JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 7737–7742 7737
DOI:10.1021/jf9019344

Preparation, Characterization, and Antiproliferative Activities of the Se-Containing Polysaccharide SeGLP-2B-1 from Se-Enriched *Ganoderma lucidum*

Dejing Shang,*,† Jianing Zhang,‡ Lei Wen,† Yang Li,† and Qiao Cui†

†Liaoning Provincial Key Laboratory of Biotechnology and Drug Discovery, Faculty of Life Science, Liaoning Normal University, Dalian 116029, China, and ‡Department of Biochemistry, Dalian Medical University, Dalian 116027, China

Inorganic selenite can be transformed into organic forms and bind to proteins and polysaccharides in Se-enriched submerged *Ganoderma lucidum* cultures. In the present study, a novel Se-containing polysaccharide, SeGLP-2B-1, was purified from the Se-enriched mycelia of *G. lucidum* and the antiproliferative activities against six human cancer cell lines were investigated. The Se content of SeGLP-2B-1 was 186.7 μg/g, which was 150-fold larger than that of the regular polysaccharide GLP-2B-1 (1.3 μg/g). SeGLP-2B-1 (1.06×10^6 Da) was composed of glucose, rhamnose, xylose, and galactose with a molar ratio of 1.000:0.652:0.443:0.227. SeGLP-2B-1 exhibited an approximately 10-fold stronger antiproliferative activity against six human cancer cell lines as compared to GLP-2B-1. Thus, Se is believed to play an important role in increasing the antiproliferative property of SeGLP-2B-1. These findings indicate that SeGLP-2B-1 may serve as a dietary Se supplement.

KEYWORDS: Selenium; polysaccharide; *Ganoderma lucidum*; antiproliferation; isolation and purification

INTRODUCTION

Selenium (Se) is an essential trace element for human beings and many other forms of life as well as an essential component of a number of Se-dependent enzymes, such as glutathione peroxidase, thioredoxin reductase, and iodothyronine 5′-deiodinase (*1*). Se deficiency induces a variety of chronic diseases, including cardiovascular disease, cancer, thyroid dysfunction, immunity impairment, Keshan disease, and anemia (*2*). Suitable Se supplements in human diets may increase antioxidant activity *in vivo* and reduce cancer risk (*3*) and are particularly necessary for populations that live in geographical regions characterized by low soil and water Se levels. Because the bioavailability, retention, and fate of Se in the human body are chemical-species-dependent, the chemical form of Se in Se supplements is important. There is consensus that organic Se from natural foods has higher bioavailability and is safer than inorganic Se species (*4*, *5*); therefore, it is crucial to develop suitable organic Se dietary sources for humans. Submerged mushroom cultures enriched with Se provide a feasible and economical approach for the production of organic Se compounds. Se-enriched mushrooms are currently being explored as a promising dietary Se supplement.

Ganoderma lucidum mushroom has been used as a traditional herbal medicine to treat various human diseases, such as chronic hepatopathy, hypertension, bronchitis, and cancer, in China and other Asian countries for thousands of years (*6*, *7*). Previous reports demonstrated that *G. lucidum* exhibited significantly higher capability to biotransform Se than *Flammulina velutipes*, *Lentinula edodes*, and *Hericium erinaceus* in Se-enriched submerged culture, and up to 5000 μg/g organic Se was found in the mycelia of Se-enriched *G. lucidum* (SeGL) (*8*). Se-enriched mycelia of *G. lucidum* absorbed 20−30% of the inorganic Se in the growth medium and biotransformed it into organic forms by preferentially integrating Se into proteins and polysaccharides mainly responsible for the storage of organic Se (*9*). A 36 600 Da glycoprotein with high Se content was isolated from SeGL, showed approximately 3-fold stronger activity for scavenging superoxide and hydroxyl radicals as compared to the water-soluble protein extract, and demonstrated antioxidant activity in a Se-dependent manner (*10*). The polysaccharide extracts from Se-enriched *G. lucidum* (SeGLPs) protected DNA from hydroxyl radical oxidative damage in a Se dose-dependent manner and exhibited high activities for scavenging superoxide, hydroxyl radicals, and other antioxidant properties (*9*). It is of great interest to investigate the antiproliferative activity of SeGLPs. In the present study, SeGLP-2B-1, a 1060 kDa polysaccharide with high Se content in SeGL, was isolated and characterized for the first time and exhibited antiproliferative activity in cancer cells.

