitory and apoptosis induction effects of this protein on the U937 tumor cell line.

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MATERIALS AND METHODS

Isolation of the Bioactive Component. Fresh fruiting bodies (200 g) of the mushroom *P. citrinopileatus* were purchased from a local supermarket, cleaned by rinsing several times in tap water, blended with acetone (1:3, w/w) for 1 min using a Waring blender (Cycle blender, Osterizer, Mexico), and then filtered through a sieve to collect the residue. The residue was soaked in 0.5 L of 10% NaCl solution at 50 °C for 1 h to form a slurry. The slurry was centrifuged at 8500*g* for 30 min to collect the supernatant. Solid ammonium sulfate was added to the supernatant to reach 40% saturation and then centrifuged to collect the supernatant. More ammonium sulfate was added into the supernatant to 80% saturation and then centrifuged again to collect the 40−80% saturation precipitate. The precipitate was dissolved in a small volume of distilled water and then dialyzed against distilled water to obtain the crude mushroom extract.

The crude extract was applied to a DEAE-Sepharose column (GE Healthcare, Uppsala, Sweden) (2.6 × 30 cm) that had previously been equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The column was then eluted with 0.05 M Tris-HCl buffer (pH 8.0) in two steps. In the first step, the column was eluted with 5 bed volumes of the Tris-HCl buffer for the unadsorbed fractions. In the second step, protein-rich fractions were collected through the elution with 5 bed volumes of 0−0.5 M NaCl gradient Tris-HCl buffer. The antiproliferative activity of each fraction was evaluated. The fraction with the highest activity was further fractionated by gel filtration in a 1.6 × 100 cm Sephacryl S-300 column (GE Healthcare) eluted with 3 bed volumes of the Tris-HCl buffer. The protein-rich subfraction that retained the highest growth inhibitory activity was freeze-dried to be the sample for the following experiments.

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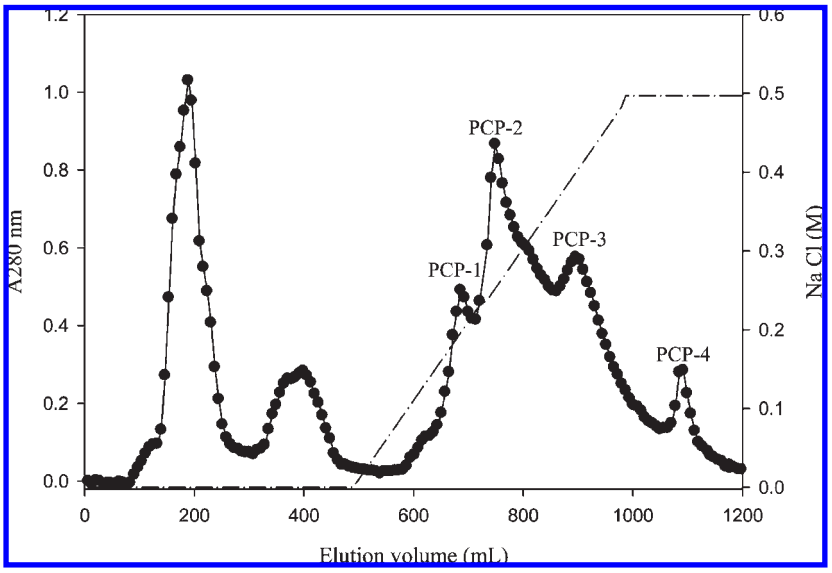


Figure 1. DEAE-Sepharose ion-exchange chromatography of 40−80% ammomium sulfate precipitates from *Pleurotus citrinopileatus*. Size of column was 2.6 cm × 30 cm. A gradient of 0−0.5 mol/L NaCl in 50 mM Tris-HCl buffer (pH 8.0) was used for elution of the four protein-rich fractions, PCP-1−4.

