Chemical Characteristics of Selenium Polysaccharide from *Pleurotus ostreatus* and Antioxidant Activities *in vitro*

Abstract

A new selenium-enriched *Pleurotus ostreatus* polysaccharide (Se-POP-11) was extracted and purified, it was a homogenous polysaccharide with an average molecular weight of 9.43 kDa, mainly composed of glucose, mannose, galactose, arabinose and xylose with molar ratio of 5.30:1.55:2.14:0.29:0.63, respectively, and the selenium content was 3.21 μg/g. Se-POP-11 presented an obvious effect to scavenge superoxide anion radical, hydroxyl radical, ABTS and DPPH radicals with an increasing concentration, the scavenging activities were close to V_C when the concentration up to 6mg/mL. Reduction in apoptosis, less sub-G1 DNA content revealed its ability to alleviate oxidative damage for PC12 cells induced by H_2O_2. These results suggest that Se-POP-11 possesses potent antioxidant and with the ability to prevent oxidative damage, it could be developed as organic selenium dietary supplement for functional food.

Keywords: Selenium-enriched polysaccharide; *Pleurotus ostreatus*; Chemical structure; Antioxidant activity; PC12 cells

Research Article

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Abbreviations: GC: Gas Chromatography; HPGPC: High Performance Gel Permeation Chromatography; IR: Infrared Radiation; FT-IR: Fourier Transform Infrared; NMR: Nuclear Magnetic Resonance; ABTS: 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate); DPPH: 2,2-diphenyl-1-picrylhydrazyl

Introduction

Pleurotus ostreatus as one of the most commercialized and popular fungi species was cultivated and consumed as traditional food for long time in many Asian countries like China, Korea and Japan [1], it contains many biological active materials, such as protein, fiber, carbohydrates, fat, trace elements and ash. Polysaccharide, one of the main bioactive components of *P. ostreatus* has received more and more academic attentions owing to its antioxidant, immunomodulatory, anti-tumor and antimicrobial properties [2-4].

Oxygen-derived free radicals in living cells are considered to contribute to various diseases, such as carcinogenesis, atherosclerosis, cancer, rheumatoid arthritis as well as in degenerative processes of aging [5,6]. Antioxidants including polyphenols and polysaccharides can potently scavenge free radicals and properly protect organism in the prevention of oxidative damages in living organisms, polysaccharide in the mushroom has been demonstrated as one of the effective antioxidants [7,8].

Selenium (Se) is an essential trace element, which is actively involved in animal physiology via a variety of selenium-dependent enzymes and selenoproteins with antioxidant Function [9]. It is generally accepted that organic selenium compounds have lower toxicity, higher bioavailability and biological functionality compared with inorganic selenium. Selenium-enriched food such as Se-enriched yeast, Se-enriched tea and Se-enriched gralic are generally considered as a major source of Se dietary supplement [10]. Microorganism fermentation with selenium technique could provides a feasible and economic approach for production of organic selenium compounds [11], mushrooms have been proven to absorb certain trace elements effectively from the cultivated medium [12], and thus *Pleurotus ostreatus* is one of the best choice for selenium accumulators.

It was reported that Selenium exopolysaccharide from *Lactococcus lactis* and Se-enriched Maitake polysaccharide displayed higher antioxidant activities than the native polysaccharide [13,14], but there is a lack of available information regarding the structural elucidation and antioxidant activity of Se-polysaccharide isolated from selenium-enriched *Pleurotus ostreatus*, therefore, it is quite necessary and significative to explore the selenium *P. ostreatus* polysaccharide.

The aim of present work was conducted to investigate the main chemical characterization and antioxidant activities of polysaccharide from selenium-enriched *Pleurotus ostreatus*. Furthermore, this study was also conducted to describe whether Se-POP-11 had protective effects on H_2O_2-induced cytotoxicity in PC12 cells.

Experimental

Materials and chemicals

The samples of *Pleurotus ostreatus* were provided by the zituan Ecological Agriculture Co., Ltd (Changzhi City, Shanxi Province, China) in September 2014. The material was authenticated by Prof An-jun Liu of Tianjin University of Science and Technology (The School of Food Engineering and Biotechnology). A voucher specimen (no. TUST2014101105) was kept in the herbarium of the university.

