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# In Vitro Antioxidant and Antiproliferative Activities of Selenium-Containing Phycocyanin from Selenium-Enriched *Spirulina platensis*

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Both selenium and phycocyanin have been reported to show potent cancer chemopreventive activities. In this study, we investigated the in vitro antioxidant and antiproliferative activities of selenium-containing phycocyanin (Se-PC) purified from selenium-enriched *Spirulina platensis*. The antioxidant activity of Se-PC was evaluated by using four different free radical scavenging assays, namely, the 2,2′-azinobis-3-ethylbenzothiazolin-6-sulfonic acid (ABTS) assay, 1,1-diphenyl-2-picryhydrazyl (DPPH) assay, superoxide anion scavenging assay, and erythrocyte hemolysis assay. The results indicated that Se-PC exhibited stronger antioxidant activity than phycocyanin by scavenging ABTS, DPPH, superoxide anion, and 2,2′-azobis-(2-amidinopropane)dihydrochloride free radicals. Se-PC also showed dose-dependent protective effects on erythrocytes against $H_2O_2$-induced oxidative DNA damage as evaluated by the Comet assay. Moreover, Se-PC was identified as a potent antiproliferative agent against human melanoma A375 cells and human breast adenocarcinoma MCF-7 cells. Induction of apoptosis in both A375 and MCF-7 cells by Se-PC was evidenced by accumulation of sub-G1 cell populations, DNA fragmentation, and nuclear condensation. Further investigation on intracellular mechanisms indicated that depletion of mitochondrial membrane potential ($\Delta\Psi_m$) was involved in Se-PC-induced cell apoptosis. Our findings suggest that Se-PC is a promising organic Se species with potential applications in cancer chemoprevention.

**KEYWORDS: Selenium; phycocyanin; antioxidant activity; antiproliferative activity; apoptosis**

## INTRODUCTION

Selenium (Se) is an essential trace element with fundamental importance to humans and animals (*1*). Se functions in the active sites of a large number of Se-dependent enzymes such as glutathione peroxidase (GPXs) and acts as an essential component of several major metabolic pathways, including thyroid hormone metabolism, antioxidant defense systems, and immune function (*2–4*). The importance of Se is also due to the requirement for selenocysteine, which was genetically encoded into specific selenoproteins and known as the 21st essential amino acid (*5*). Selenium can also be incorporated nonspecifically into proteins in the form of selenomethionine (*6*). Many epidemiological, preclinical, and clinical studies have supported the role of Se compounds as potent cancer chemopreventive agents (*4*). Se supplementation was effective in reducing the incidence of cancers including prostate, lung, colon, and liver cancers (*7, 8*). The dose and the chemical form were the critical factors for cancer chemopreventive activities of Se compounds (*9, 10*). Recently, a novel selenadiazole derivative was shown to exhibit anticancer activities (*11*). Apoptosis, an active mode of cell death, has been postulated to be a critical mechanism for cancer chemoprevention by Se compounds (*4*). A number of epidemiological investigations revealed that Se deficiency would put people at risk of many chronic diseases, such as cardiomyopathy, cancer, endemic osteoarthropathy, and anemia (*1, 5*). Se deficiency is a worldwide problem. A variety of Se-enriched functional foods, such as garlic, yeast, green tea, algae, and rice have been commercialized for Se supplementation. Interestingly, it was found that Se incorporation could enhance the antioxidant activities of aqueous extracts of green tea and rice (*12, 13*). Se-containing proteins extracted from Se-enriched *Ganoderma lucidum* were also found to exhibit much higher antioxidant activities than those without Se (*14*).

*Spirulina platensis*, a blue-green microalga, has long been grown photoautotrophically for the production of functional food products because of its high contents of protein and other nutritional elements. Our previous works demonstrated that *S. platensis* was a good candidate for Se enrichment (*15–17*). Others also reported that Se-enriched *S. platensis* was a promising source for dietary Se supplementation (*18, 19*). *S. platensis* is an important source of phycocyanin (PC), a blue photosynthetic pigment that has been described as a novel natural antioxidant and antiproliferative agent (*20–27*). Recent studies suggested that PC inhibited cancer cell growth via the