Molecular Mass Estimation and Isoelectric Focusing (IEF). To confirm the purity and to reveal the quaternary structure, the bioactive sample was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) followed with Sypro Ruby staining (Sigma, St. Louis, MO). Low molecular weight protein markers (97.0, 66.0, 45.0, 30.0, 20.1, and 14.4 kDa) were purchased from GE Healthcare, Buckinghamshire, U.K. Periodic acid Schiff (PAS) staining was applied to check the presence of a carbohydrate moiety in the sample, referring to the method reported by Segrest and Jackson (*16*).

IEF was performed on a pH 3−10 Ready Gel IEF in a Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, Hercules, CA) following the manufacturer's recommended procedure. The molecular mass and p*I* values of the protein were calculated using a Syngene GeneGenius gel documentation system and GeneTools software (Cambridge, U.K.).

The native molecular mass of the bioactive sample was determined by gel filtration chromatography on Sephacryl S-300 gel, referring to the methods reported by Whitaker (*17*) and Andrews (*18*). The gel filtration molecular mass protein markers (669, 440, 158, 75, and 43 kDa) were purchased from GE Healthcare.

Hemagglutinating Assay. The bioactive sample was dissolved in pH 7.2 phosphate-buffered saline (PBS; 8.00 g of NaCl/1.15 g of Na_2HPO_4/0.20 g of KH_2PO_4/0.20 g of KCl/L) and then diluted in 2-fold series. Each 50 μL aliquot of the sample solution was pipetted into a well on a microtiter U-plate, and then 50 μL of a PBS suspension of rabbit or human erythrocytes (2%) was added. The plates were incubated at 37 °C. The hemagglutination titer was recorded 2 h later when complete precipitation was observed in the control that contained no bioactive protein.

Cell Culture. Human myeloid leukemic U937 cells were purchased from ATCC (Manassas, VA) and cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were harvested and resuspended in the medium at a density of 1×10^5 cells/mL. The sample was diluted with PBS and then added to the cell suspension at 50, 25, and 12.5 μg/mL concentrations. Each 5 mL aliquot of the cell suspension was then seeded in a 35 mm Petri dish. The cultures were incubated at 37 °C. The cells were harvested in 24, 48, and 72 h. The total cell count was performed using a hemocytometer. The count of viable cells was done using a Trypan Blue Dye exclusion test (*19*). The growth inhibitory activity was calculated as follows: growth inhibitory activity (%) = (1 − population density of viable cells in the treated culture/population density of viable cells in the control) × 100%.

Flow Cytometry Analysis. Propidium iodide (PI) staining and flow cytometry (FACSCaliber, Becton Dickinson, CA) were used to analyze DNA content, referring to the method of Nicoletti et al. (*20*). U937 cells were transferred to a Petri dish at 1×10^6 cells/dish initial concentration, incubated at 37 °C, and treated with the bioactive sample (12.5 μg/mL) for 0, 12, 24, and 36 h. The cell suspension was centrifuged at 200*g* for 5 min to collect the cells. The cells were resuspended in ice-cooled 70% ethanol and then stored at −20 °C overnight to be fixed. The ethanol suspension was centrifuged at 500*g* for 5 min to collect the fixed cells, which were resuspended in 1 mL of PBS containing 0.1 μg/mL RNAase A, 50 μg/mL PI, and 0.1% Triton X-100 at room temperature and then set aside in the dark for 30 min for DNA staining. A flow cytometeter was then used to evaluate the cell cycle distribution and apoptosis. Ten thousand cells were collected and analyzed by ModFit^{LT} software (Becton Dickinson).

Statistical Analysis. The results were expressed as means ± standard deviations. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Duncan's multiple-comparison test. Differences were considered to be significant when the p values were < 0.05.