MATERIALS AND METHODS

Reagents and Cell Lines. Sodium selenite and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (Sigma-Aldrich Co., Shanghai, China). RPMI-1640 medium and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). All of the solvents used were of high-performance liquid chromatography (HPLC) grade. The water used for all experiments was supplied by a Milli-Q water purification system from Millipore (Millipore Co., Beijing, China). Human malignant breast carcinoma (MCF-7), human erythroid

*To whom correspondence should be addressed. Telephone: +86-411-84258575. Fax: +86-411-82156111. E-mail: djshang@lnnu.edu.cn.

Published on Web 08/13/2009

chronic myeloid leukemia (K562), human hepatocarcinoma (HepG2 and 7721), human cervical cancer (HeLa), and human ovarian cancer (SKOV4) cells were obtained from Dalian Medical University (Dalian, China). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2, and the medium was changed every 2 days. After confluence, the cells were subcultured following trypsinization.

Se-Enriched Submerged Culture of *G. lucidum*. The Se-enriched submerged culture of *G. lucidum* was carried out according to Shang et al. (*11*). Briefly, mycelia were first incubated in an agar-potato-dextrose solid medium at 28 °C for 10 days. A small piece (1 cm^2) of mycelia from solid medium was then transferred to the liquid medium containing 400 μg/mL of sodium selenite in 500 mL flasks and incubated with a shaking rate of 180 rpm at 28 °C for 7 days. Regular mycelia were grown in medium without sodium selenite (*11*).

Isolation and Purification of SeGLP-2B-1. The mycelia of Se-enriched *G. lucidum* were collected by centrifugation at 4500*g* for 20 min and dried to give powder. The powdered mycelia (10 g) were extracted first with water (1:20, w/v) at 50 °C for 24 h and then with 1.0 M NaOH (1:20, w/v) at 50 °C for 24 h. The extracted solution was treated with Sevag agent (1:4 chloroform/*n*-butanol) to remove the proteins and concentrated to 10% of the original volume by a rotary evaporator under reduced pressure. Then, ethanol was added to the concentrated solution to 75% (v/v) and placed at 4 °C for 24 h. The sediment was centrifuged (5000*g* for 10 min) and vacuum freeze-dried, and crude Se-containing polysaccharides (SeGLP-1) was obtained. In addition, the supernatant was added in ethanol until 85% (v/v) of ethanol and then placed at 4 °C for 24 h. The sediment was centrifuged (5000*g* for 10 min) and vacuum freeze-dried, and crude SeGLP-2 was obtained. SeGLP-2 was fractionated separately over a DEAE-52 column (2.6 × 50 cm) eluting with distilled water and a linear gradient of 0−0.5 M NaCl, respectively, at a flow rate of 0.5 mL/min. The carbohydrate content of each fraction was determined by the phenol−sulfuric acid method (*12*). The SeGLP-2B fraction was collected and purified on a TSK-G5000PW HPLC column (1.6 × 50 cm), eluting with distilled water at a flow rate of 0.5 mL/min and column temperature of 25 °C. The collected SeGLP-2B-1 fraction was further purified on a SHIM-PACK HPLC column, eluting with acetonitrile/distilled water (60:40) at a flow rate of 1.0 mL/min. The isolation and purification of the regular polysaccharide (GLP-2B-1) from the non-Se-enriched mycelia of *G. lucidum* was the same with SeGLP-2B-1 in procedure. The polysaccharides were detected by an evaporative light-scattering detector, ELSD (SEDEX 75, Sedere Co., Cedex, France), vaporized at 45 °C and 3.5 MPa. The molecular weights of SeGLP-2B-1 and GLP-2B-1 were determined by HPSEC on a TSK-G5000PW column (1.6 × 50 cm) at 40 °C, eluting with distilled water. The standard dextran series of different molecular weights (T_{10}, T_{40}, T_{70}, and T_{110}, Pharmacosmos Company, Holbaek, Denmark) was chosen as the calibration standard to obtain a regression equation. The relative molecular masses of SeGLP-2B-1 and GLP-2B-1 can be derived from the regression equation.