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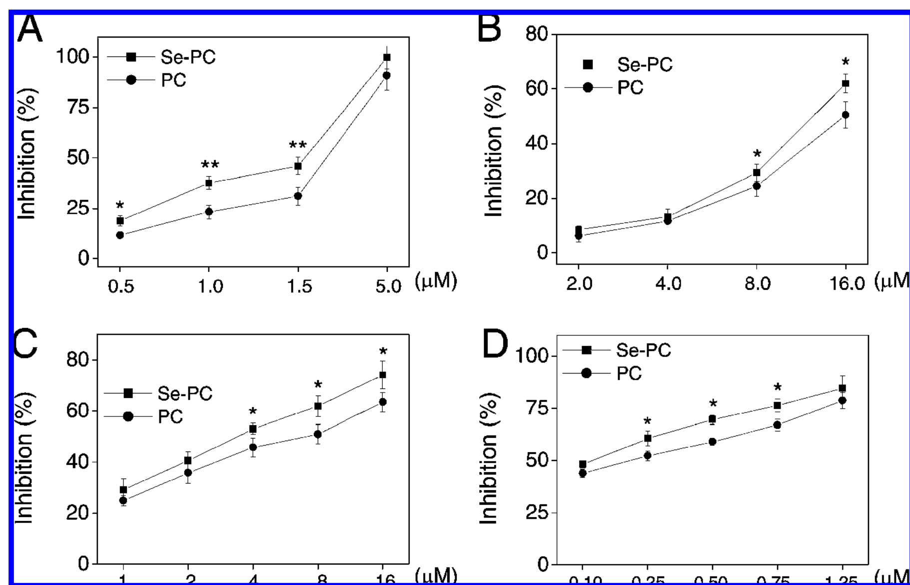


**Figure 1.** Antioxidant activities of Se-PC as determined by ABTS assay (**A**), DPPH assay (**B**), superoxide anion scavenging assay (**C**), and erythrocyte hemolysis assay (**D**). Values expressed are means ± SD of triplicates. The significant difference between Se-PC and PC treatments at the same concentration is indicated at $P < 0.05$ (*) or $P < 0.01$ (**) level.

induction of apoptosis (*22–27*). However, very limited information on the antiproliferative activity of selenium-containing phycocyanin (Se-PC) and the underlying mechanism is available.

Our previous studies showed that over 85% of Se in *S. platensis* existed in organic forms, and most of them were incorporated into the protein components (*16*). In addition, selenomethionine was identified as one of the major organic Se compounds in Se-enriched *S. platensis* (*28*). We recently purified and characterized the Se-PC from Se-enriched *S. platensis* (*29*). The objectives of this study were to evaluate the antioxidant and antiproliferative activities of Se-PC and to investigate Se-PC-induced apoptotic cell death in selected cancer cells.

## MATERIALS AND METHODS

**Chemicals.** Sodium selenite and 6-hydroxy- 2,5,7,8-tetramethyl-chromane-2-carboxylic acid (trolox) were obtained from Fluka. ABTS (2,2′-azinobis-3-ethylbenzothiazolin-6-sulfonic acid), DPPH (1,1-di-phenyl-2-picryhydrazyl), β-nicotinamide adnine dinucleotide, reduced form (NADH), nitrotetrazolium blue chloride (NBT), phenazine metho-sulfate (PMS), ascorbic acid, thiazolyl blue tetrazolium bromide (MTT), propidium iodide (PI), solid JC-1, and 4′,6-diamidino-2-phenyindole (DAPI) were obtained from Sigma. AAPH [2,2′-azobis-(2-amidino-propane)dihydrochloride] and hydrogen peroxide were obtained from Wako and BDH, respectively. A reagent kit for single cell gel electrophoresis assay (Comet Assay) was purchased from Trevigen, Inc. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, bovine calf serum, and the antibiotic mixture (penicillin−streptomycin) were purchased from Invitrogen (Carlsbad, CA). All of the solvents used were of high-performance liquid chromatgraphy (HPLC) grade. The water used for all experiments was supplied by a Milli-Q water purification system from Millipore.

**Purification of Se-PC.** The culture of Se-enriched *S. platensis* was carried out by using a stepwise Se addition method, where Se was added to the medium on day 7 (100 mg/L), day 8 (150 mg/L), and day 9 (200 mg/L), respectively, with an accumulative concentration of 450 mg/L (*16, 17*). Se-PC and PC were extracted from *S. platensis* by ultrasonication and purified by liquid chromatography as described by Chen et al. (*28*). The fractions with purity ratios ($A_{620}/A_{280}$) > 4.5 were collected and lyophilized to solid powder for further use. The total Se concentration in the purified Se-PC was determined as 496.5 μg/g by inductively couple plasma-atomic emission spectroscopy method (*29*). The molecular masses of Se-PC and PC were determined as 35682.4 and 35671.8 Da, respectively, by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight analysis (*29*).

**Trolox Equivalent Antioxidant Capacity (TEAC) Assay.** $ABTS^{\bullet+}$ free radical scavenging activities of antioxidants were measured according to the method described by Miller et al. (*30*) with some modifications. The ABTS reagent was prepared by passing the ABTS stock solution (5 mM) through manganese dioxide to allow the completion of radical generation, and then, the solution was passed through a 0.2 μM PVDF syringe filter. After that, the filtrate was diluted with 5 mM phosphate-buffered saline (PBS) buffer (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm recorded on a spectrophotometer (Genesys5, Spectronic Instruments, Rochester, NY). To determine the antioxidant activity, 1 mL of ABTS reagent was mixed with 50 μL of sample or positive controls. The absorbance was then measured at 734 nm, 6 min after the initiation of mixing (*31*).