RESULTS AND DISCUSSION

Isolation of Bioactive Component. Four protein-rich fractions (PCP-1−4) were recovered from the crude extract of *P. citrinopileatus* by the DEAE-Sepharose ion-exchange column eluted with a NaCl gradient (**Figure 1**). Among them, fraction PCP-3, which corresponded to 0.37 mM NaCl elution concentration, was found to have the highest growth inhibitory activity. This fraction was further separated by the Sephacryl S-300 gel filtration column into two subfractions (PCP-3A and PCP-3B) (**Figure 2**). The former was stronger than the latter in growth inhibitory activity. Therefore, PCP-3A was taken as the bioactive component for subsequent analyses. The recovery of PCP-3A was about 0.023 mg/g of dry fruiting body powder.

Molecular Mass Estimation and IEF. Electrophoresis was conducted to determine the biochemical characteristics of PCP-3A. The apparent molecular mass of PCP-3A, as determined by gel filtration chromatography, was 450.0 kDa. SDS-PAGE revealed a single band at 44.5 kDa molecular mass (**Figure 3A**), indicating that PCP-3A is composed of 10 44.5 kDa subunits.

IEF and PAS staining assay were performed to determine the p*I* value of PCP-3A and to check whether it is a glycoprotein, respectively. The IEF of PCP-3A on polyacrylamide gel in the pH range 3−10 revealed a single band at pH 6.85 as the p*I* (**Figure 4**). PAS staining of the SDS-PAGE gel revealed an apparent single band, indicating that PCP-3A is a glycoprotein (**Figure 3B**).

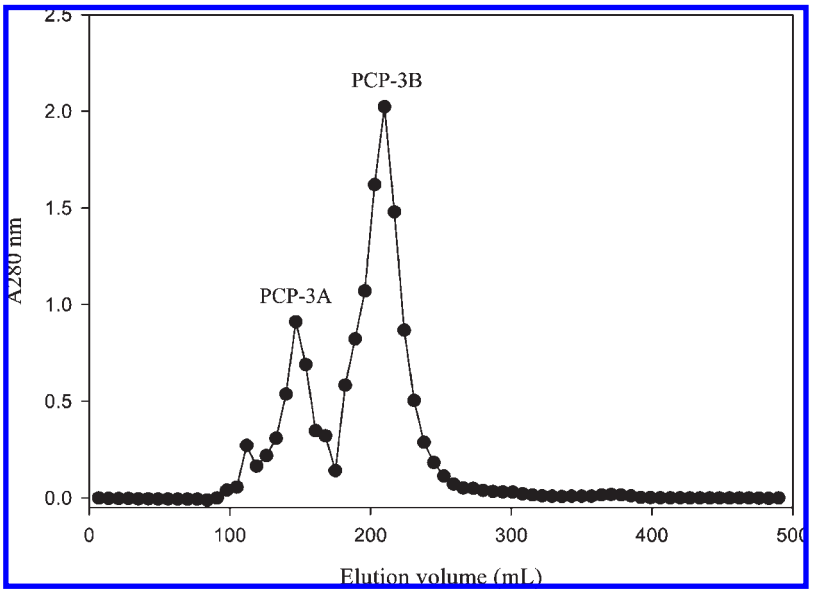


Figure 2. Sephacryl S-300 gel filtration chromatogram of fraction PCP-3 from DEAE-Sepharose CL-6B ion-exchange chromatography. Size of the gel filtration column was 1.6 × 100 cm. The column was eluted with Tris-HCl buffer (pH 8.8) at a flow rate of 0.5 mL/min. Two protein-rich subfractions (PCP-3A and PCP-3B) were obtained.