PC12 cell was obtained from the Key Laboratory of Food Nutrition and Safety (Ministry of Education, China), College of Food Science and Biotechnology (Tianjin University of Science and Technology, Tianjin, China), the assay kits were bought from Nanjing Jiancheng Bioengineering Institute (Nangjing, China). All other chemicals were of the highest purity and commercially grade available.

Extraction and purification of polysaccharides

Defatted and dried samples of *P. ostreatus* was mixed distilled water with stirring for 3h at 90˚C, then the supernatant was collected and concentrated under reduced pressure, 4 volumes of ethanol were added and kept at 4˚C overnight. Polysaccharide precipitate was obtained by centrifugation at 4000 rpm for 10 min, and deproteinated with Sevag reagent, dialyzed against water, then the crude Se-POP was applied to DEAE-52 Sepharose Fast Flow column (2.6×40 cm) and Sephadex G-100 (30×2.6 cm) column, one main fraction (Se-POP-11) was collected and lyophilized for further study.

Molecular weight distribution

The average molecular weight (Mw) of Se-POP-11 was determined by a HPGPC (Agilent-1200, USA) equipped with a TSK-gel G4000 PW×L column (7.8×300 mm, column temperature 30˚C) and Refractive Index Detector (RID, detecting temperature 40˚C) [15].

FT-IR and NMR analysis

1 mg polysaccharide with 150 mg dried KBr powder was ground and pressed into a pellet, the analysis at the absorbance mode was conducted on a Fourier transformed IR spectrophotometer with scanning range of 4000-400 cm^{-1} (VECTOR-22) [16].

The sample was dissolved in D_2O, exchanged three times, lyophilized and redissolved in D_2O, then 1H NMR and 13C NMR spectra were recorded with a Bruker Spectrometer (600 MHz) at a probe temperature of 298 K [17].

Monosaccharide composition analysis

Sample was dissolved with trifluoroacetic acid (TFA) and hydrolyzed in a oil bath at 110˚C for 5 h, then was acetylated at 90˚C for 1 h, the acetylated sample was dissolved in dichloromethane for further GC (GC2010, Shimadzu, Japan) analysis [18].

Antioxidant activities assay

DPPH radical scavenging capabilities of Se-POP-11 were assayed according to method described previously by Ye et al. [19]. Hydroxyl radical scavenging activity was measured according to the Fenton reaction described by Li et al. [20]. The ferrous ion reducing power was determined using the reported method proposed by Li et al. [21]. The ability of Se-POP-11 to scavenge the ABTS radical was performed with the method described by Sun et al. [22]. The superoxide radical scavenging assay was determined by the method reported by Chen et al. [23].

Protective effect of Se-POP-11 on PC12 cells injured by H_2O_2

PC12 cells were seeded in 6-well plates or in cell culture bottles at a density of 1×10^5/mL. Prior to exposure to freshly prepared H_2O_2 with concentration of 400 μmol/L for 2 h, cells were pretreated with 400 μg/mL of Se-POP-11 and 200 μg/mL of V_C for 24 h, respectively. The cell apoptosis and cell cycle were analyzed using a flow cytometer [24,25], cells changes in nuclear morphology were observed on a fluorescence microscope [26].

Statistical analysis

Results were reported as the mean±SD (standard deviation). SPSS version 16.0 software was used for all statistical calculations (SPSS Inc., Chicago, USA). An ANOVA was used to determine the differences between the sample results. All values with $p<0.05$ were considered significantly different.