**DPPH Free Radical Scavenging Assay.** The DPPH free radical scavenging activities were determined according to the method described by Okada and Okada (*32*). Briefly, 1 mL of test sample was mixed with 0.5 mL of DPPH solution with a final concentration at 0.2 mM. The mixture was shaken vigorously and left to stand for 6 min, and then, the change in absorbance at 517 nm was measured.

**Superoxide Anion Scavenging Activity.** The measurement of superoxide anion scavenging activity was based on the method described previously (*33, 34*). Superoxide anions were generated nonenzymatically in a PMS−NADH system by the oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide radicals were generated in 1 mL of Tris−HCl buffer (16 mM, pH 8.0) containing NBT (50 μM) and NADH (78 μM). The reaction was started by adding PMS solution (10 μM) to the mixture. The reaction mixtures were incubated at room temperature for 5 min, and the absorbance at 560 nm was taken against the blank. A decrease in absorbance of the reaction mixtures indicated an increase in superoxide anion scavenging activities. The percentage inhibition of superoxide anion was calculated as follows:

$$\% \text{ inhibition} = (1 - A/B) \times 100$$

where *A* and *B* are the absorbances of the treated sample and the control, respectively.

**Erythrocyte Hemolysis Assay.** The AAPH free radical scavenging activities of antioxidants were measured as the inhibition of erythrocyte hemolysis according to the procedures described by Cheung et al. (*35*) with some modifications. Briefly, the blood was obtained using heparinized syringes from male Sprague−Dawley rats at body weights of 150−200 g. Erythrocytes were separated from the plasma by centrifugation at 1500*g* for 10 min and then washed three times with PBS buffer (pH 7.4). An aliquot of 0.1 mL of 20% erythrocyte suspension was mixed with 0.2 mL of 200 mM AAPH and 0.1 mL of PBS buffer (absorbance *A*) or 0.1 mL of test compounds (absorbance *B*). The mixture was shaken gently and incubated at 37 °C for 3 h.

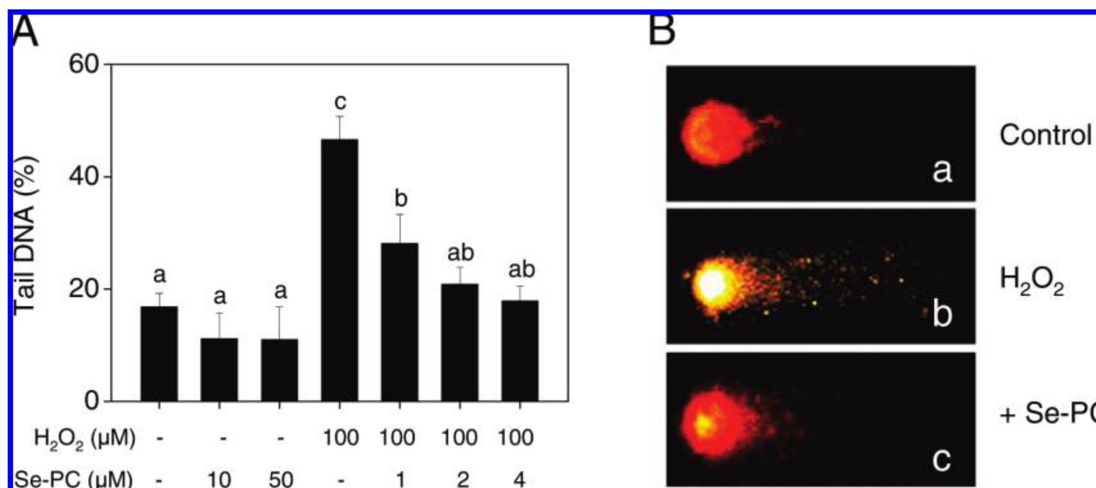


**Figure 2.** Protective effects of Se-PC on blood cells against $H_2O_2$-induced DNA damage as assessed by the Comet assay (**A**). Values expressed are means ± SD of three replicates. Data with the same letters are not significantly different ($P > 0.05$). (**B**) Representative photomicrographs of blood cells under different treatments (a, control; b, 100 $\mu$M $H_2O_2$; and c, 100 $\mu$M $H_2O_2$ + 2 $\mu$M Se-PC).

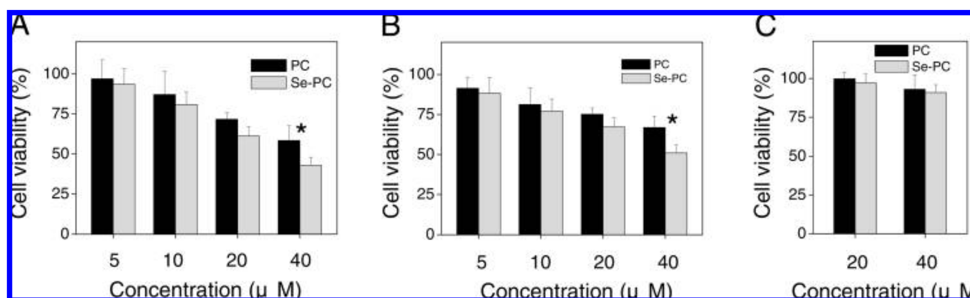


**Figure 3.** Effects of Se-PC and PC on the growth of A375 cells (**A**), MCF-7 cells (**B**), and Hs68 cells (**C**). Cells were treated with different concentrations of Se-PC or PC for 72 h. Cell viability was determined by the MTT assay as described in the Materials and Methods. The significant difference between Se-PC and PC treatments at the same concentration is indicated at the $P < 0.05$ (*) level.