Determination of Selenium and Polysaccharide Content. The polysaccharide content of SeGLP-2B-1 and GLP-2B-1 was determined by the phenol−sulfate method using glucose as the standard. In brief, 2 mL of sample solution was vortex-mixed with 1 mL of 5% phenol in water before rapidly adding 5 mL of concentrated sulfuric acid from a glass dispenser. After standing for 20 min at room temperature, the absorbance of the sample solution was measured at 490 nm against the blank (prepared by substituting distilled water for the sample solution). The concentrations of glucose for the standard were 160, 80, 40, 20, 10, and 5 μg/mL. The Se content in SeGLP-2B-1 and GLP-2B-1 was determined respectively by inductively coupled plasma−atomic emission spectroscopy (ICP−AES) (Plasma-Spec-II, Leeman Laboratories, Hudson, NH) as reported (*13*). Standard bovine liver powder was the normal control. The operation conditions and parameters were as follows: the pressure of carrier gas was 40 psi, flux was 0.5 L/min; the pressure of coolant gas was 5 psi, flux was 12 L/min; and the pressure of auxiliary gas was 5 psi, flux was 0.5 L/min.

Compositional Analysis of SeGLP-2B-1 and GLP-2B-1. Monosaccharide constituents of SeGLP-2B-1 and GLP-2B-1 were analyzed by gas chromatography (GC-16A, Shimadzu Co., Kyoto, Japan) with a fused silica capillary column (N_2 form, 300 × 0.53 mm) and a flame ionization detector. The acetylation of standard monosaccharide: glucose, xylose, rhamnose, mannose, and galactose and GC analysis of alditol acetates derivatives were carried out as described by Shang et al. (*14*). The repeatability for three measurement results was 0.94%.

Cell Viability Assay. Cell viability was assessed by the MTT assay based on the reduction of MTT into formazan dye by active mitochondria (*15*). Briefly, all human cancer cell lines were incubated in RPMI 1640 medium with 10% (v/v) heat-inactivated fetal calf serum in 96-well microtiter plates for 24 h, and cell viability was assessed by measuring trypan blue exclusion. After the cells had attached, 4 μL of various concentrations of SeGLP-2B-1 or GLP-2B-1 was added to each well to give final concentrations ranging from 0.045 to 0.36 μM and incubated at 37 °C in 5% CO_2 for 24, 48, and 72 h. Then, 20 μL of MTT solution (5 mg of MTT/mL in PBS) was added to each well and incubated for an additional 4 h. After rinse and centrifugation, 150 μL of dimethylsulfoxide (DMSO) was added to dissolve the MTT formazan crystals. The spectrophotometric absorbance at 592 nm was determined using a scanning multiwell spectrophotometer. The IC_{50} of Se-GLP-2B-1 and GLP-2B-1 was taken as the mean concentration of the sample causing death of 50% of cells incubated for 72 h as measured by absorbance at 592 nm compared to the control values. In each experiment, the MTT assay was performed in eight replicates and repeated 3 times.

Morphological Observation of K562 Cells. K562 cells were treated with 0.09 μM of SeGLP-2B-1 or GLP-2B-1 for 72 h. Cells (5×10^4/mL) were washed twice with PBS to remove dead cells and then fixed in an ethanol solution containing 2.5% glutaraldehyde and 1% osmic acid for 15 min. The cells were then rehydrated with several washes in a sodium phosphate buffer (10 mM, pH 7.9) for 1 h and stained by applying a solution containing uranyl acetate and lead citrate. The morphological changes of the cells were observed using a JEM-200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan). Photographs of cell were taken with a CONTAX 167MT camera (Kyocera Co., Tokyo, Japan) and Kodak 100 Elite Chrome film (Eastman-Kodak, Japan).

Cell Cycle Analysis. K562 cells (1×10^5/mL) were incubated with 0.09 μM SeGLP-2B-1 or GLP-2B-1 for 24, 48, and 72 h. The cells were collected, washed, suspended in cold 1× PBS, then fixed in 95% icy ethanol, and stained with propidium iodide (*16*). Cell cycle analysis was performed using the EPICSXL (Beckman Coulter, Fullerton, CA) flow cytometer, and the results were analyzed by the system-III software.

Statistical Analysis. The data were analyzed using one-way analysis of variance and Duncan's test. All experiments were carried out in triplicate. The statistical significance was established at $p \leq 0.05$.