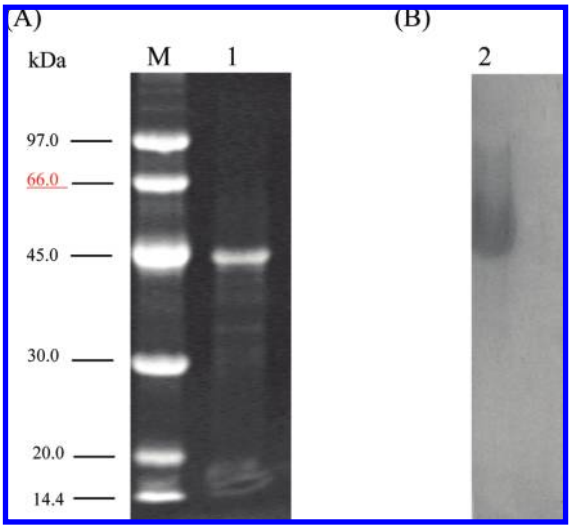


Figure 3. 12.5% SDS-PAGE electrophoregram of subfraction PCP-3A from Sephacryl S-300 gel filtration as visualized by (**A**) Sypro Ruby staining and (**B**) PAS staining. Lanes: 1 and 2, PCP-3A; M, molecular standards, from top downward, 94, 66, 45, 30, 20, and 14.4 kDa.

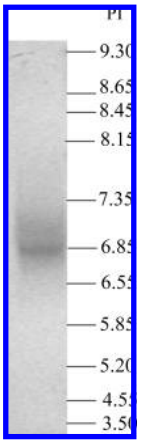


Figure 4. Isoelectric focusing of PCP-3A. The gel was stained with CBR reagent.

Hemagglutinating Assay. Most bioactive mushroom proteins are lectins. A lectin is a glycoprotein able to agglutinate mammalian erythrocytes (*21*). Hemagglutinating assay demonstrated that PCP-3A was unable to agglutinate rabbit and human erythrocytes (**Figure 5**). The positive control, which is the hemagglutinating protein PHA purified from *Phaseolus vulgaris*, agglutinated rabbit and human erythrocytes readily at a concentration as low as 125 μg/mL. On the contrary, PCP-3A failed to demonstrate any hemagglutinating activity at all at a concentration as high as 1000 μg/mL. On the basis of the results in the agglutination test, we concluded that PCP-3A is not a lectin.

Many bioactive proteins derived from the *Pleurotus* genus have been reported previously. For example, Sumisa et al. (*22*) isolated a 40 kDa lectin from the mycelial aggregate of *Pleurotus cornucopiae*. Oguri et al. (*23*) isolated a lectin-related 16.5 kDa protein from *P. cornucopiae*. Wang and Ng (*24*) isolated a 12.5 kDa translation-inhibitory ribonucleolytic protein from *Pleurotus ostreatus*. Brechtel et al. (*25*) reported a phosphatase-activating protein from *P. ostreatus*, which is a lectin consisting of two subunits (41 and 44 kDa) with affinity to α/β-galactose. All of these bioactive protein from the *Pleurotus* genus are glycoproteins.

Both lectins and nonlectin bioactive proteins have been previously found in the mushroom being investigated in the present study, *P. citrinopileatus*. Sheu et al. (*12*) isolated a 15.0 kDa nonlectin immunomodulatory protein from this mushroom. Cui et al. (*9*) found an alkaline protease from its fresh fruiting bodies. Li et al. (*4*) reported a novel lectin with potent antitumor, mitogenic, and HIV-1 reverse transcriptase inhibitory activities. The biochemical characteristics and the hemagglutinating activity suggested that PCP-3A is a new bioactive nonlectin glycoprotein never reported before.

Cell Culture. There are two ways to evaluate the inhibitory effect of a natural product on leukemic cells, either by direct inhibition on the proliferation of these cells or by stimulating the secretion of differentiation-inducing factors from immune cells to act on leukemic cells indirectly (*26*). The present study used