Results

Chemical contents and monosaccharide compositions

The polysaccharide with yield of 5.37% was obtained from the fruit body of *Pleurotus ostreatus* by hot water extraction, with 80% ethanol precipitation and sevag reagent deproteination, and then the sample was purified by DEAE-52 Sepharose Fast flow column and Sephadex G-100 column chromatography. Relative high content (53.68 %) of one fraction (named Se-POP-11) with yield of 1.27 % was gathered. Contents of total carbohydrate, protein, uronic acid, sulfate and selenium of Se-POP-11 were 85.26%, 7.41%, 1.89%, 4.32%, 3.21 μg/g, respectively. The selenium content was higher than the native *Pleurotus ostreatus* polysaccharide, similar to the content of 2.14 μg/g and 4.50 μg/g of (Se)-polysaccharide from Ziyang green tea and Se-enriched Maitake polysaccharide (Se-GP33), respectively [27,14]. But lower than the Se-polysaccharide from *Catathelasma ventricosum* and *Coprinus comatuson* with organic selenium content of 41.77 μg/g and 15.21 μg/g, respectively [28,29].

Monosaccharide composition was detected by GC and the results recorded in Figure 1, six standard monosaccharides of L-rhamnose, D-arabinose, D-xylose, D-mannose, D-glucose and D-galactose were used as standard monosaccharides and their peaks were indentified separately within 20 min as shown in Figure 1(1). The sample was identified by matching their retention time with those of monosaccharide standards under the same analytical conditions, as shown in Figure 1(2), the monosaccharide composition showed that Se-POP-11 mainly consisted of glucose, mannose, galactose, arabinose and xylose with molar ratio of 5.30:1.55:2.14:0.29:0.63, respectively.

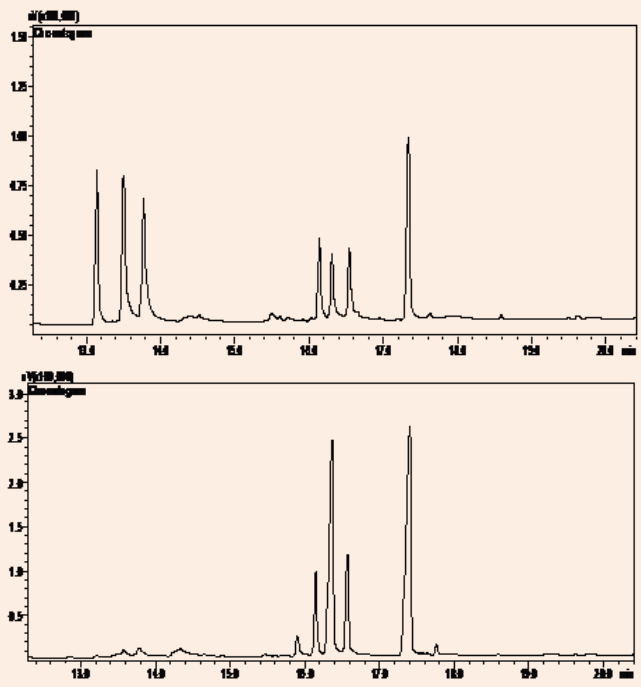

Figure 1: GC chromatography of standard monosaccharide (1) and Se-POP-11 (2), Peaks: rhamnose (1) arabinose (2) xylose (3) mannose (4) glucose (5) galactose (6) internal standard (7).

IR and NMR analysis

FT-IR spectroscopy is usually used for identification of characteristic organic groups in the polysaccharides, the IR absorbance of Se-POP-11 was shown in Figure 2, the band of absorbance at 3339 cm^{-1} resulted from the stretching of the hydroxyl groups whereas an intense ring and (COH) side group band at 1048 cm^{-1} dominated the spectrum of xylan with β (1→4) backbone, the peak at 2933 cm^{-1} was represented the C-H vibration, the characteristic absorbance at around 1606 cm^{-1} was attributed to N-H stretching, which suggest the protein existed in the polysaccharides. Two stretching peaks, at 1408 and 1161 cm^{-1} in the IR spectra suggested the presence of C–O bonds, the signals at 1408.79 cm^{-1} indicated the absence of symmetric stretching of carboxylate anion group (C=O), the absorption at 1078.36 cm^{-1} was designated to a pyranose form of sugars. These common peaks suggested that introduction of selenium does not affect the main structure of the polysaccharide which in accordance with other results [29]. Ding et al. [30] found a striking peak observed at 794 cm^{-1} for the (Se=O) stretching vibration in the selenium-containing exopolysaccharide synthesized from Rhizobium sp. N613 polysaccharide and selenious acid [30], the 802 cm^{-1} in our observation may also suggested the group (Se=O) existed.