RESULTS

Isolation, Purification, and Physicochemical Properties of SeGLP-2B-1. SeGLP-2 was separated into two fractions, SeGLP-2A and SeGLP-2B, on DEAE-52 (**Figure 1A**). The SeGLP-2B fraction was sequentially purified through a TSK-G5000PW HPLC column, giving two elution peaks, SeGLP-2B-1 and SeGLP-2B-2 (**Figure 1B**). The yields of SeGLP-2B-1 and SeGLP-2B-2 were about 22.3 and 76.2%, respectively. Peak SeGLP-2B-1 was ultimately loaded on a Hypersil SHIM-PACK C18 reverse-phase HPLC column, and SeGLP-2B-1 was purified into a single-elution peak (**Figure 1C**). The polysaccharide content of SeGLP-2B-1 was 98.2%, with a molecular mass of 1.06×10^6 Da.

The polysaccharide isolated and purified from *G. lucidum* cultured without Se (GLP-2B-1) had a polysaccharide content of 99.5% and a molecular mass of 0.93×10^6 Da. The two polysaccharides had very different monosaccharide molar percentages, although they had the same monosaccharide composition. Monosaccharide composition analysis showed that SeGLP-2B-1 was composed of 31.7% glucose, 7.2% galactose, 14.0% xylose, and 47.1% rhamnose but GLP-2B-1 was composed of 35.2% glucose, 9.3% galactose, 32.6% xylose, and 22.9% rhamnose. The molar percentage of rhamnose in SeGLP-2B-1 was larger than that in GLP-2B-1. In contrast, the molar percentage of xylose in SeGLP-2B-1 was lower than that in GLP-2B-1. No

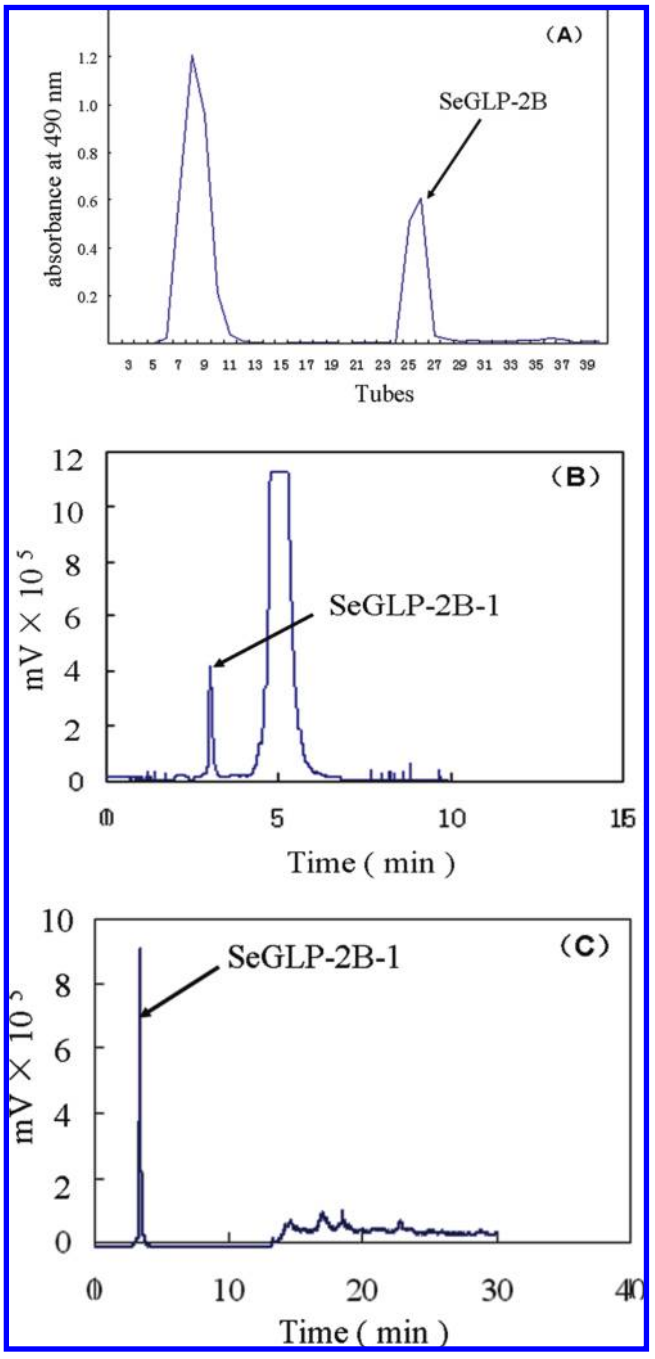

Figure 1. Fractionation of selenium-enriched polysaccharides. (A) Gel filtration of selenium-containing polysaccharides from selenium-enriched *G. lucidum* mycelia from the DEAE−cellulose column. (B) SeGLP-2B fraction from the DEAE−cellulose column was purified on a TSK-G5000PW HPLC column. (C) SeGLP-2B-1 fraction was purified on a SHIM-PACK C18 RP-HPLC column. SeGLP-2B and SeGLP-2B-1 are indicated by arrows.