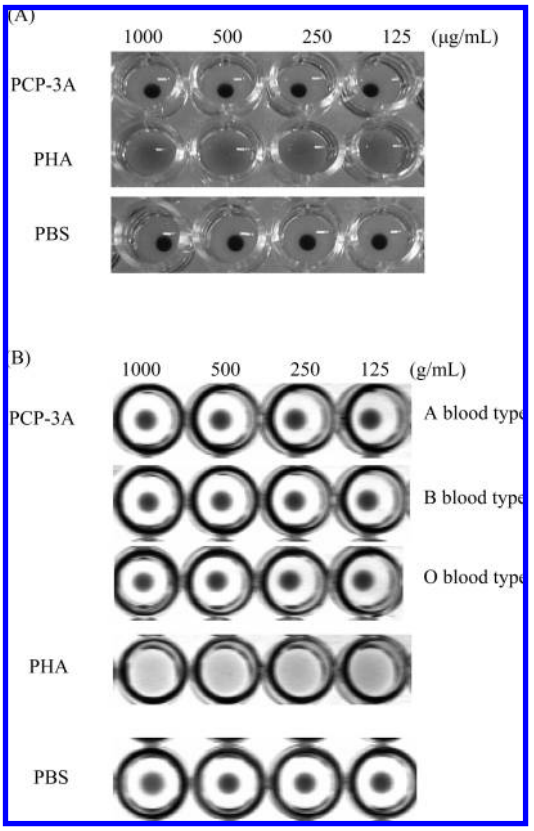


Figure 5. Hemagglutinating activity of PCP-3A: (**A**) rabbit erythrocytes; (**B**) human erythrocytes. The hemagglutinating protein PHA served as positive control. The vehicle control was treated with PBS without protein.

PCP-3A from *P. citrinopoleatus* to inhibit the growth of U937 cells in a direct model. The results are shown in **Figure 6A**. PCP-3A at 50 μg/mL concentration inhibited the growth of U937 cells by as much as 97.7.0 $\pm$ 2.31%. The growth inhibitory activity of PCP-3A at 12.5 μg/mL concentration increased with the duration (from 24 to 72 h) (**Figure 6B**). The time dependence of the growth inhibitory effect of PCP-3A on U937 cells is thus observed.

Flow Cytometry Analysis. The regulation on cell cycle and apoptosis is an important way to maintain cellular homeostasis between cell division and cell death (*27*). The induction of cell cycle arrest and apoptosis is commonly recognized as a good strategy for cancer treatment. Many studies were performed on mushrooms in search of bioactive components that may induce cell cycle arrest and apoptosis. For example, Zhao et al. (*5*) isolated an apoptosis-inducing lectin from *Agrocybe aegerita*, and Hsieh et al. (*28*) reported the induction of cell cycle arrest and apoptosis by water extracts from *Coriolus versiclor*.

The result of cell cycle analysis in samples treated with PCP-3A at 12.5 μg/mL is shown in **Figure 7**. The PCP-3A treatment apparently altered the cell cycle of U937 cells. The percentage of apoptotic cells increased while that of cells in the G2/M phase reduced. The percentage of cells in the G1/G0 phase did not change. PCP-3A at 25 μg/mL or higher concentrations induced necrosis on U937 cells (data not shown). We conclude that the presence of this protein exceeding a certain concentration can be cytotoxic, whereas at a proper concentration it is capable of inhibiting the growth of cancer cells by way of S-phase arrest and apoptotic induction.

There are reports pertaining to cell cycle arrest by extracts of mushrooms, including *Ganoderma lucidum*, *Lentinus edodes*, and *Coprinus comatus* (*29*−*31*). However, no bioactive proteins were