After incubation, the mixture was diluted with 8 mL of PBS, while complete hemolysis was achieved by dilution with 8 mL of distilled water. The mixture was centrifuged at 1500*g* for 10 min, and the absorbance of the supernatant was measured at 540 nm. Ascorbic acid and trolox were used as positive controls. The % hemolysis inhibition was calculated as follows:

$$\% \text{ hemolysis inhibition} = (1 - A/B) \times 100\%$$

**Comet Assay (Single Cell Gel Electrophoresis).** Blood cells collected from Sprague−Dawley rats were incubated with 100 $\mu$M $H_2O_2$ in the presence or absence of Se-PC for 1 h at 37 °C. Treated cells were then harvested by centrifugation at 1500*g* for 10 min. A Comet assay reagent kit for single-cell gel electrophoresis (Trevigen, Inc.) was used for detection of DNA damage according to procedures provided by the manufacturer. Briefly, cells were combined with molten LMAgarose (at 37 °C) at a ratio of 1:10 (v/v) and immediately pipetted onto the slides, which were subsequently placed in a refrigerator for 30 min until clear rings appeared at the edge of the slide area. The slides were then immersed in prechilled lysis solution (containing 10% DMSO) and left on ice for 60 min followed by immersion in freshly prepared alkali solution (300 mM NaOH, 1 mM EDTA, pH > 13) for 60 min on ice in the dark. After DNA unwinding, slides were subjected to alkaline solution for electrophoresis, which was performed for 30 min in a Savant ps 250 system set at 300 mA and 1 V/cm. After electrophoresis, slides were dipped into $H_2O$ several times, then immersed in 70% ethanol for 5 min, and dried in the air. Finally, DNA was stained with diluted SYBR Green I (Trevigen, Inc.) for epifluorescence microscopy. The slides were mounted, sealed with nail polish, and viewed immediately on a fluorescence microscope (Nikon, Eclipse E-600). Fifty cells per slide were selected randomly, and their tail DNA was determined using an image analysis system (Komet 3.1 from Kinetics Imaging Ltd., Liverpool) linked to a CCD camera.

**Cell Culture.** The cell lines used in this study, including human melanoma A375, human breast adenocarcinoma MCF-7, and human fibroblast Hs68, were obtained from American Type Culture Collection (ATCC, Manassas). All cell lines were cultured in either RPMI 1640 or DMEM medium supplemented with fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (50 units/mL) at 37 °C in a humidified incubator with 5% $CO_2$ atmosphere.

**Cell Viability Assay.** Cell viability was determined by measuring the ability of the cells to metabolize 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan dye according to our previous method (*36*). Briefly, cells were placed into 96-well plates at a density of 2.0 $\times 10^3$ cells/well. After 24 h, different concentrations of Se-PC or PC were added and incubated for 72 h. Then, 20 $\mu$L/well of MTT solution (5 mg/mL in PBS buffer) was added and incubated for another 5 h. The medium was removed and replaced with 150 $\mu$L/well of DMSO to dissolve the formazan crystals. Absorbance at 570 nm was taken with a 96-well microplate reader.

**Flow Cytometry.** Flow cytometric analysis was carried out according to our previous method (*37*). Briefly, cells exposed to Se-PC were harvested by centrifugation and washed with PBS. Cells were stained with PI after fixation with 70% ethanol at −20 °C overnight. The DNA content was analyzed with a Beckman Coulter Epics XL MCL flow cytometer (Miami, FL). The cell cycle distribution was analyzed using a MultiCycle software (Phoenix Flow Systems, San Diego, CA). The proportions of cells in G0/G1, S, and G2/M phases were represented as DNA histograms. Apoptotic cells with hypodiploid DNA contents were measured by quantifying the sub-G1 peak. For each experiment, 10000 events per sample were recorded.

**TUNEL Assay and DAPI Staining.** DNA fragmentation was examined by fluorescence staining using the TUNEL apoptosis detection kit (Roche). Briefly, cells cultured in chamber slides were fixed with 3.7% formaldehyde and permeablized with 0.1% Triton X-100 in PBS.