absorption at 280 nm indicated that SeGLP-2B-1 and GLP-2B-1 did not contain any peptide. The selenium content in SeGLP-2B-1 and GLP-2B-1 was 186.7 and 1.3 μg/g, respectively.

Antiproliferation Activity of SeGLP-2B-1 in Cancer Cell Lines. The antiproliferation activity induced by SeGLP-2B-1 was investigated in six human cancer cell lines using the MTT assay. All tested cells were treated with SeGLP-2B-1 or GLP-2B-1 at various concentrations for 72 h. As shown in **Figure 2**, the number of metabolically active cells decreased when treated with the samples at various concentrations for 72 h as compared to the

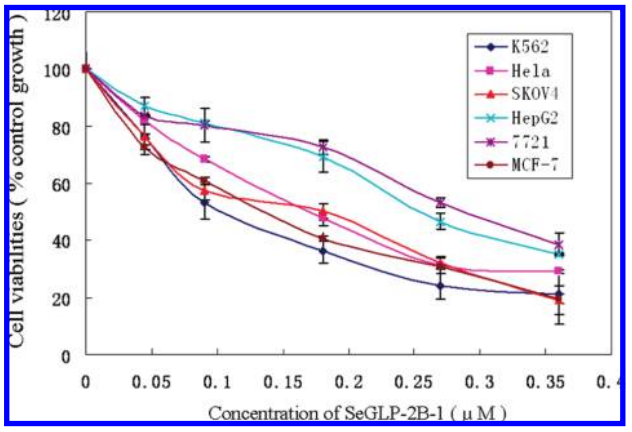

Figure 2. Effects of SeGLP-2B-1 on tumor cell growth. Human malignant breast carcinoma (MCF-7), human erythroid chronic myeloid leukemia (K562), human hepatocarcinoma (HepG2 and 7721), human cervical cancer (HeLa), and human ovarian cancer (SKOV4) cells were used. Cells of each line were treated with SeGLP-2B-1 (0.045, 0.09, 0.18, 0.27, and 0.36 μmol) for 72 h. Results represent the mean ± SD of three different experiments performed in triplicate.

untreated cells. The fact that cell survival decreased as the concentration of SeGLP-2B-1 increased showed that inhibition by SeGLP-2B-1 was concentration-dependent. SeGLP-2B-1 had the highest antiproliferation activity against human leukemia K562 cells, whereas the inhibitory activity against the two human hepatocarcinoma cell lines (HepG2 and 7721) was low. The IC_{50} of SeGLP-2B-1 was 0.1−0.3 μM (**Table 1**). The antiproliferative activity of SeGLP-2B-1 was significantly 10−25-fold higher than that of GLP-2B-1.

To further validate these results, the morphology of human leukemia K562 cells treated with 0.09 μM SeGLP-2B-1 or GLP-2B-1 for 72 h was evaluated using microscopy and electron microscopy. Our experiments indicated that the cancer cells treated with SeGLP-2B-1 or GLP-2B-1 exhibited a lower rate of survival than the untreated cells (**Figure 3**). These results were consistent with the reduced rate of growth and proliferation and increased cell death when treated with SeGLP-2B-1.

G1/S Arrest Induced by SeGLP-2B-1. Cell cycle analysis was performed for K562 cells treated with 0.09 μM SeGLP-2B-1 or GLP-2B-1 for 24, 48, and 72 h. After treatment with SeGLP-2B-1 for 48 h, the DNA content of cells in the S phase was the same as that in the G1 phase, which indicated that DNA synthesis was blocked and instead presented as sub-G1/G0 DNA content (**Figure 4**). Cells in the sub-G1/G0 phase were considered apoptotic, and the degree of apoptosis was positively correlated with treatment time. Results revealed a G1/S arrest. No increase of cells treated with SeGLP-2B-1 were in the S phase from 48 h, which indicated that the cell cycle was delayed at the key point between G1 and S phases (**Figure 5**). Cells treated with GLP-2B-1 did not occur during the sub-G1/G0 phase (apoptotic peak).