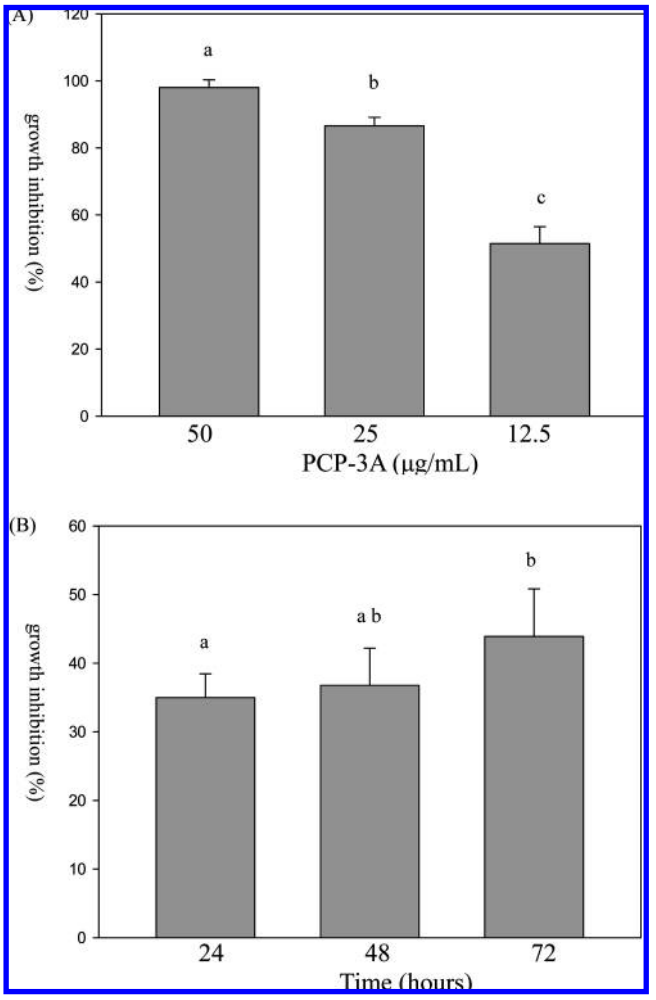
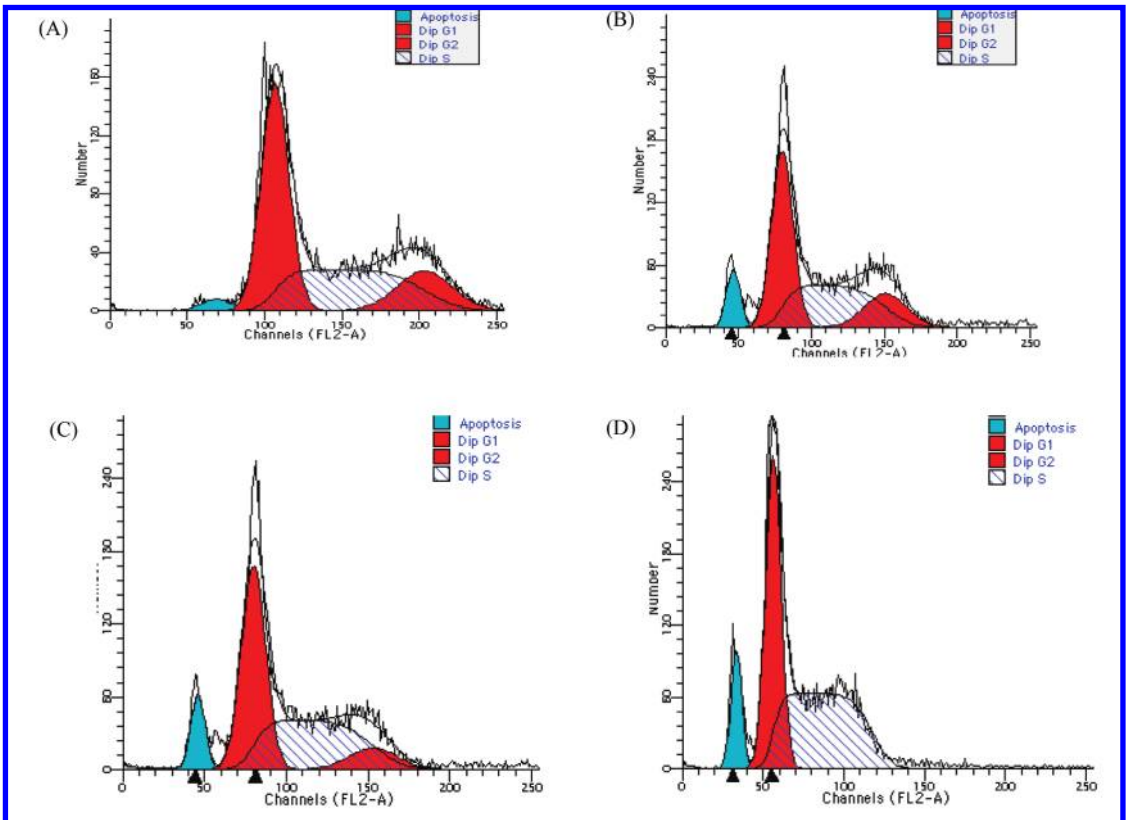