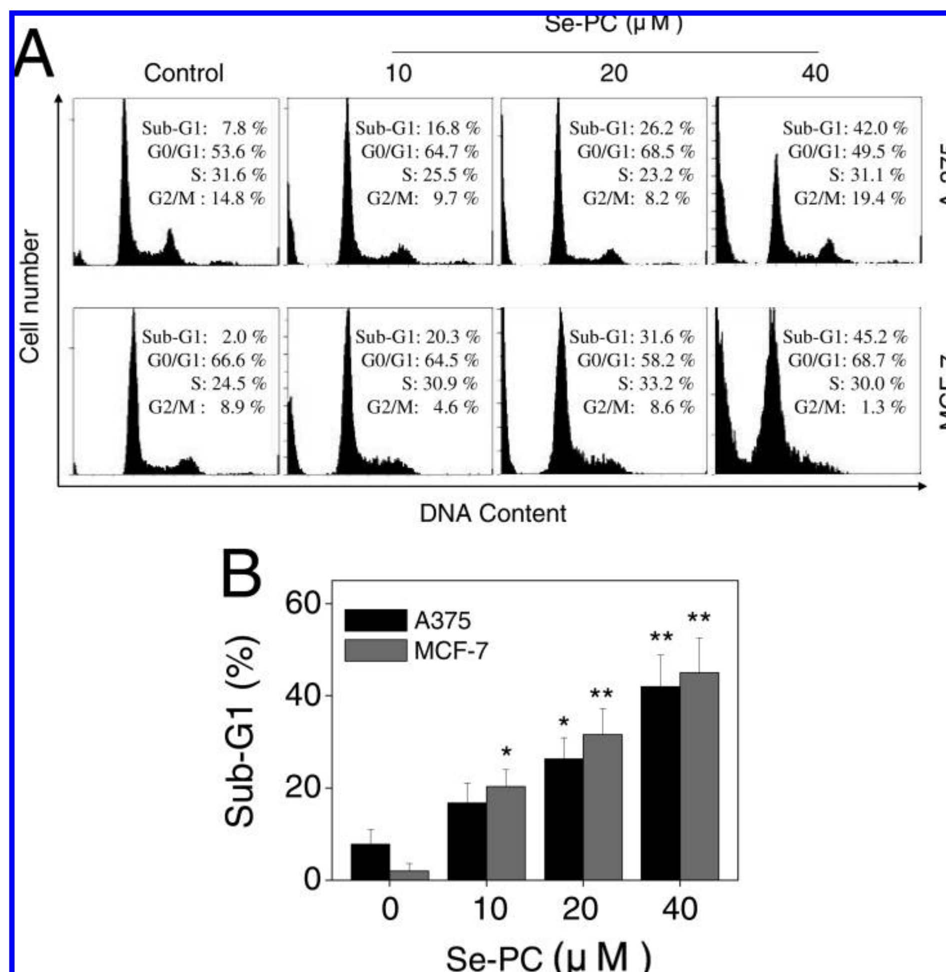


**Figure 4.** Effects of Se-PC on cell cycle distribution of A375 and MCF-7 cells. (**A**) Cells treated with different concentrations of Se-PC for 72 h were collected and stained with PI after fixation by 70% ethanol. Following flow cytometry, cellular DNA histograms were analyzed by the MultiCycle software. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak. Each value represents the mean of three independent experiments. (**B**) Quantitative analysis of Se-PC-induced apoptotic cell death by measuring the sub-G1 cell population. The significant difference between the Se-PC treatment and the control is indicated at $P < 0.05$ (*) or $P < 0.01$ (**) levels.

The cells were incubated with TUNEL reaction mixture for 1 h. For nuclear staining, cells were incubated with 1 $\mu$g/mL DAPI for 15 min at 37 °C. After then, cells were washed with PBS and examined with a fluorescence microscope (Nikon Eclipse 80i) (magnification, 200×). The apoptotic percentage was calculated by dividing the TUNEL-positive cell number by the total cell number (DAPI-positive) within the same area.

**Evaluation of Mitochondrial Membrane Potential ($\Delta\Psi_m$).** Cells cultured in six-well plates were trypsinized and resuspended in 0.5 mL of PBS buffer containing 10 $\mu$g/mL JC-1. After incubation for 10 min at 37 °C, the cells were immediately centrifuged to remove the supernatant, and the cell pellets were suspended in PBS and then analyzed by flow cytometry. The percentage of green fluorescence from JC-1 monomers was used to represent the cells that lost $\Delta\Psi_m$.

**Statistical Analysis.** Experiments were carried out at least in triplicate, and results were expressed as means ± standard deviations (SDs). Statistical analysis was performed using SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc. Chicago, IL). The difference between two groups was analyzed by two-tailed Student's *t* test, and that between three or more groups was analyzed by one-way analysis of variance multiple comparisons. Differences with $P < 0.05$ (*) or $P < 0.01$ (**) were considered statistically significant.