DISCUSSION

The fundamental importance of Se to human health has received considerable attention. In comparison to inorganic selenium compounds, organic selenium compounds have high bioavailability, high retention, and low toxicity to the body and are considered to be better sources of Se. These compounds are found in a variety of Se-enriched functional foods, such as garlic, yeast, green tea, algae, and edible mushrooms. Previous studies confirmed that Se-containing proteins were mainly responsible for the storage of organic Se in Se-enriched edible

Table 1. Inhibition Concentrations (IC_{50}) (μM) of SeGLP-2B-1 against Cancer Cells[a]

	IC_{50} (μM)					
	K562	MCF-7	HeLa	SKVO4	HepG2	7721
SeGLP-2B-1	0.11 ± 0.81^b	0.14 ± 1.02^b	0.17 ± 0.77^b	0.18 ± 0.63^b	0.26 ± 1.78^b	0.29 ± 1.23^b
GLP-2B-1	2.10 ± 1.53	1.26 ± 0.61	1.58 ± 1.88	4.20 ± 1.63	4.20 ± 0.77	6.30 ± 2.22

[a] Values are mean $\pm$ standard deviation (SD) ($n = 3$). [b] $p < 0.01$ compared to the GLP-2B-1 group.

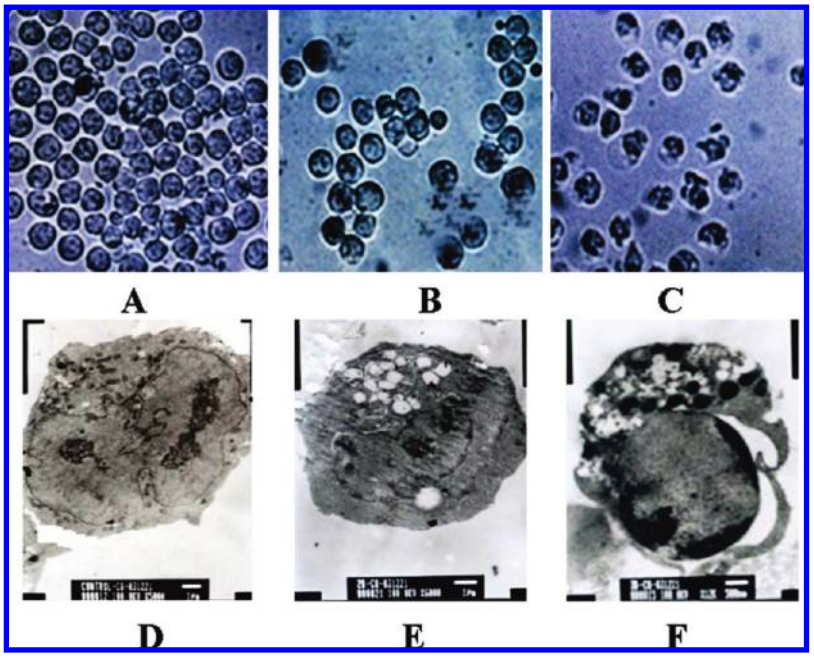

Figure 3. Effect of SeGLP-2B-1 or GLP-2B-1 on the cell morphology and growth of K562 cells. (A and D) control, untreated K562 cells. (B and E) K562 cells treated with 0.09 μM of GLP-2B-1 for 72 h. (C and F) K562 cells treated with 0.09 μM of SeGLP-2B-1 for 72 h. Cells treated with SeGLP-2B-1 exhibited typical characteristics of apoptosis with depauperation of the nucleus and cytoplasmic hypervacuolization, condensation and degradation of chromatin, and nuclear margination.