Figure 6. Growth inhibitory effect of PCP-3A on human myeloid leukemic U937 cells. (**A**) Cells were incubated with 12.5, 25, or 50 μg/mL PCP-3A for 72 h. For the control sample, cells were incubated without the presence of PCP-3A (0 μg/mL). (**B**) Human myeloid leukemic U937 cells were incubated with 12.5 μg/mL PCP-3A for 24, 48, and 72 h. For the control sample, cells were incubated without the presence of PCP-3A (0 μg/mL). The number of viable cells was determined by a Trypan Blue Dye exclusion test at the end of incubation. Data are from three independent experiments. Columns affixed with different letters (a−d) are significantly different ($p < 0.05$).

isolated in these studies. Some other reports on mushrooms described the presence of nonprotein bioactive components that promote cell cycle arrest; for example, Hui et al. (*32*) found polysaccharide peptides from *Coriolus versicolor*, Wang et al. (*33*) found irofulven from *S. preclinical*, Ye et al. (*34*) found grifolin from *Albatrellus confluens*, and Kobori et al. (*35*) found 9,11-dehydroergosterol peroxide (9(11)-DHEP)) from *Sarcodon aspratus*. The present study constitutes the first report on the induction of S-phase arrest by protein from mushroom.

In conclusion, we isolated a novel nonlectin glycoprotein (PCP-3A) from the fruiting body of edible mushroom *P. citrinopoleatus* and proved the inhibitory activity of this protein against the growth of human myeloid leukemic U937 cells through the induction of S-phase cell cycle arrest and apoptosis. Further investigation into the mechanism of signal transduction in the induction of apoptosis and cell cycle arrest, using techniques such as proteomics experiments by two-dimentional electrophoresis and protein identification by mass spectrometry, will be worthwhile.



Time (hr)	Go/G1	S	G2/M	Apoptosis
0	47.96±4.52%^{a}	36.00±4.12%^{a}	15.31±1.32%^{a}	3.20±0.33%^{a}
12	50.09±6.13%^{a}	37.15±5.31%^{ab}	12.76±0.92%^{b}	4.97±0.12%^{b}
24	46.51±5.31%^{a}	43.49±1.23%^{b}	10.00±1.10%^{c}	8.96±1.22%^{c}
36	45.10±4.59%^{a}	54.00±2.30%^{c}	0.00±0.00%^{d}	10.67±0.59%^{d}

Figure 7. Cell cycle distribution of U937 cells after incubation with PCP-3A at 12.5 μg/mL for (**A**) 0 h as the control, (**B**) 12 h, (**C**) 24 h, and (**D**) 36 h. After U937 cells were grown in medium with or without PCP-3A at the indicated concentrations, cells were harvested and washed with PBS, fixed with icy-cold 70% ethanol, stained with PI, and then treated with RNase A. Cell cycle distribution was analyzed by flow cytometry. Data shown are a representative result among three independent experiments. Means in the same column with different letters (a−d) are significantly different ($p < 0.05$).

The antitumor effect of proteins is usually attributed to their immunomodulatory activity (*2*, *7*, *14*, *25*). The present study found the possibility for apoptosis induction and cell cycle arrest to be an alternative mechanism. Further investigation into the structure, toxicity, pharmacokinetics, and reaction mechanism of PCP-3A will be among the next steps in developing it into an antitumor agent.

ABBREVIATIONS USED

IEF, isoelectric focusing; CBR, Coomassie Brilliant Blue R-250; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PAS, periodic acid Schiff; PBS, phosphate-buffered saline; LDL, low-density lipoprotein; PI, propidium iodide; ANOVA, one-way analysis of variance.

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