## RESULTS AND DISSCUSION

**Antioxidant Activity of Se-PC.** *ABTS Assay.* In this assay, the relatively long-lived $ABTS^{\bullet+}$, generated by the direct oxidation of ABTS with manganese dioxide, is decolorized during the reaction with hydrogen-donating antioxidants (*38*). This assay provides a direct comparison of the antioxidant activities of tested samples as expressed in terms of percentage inhibition. As shown in **Figure 1A**, concentration−response curves of Se-PC and PC were obtained at final concentrations ranging from 0.5 to 5.0 $\mu$M. Se-PC inhibited ABTS oxidation by 18.8% at 0.5 $\mu$M and up to 46.0% at 1.0 $\mu$M, which were higher than those of PC (11.7 and 31.1% respectively), suggesting a stronger antioxidant activity of Se-PC under the hydrophilic condition. In addition, Se-PC and PC showed significantly ($P < 0.05$) higher inhibition on ABTS oxidation than two well-known antioxidants, namely, ascorbic acid and trolox (data not shown).

*DPPH Assay.* The stable radical DPPH has been widely used for the determination of primary antioxidant activities of pure antioxidant compounds, plant and fruit extracts, and food materials. The assay is based on the reduction of DPPH radicals in methanol, which causes a decrease in absorbance at 515 nm. The potency of a molecule to scavenge DPPH radicals is due to the number of hydrogens available for donation by the hydroxyl groups (*39*). The DPPH radical scavenging activities of Se-PC and PC are shown in **Figure 1B**. Se-PC inhibited DPPH radical by 58.9% at concentration of 16.0 $\mu$M, which is

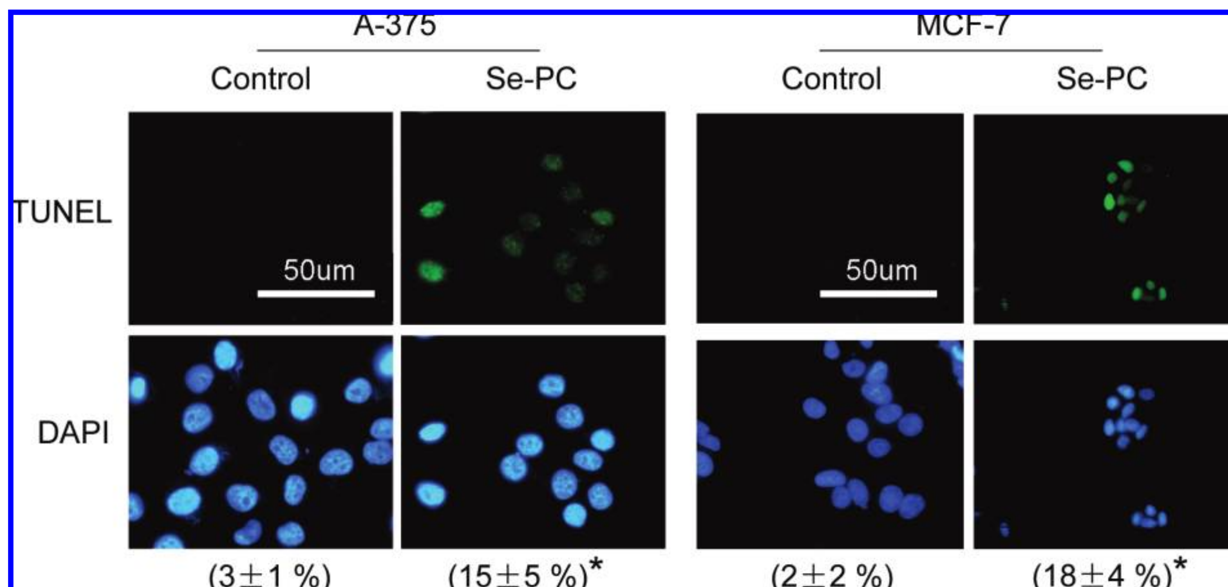


**Figure 5.** Representative images of Se-PC-induced DNA fragmentation in A375 and MCF-7 cells as detected by TUNEL assay and DAPI staining (magnification, 200×). Cells were cultured with or without 10 $\mu$M Se-PC for 24 h. The apoptotic percentages, as calculated by dividing the TUNEL-positive cell number by the total cell number (DAPI-positive) within the same area, were listed in the parentheses. The values are expressed as means ± SD of three independent experiments. The significant difference between the Se-PC treatment and the control is indicated at the $P$ < 0.05 (*) level.