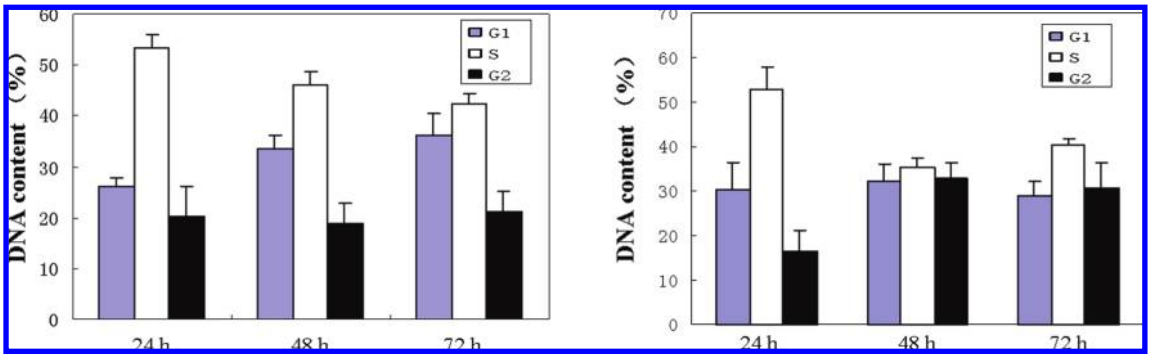

Figure 4. DNA distribution (%) of the cell cycle of K562 cells treated with (A) GLP-2B-1 or (B) SeGLP-2B-1.

mushrooms (*17*, *18*). Furthermore, Se-containing protein extracted from Se-enriched *G. lucidum* and *Spirulina platensis* exhibited much higher antioxidant activities than their non-Se-containing counterparts (*19*, *20*). On the other hand, Se from the growth medium is incorporated into polysaccharides of Se-enriched edible mushrooms (*21*). In the present study, we describe the purification of SeGLP-2B-1, a Se-containing polysaccharide from Se-enriched *G. lucidum* mycelia. The isolation and purification of SeGLP-2B-1 were carried out using three main procedures: fractional precipitation with 75 and 85% ethanol, ion-exchange chromatography on DEAE-cellulose, and TSK column fractionation by HPLC. GLP-2B-1 is a polysaccharide with similar properties purified from *G. lucidum* mycelia cultured without Se. SeGLP-2B-1 was purified to a homogeneous polysaccharide with a molecular mass of 1.06×10^6 Da. The selenium content in SeGLP-2B-1 was 150 times higher than that of GLP-2B-1.

Previous studies showed that the polysaccharides from fruiting bodies, submerged cultured mycelium, or spores of *G. lucidum* were dominantly composed of glucose residues in the main chain, sometimes accompanied with galactose, mannose, rhamnose, arabinose, and fucose residues in the side chain, and glucose was often more than 80% (*22*−*24*). In this study, the monosaccharide composition of GLP-2B-1 from submerged cultured mycelium was composed of 35.2% glucose, 9.3% galactose, 32.6% xylose, and 22.9% rhamnose. In addition, SeGLP-2B-1

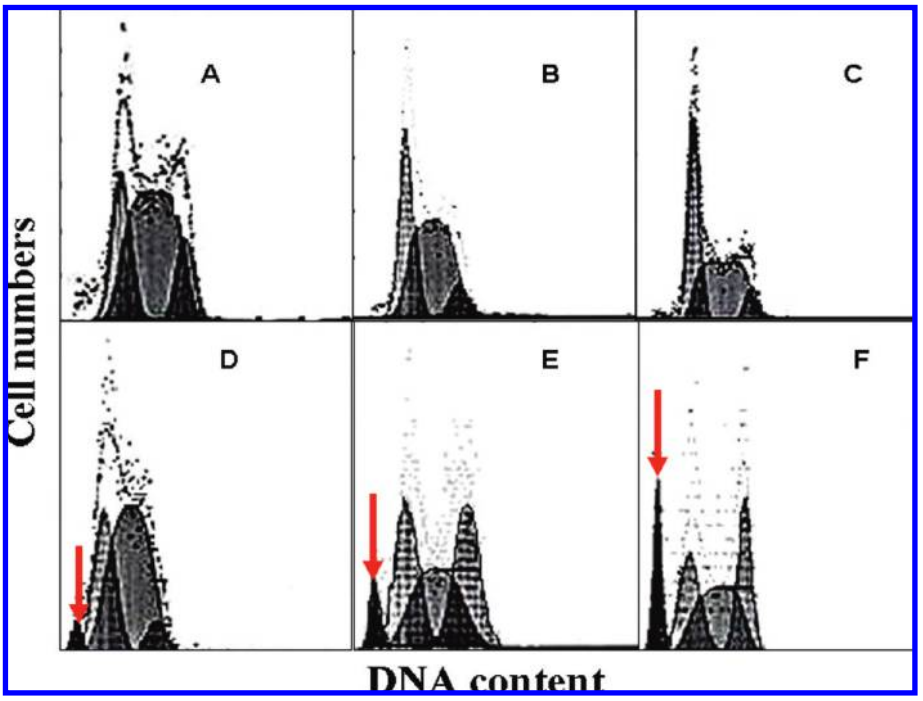

Figure 5. Flow cytometric analysis of K562 cell apoptosis. K562 cells were treated with 0.09 μM GLP-2B-1 for (A) 24 h, (B) 48 h, and (C) 72 h or SeGLP-2B-1 for (D) 24 h, (E) 48 h, and (F) 72 h. The arrow indicates the sub-G0/G1 phase (apoptosis peak).