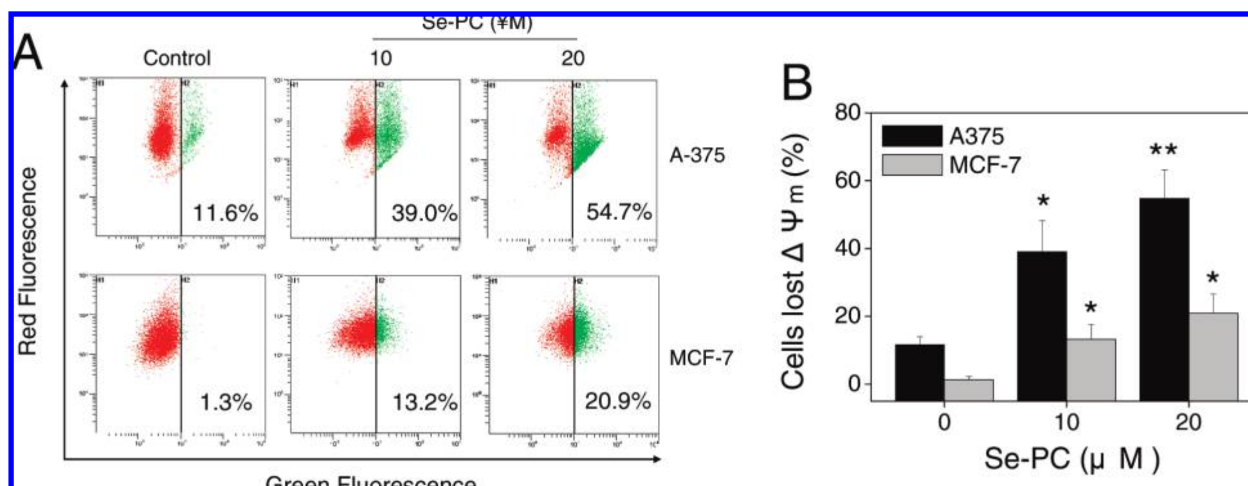


**Figure 6.** Loss of mitochondrial membrane potential ($\Delta\Psi_m$) in A375 and MCF-7 cells induced by Se-PC. (**A**) Cells treated with Se-PC were harvested and stained with JC-1 and then analyzed by flow cytometry. The number in the right region of each dot plot represents the percentage of cells that emit green fluorescence due to the loss of $\Delta\Psi_m$. (**B**) Percentages of cells that lost $\Delta\Psi_m$ in response to Se-PC treatment. The significant difference between the Se-PC treatment and the control is indicated at $P$ < 0.05 (*) or $P$ < 0.01 (**) levels.

significantly ($P$ < 0.05) higher than that of PC (50.5%), indicating a better free radical scavenging potential of Se-PC under the hydrophobic condition.

*Superoxide Anion Scavenging Activity.* Superoxide anion is considered to be biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals. In the PMS/NADH−NBT system, superoxide anions are derived from dissolved oxygen by a PMS/NADH coupling reaction, which can specifically reduce NBT. The decrease of absorbance at 560 nm indicates the consumption of superoxide anions in the reaction mixture. As shown in **Figure 1C**, Se-PC and PC effectively inhibited superoxide anions in a dose-dependent manner at the range of 1−16 $\mu$M. For instance, Se-PC inhibited superoxide anions by 29.1% at 1 $\mu$M and up to 71.2% at 16 $\mu$M, which were higher than those of PC (24.9 and 67.5%, respectively).

*Erythrocyte Hemolysis Assay.* In this assay, AAPH radicals attack erythrocyte membrane components, including proteins and lipids, and cause changes in the structure and function of the membranes (*40*). As shown in **Figure 1D**, Se-PC and PC showed dose-dependent protection on erythrocytes against AAPH-induced hemolysis. For instance, Se-PC inhibited hemolysis by 50.2% at 0.1 $\mu$M and up to 84.6% at 1.25 $\mu$M, which were higher than those of PC (43.9 and 78.7%). The difference in antioxidant activities between Se-PC and PC obtained by this assay was quite consistent with those obtained by ABTS, DPPH, and superoxide anion scavenging assays.

**Protective Effects of Se-PC on $H_2O_2$-Mediated DNA Damage.** Reactive oxygen species (ROS) are constantly generated and eliminated in all biological systems, and they play important roles in a variety of normal biochemical functions and abnormal pathological processes. Excess intracellular ROS may attack cellular

membrane lipids, proteins, and DNA, inhibit their normal functions, and finally cause oxidative damage. $H_2O_2$ is one of the reactive products of oxygen metabolism. Accumulation of $H_2O_2$ is suggested to cause DNA strand breakage by the generation of hydroxyl radicals ($OH^{\bullet}$) close to a DNA molecule through the Fenton or Fenton-like reactions (*41*). Exposure of DNA to oxidative stress leads to more than 20 different types of base damage, producing oxidized and ring-fragmented nitrogen bases (*42*). The Comet assay provides a simple and effective method for evaluating DNA damage at the single-cell level. The assay is based on the ability of denatured or cleaved DNA fragments to migrate out of the cell under the influence of an electric field. The alkaline electrophoresis used in this experiment could detect single-stranded DNA breaks, double-stranded DNA breaks, and the majority of apurinic sites and apyrimidinic sites as well as alkali labile DNA adducts, for example, phosphoglycols and phosphotriesters (*43*). **Figure 2** shows the protective effects of Se-PC against $H_2O_2$-induced DNA damage in blood cells as assessed by the alkali Comet assay. $H_2O_2$ alone induced the occurrence of tail DNA by 46.6% at final concentration of 100 $\mu$M. In contrast, exposure of blood cells to Se-PC at concentrations of 10 or 50 $\mu$M induced no significant increase in DNA damage as compared with the control (PBS). The results revealed that Se-PC showed no genotoxicity at the concentrations tested. Interestingly, $H_2O_2$-mediated DNA damage was significantly ($P < 0.05$) inhibited by Se-PC at concentrations of 1, 2, and 4 $\mu$M, which led to a significant ($P < 0.05$) decrease in tail DNA from 46.6 ($H_2O_2$ treatment) to 28.1, 20.9, and 17.9%, respectively.

**Antiproliferative Activity of Se-PC.** As the balance between therapeutic potential and toxic side effects of a compound is very important when evaluating its usefulness as a pharmacological drug, experiments were designed to investigate the in vitro cytotoxicity of Se-PC against several cancer and normal cell lines. The antiproliferative activities of Se-PC and PC were first screened against melanoma A375 cells and breast adrenocarcinoma MCF-7 cells by the MTT assay. As shown in **Figure 3A**,**B**, both Se-PC and PC showed potent antiproliferative activities against the selected cancer cell lines. Furthermore, Se-PC exhibited stronger inhibitory effects on the growth of A375 and MCF-7 cells than PC did at the concentrations ranging from 5 to 40 $\mu$M. Despite this potency, Se-PC and PC at concentrations of 20 and 40 $\mu$M showed no significant inhibition on the growth of normal Hs68 cells (**Figure 3C**). These results suggest that Se-PC and PC possess selectivity between cancer and normal cells and display potential applications in cancer chemoprevention and chemotherapy.

**Cell Apoptosis Induced by Se-PC.** Apoptosis is characterized by cell shrinkage, cytoplasmic and nuclear condensation, membrane blebbing, DNA fragmentation, and finally the formation of apoptotic bodies followed by secondary necrosis. Apoptosis plays essential roles in the development of multicellular organisms (*44*). Nowadays, the roles of apoptosis in the action of anticancer drugs have become more and more clear (*45*). Accumulative evidence support that apoptosis is a critical mechanism for cancer chemoprevention by Se compounds (*4*). Thus, the mode of cell death-induced Se-PC was elucidated in the present study. First, we used flow cytometric analysis to examine whether apoptosis was involved in the cell death induced by Se-PC. The results in **Figure 4** revealed that exposure of A375 and MCF-7 cells to different concentrations of Se-PC led to a dose-dependent increase in the cell proportion at the sub-G1 phase. However, no significant changes in cell cycle distribution were observed in treated cells. These results indicated that cell death induced by Se-PC was mainly caused by induction of apoptosis. This finding was further confirmed by the observation of DNA fragmentation as examined by the TUNEL assay. As shown in **Figure 5**, Se-PC treatment induced a significant increase in nuclear DNA fragmentation (TUNEL-positive) in both A375 and MCF-7 cells.

**Mitochondria Dysfunction Induced by Se-PC.** Mitochondria act as a point of integration for apoptotic signals originating from both the extrinsic and the intrinsic pathways. Disruption of mitochondrial membrane potential ($\Delta\Psi_m$) and release of apoptogenic factors are critical events in both caspase-dependent and -independent apoptotic pathways (*46*). Thus, the status of $\Delta\Psi_m$ was investigated in Se-PC-treated A375 and MCF-7 cells by JC-1 flow cytometric analysis. As shown in **Figure 6**, Se-PC induced a dose-dependent increase in the depletion of $\Delta\Psi_m$ in both cell lines, as evidenced by the shift of fluorescence from red to green. The percentage of depolarized mitochondria in A375 cells exposed to 10 and 20 $\mu$M Se-PC increased from 11.6 (control) to 39.0 and 54.7%, respectively. Similarly, Se-PC induced the increase of depolarized mitochondria in MCF-7 cells from 1.3 (control) to 13.2 and 20.9%, respectively. These results indicated that Se-PC induced apoptotic cell death via the mitochondria-mediated pathway.

In summary, Se-PC exhibits stronger antioxidant activities by scavenging the ABTS, DPPH superoxide anion, and AAPH free radicals in comparison with PC. Se-PC also showed a protective effect on blood cells against $H_2O_2$-induced DNA damage. Furthermore, Se-PC was found effective to inhibit the growth of melanoma A375 cells and breast adenocarcinoma MCF-7 cells via triggering the mitochondria-mediated apoptosis. The enhancement of the antioxidant and antiproliferative activities of Se-PC could be possibly, at least partly, due to the incorporation of Se into the peptides of PC in the form of selenoamino acids, such as selenocysteine or selenomethionine. Further works are needed to reveal the exact Se forms in Se-PC responsible for the enhancement of biological activities. Because of its antioxidant and antiproliferative activities, Se-PC is suggested as a novel organic Se species with potential applications in cancer chemoprevention.

## ACKNOWLEDGMENT

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