was the same as GLP-2B-1, but the monosaccharide ratios were markedly distinct. However, the rhamnose ratio significantly increased from 22.9 to 47.1%, and the xylose ratio significantly decreased from 32.6 to 14.0% in Se-GLP-2B-1. This result was similar to the selenium-containing polysaccharide SeGLP-1 from Se-enriched mycelium of *G. lucidum* (*25*). It is unclear how selenium induces changes in the rhamnose and xylose molar percentage in Se-containing polysaccharides. Se-containing polysaccharides with rhamnose and xylose have not been reported thus far. The Se-containing polysaccharide (Se-GPS) from Hubei Enshi garlic (*Allium sativum*) is a mannan, and the selenoastragalan II is the D-dextran containing galactose and arabinose.

The polysaccharides isolated from *G. lucidum* have been demonstrated to be the major bioactive components of *G. lucidum* and possess antioxidative, antihypertensive, and anticancer effects (*26*, *27*). The *G. lucidum* polysaccharides could inhibit the proliferation of human cancer cells by the downregulation of expression of cyclin B and Cdc2 and the upregulation of p21 expression and, furthermore, induce apoptosis of cancer cells (*26*). However, Zhang et al. indicated that *G. lucidum* polysaccharides did not directly induce cancer cell apoptosis. The apoptosis induced by *G. lucidum* polysaccharides appeared to be due to the promotion of the expression of TNFα and IFNγ (*28*). In this present study, the polysaccharides from *G. lucidum* GLP-2B-1 exhibited antiproliferative effects on the six human cancer cell lines. The effective dose, which inhibited 50% cell growth (IC_{50}) was ranged between 1.26 and 6.30 μM. However, the apoptotic features induced by GLP-2B-1 had not been found in K562 cells. SeGLP-2B-1 is a Se-containing polysaccharide isolated from the Se-enriched *G. lucidum*, and the Se content of SeGLP-2B-1 was significantly higher than that of GLP-2B-1. The incorporation of Se in polysaccharides of Se-enriched mycelia of *G. lucidum* provided significantly higher antiproliferative activities. SeGLP-2B-1 inhibited proliferation of six human cancer cell lines at low concentration, and the inhibitory effect of SeGLP-2B-1 was concentration-dependent. SeGLP-2B-1 had the highest antiproliferation activity against human leukemia K562, whereas low inhibitory activity was found against the two human hepatocarcinoma cell lines, HepG2 and 7721. SeGLP-2B-1 exhibited approximately 10−25 times stronger inhibitive activity as compared to GLP-2B-1. Thus, Se is believed to play an important role in increasing the antiproliferative property of SeGLP-2B-1. Our previous studies showed that Se bonded to polysaccharide in a covalent bond in SeGLP-2B-1 and the selenious group substituted a methyl group (CH_3) in the methoxy ($-OCH_3$) group of rhamnose (*29*). A further study about the chemical form of Se that exists in polysaccharides and the way it binds with them is currently in progress in our laboratory.

Although we still have not identified the mechanism of SeGLP-2B-1 on the antiproliferative effects against the six cancer cell lines, the morphologic analysis of K562 cells treated with SeGLP-2B-1 for 72 h showed that the typical characteristics of apoptosis displayed in cancer cells as cell shrinkage, membrane blebbing, condensation and margination of nuclear chromatin, and cytoplasm vacuolization. In addition, the emergence of a distinct, subdiploid apoptotic peak, reflecting apoptotic nuclear fragmentation, further revealed the apoptosis induced by SeGLP-2B-1. It has been postulated that SeGLP-2B-1 may inhibit the proliferation of cancer cells by inducing apoptosis.

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Received June 8, 2009. Revised manuscript received July 29, 2009. Accepted July 31, 2009. The work was supported by the National Natural Science Foundation of China (Grant 30770276).