NMR analysis involving 1H NMR and 13C NMR experiments, Figure 3 which was employed to assign the chemical shifts of the sugar residues presented in the repeating units. For chemical shifts of all residues H-1 protons at δ 5.203 were higher than 4.9 ppm, which confirmed that the anomeric configurations was linked as (1→4)-α-D Glcp. The chemical shifts appeared at 3.2-4.1 ppm were assigned to protons signals of H-2 to H-6 [31]. Also the strong signals at 3.6–3.9 ppm were consistent with the presence of β-galactopyranose (β-Galp) linkages. In addition, the 13C NMR spectrum in the range of 60-80 ppm showed the carbohydrate carbon signals of C-2 to C-6. Besides, there was no carbon signal at 80-90 ppm to illustrate the Se-POP-11 was not furanose. According to the available data in the literature [32], the resonances in the region of 98-109 ppm in13 C NMR were attributed to the anomeric carbon atoms of polysaccharide.

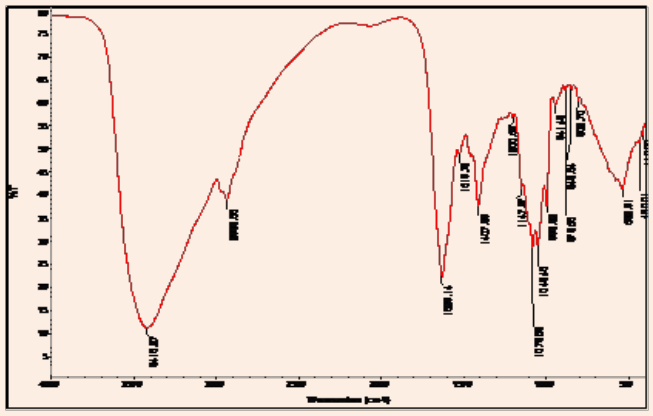

Figure 2: FT-IR spectra of Se-POP-11.

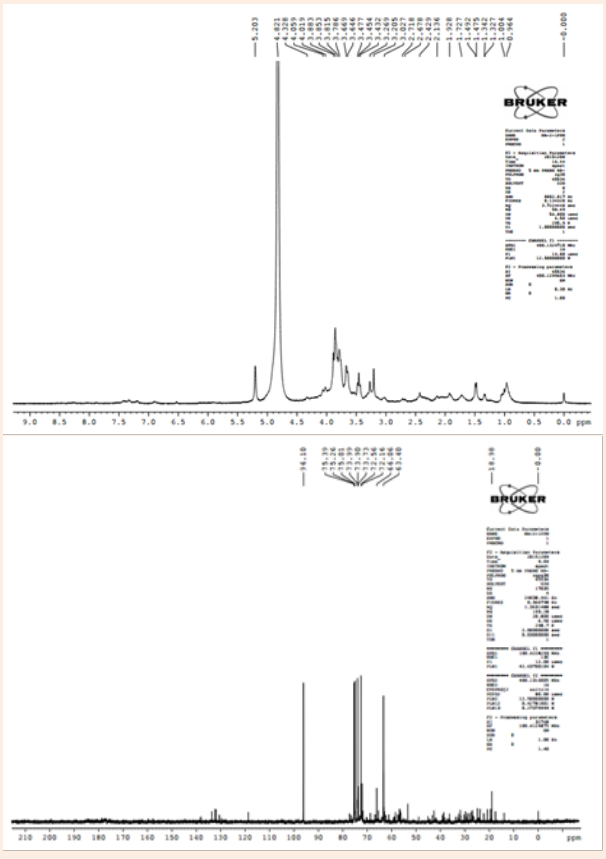

Figure 3: 1H NMR spectra (1) and 13C NMR spectra (2) of Se-POP-11 in D_2O/

Molecular weight properties

The calibration curve was obtained by using various Dextran T-series standards of known molecular weights, log Mw = 8.6414-0.3806t, R^2 = 0.9945 (Mw is molecular weight and t is retention time). As shown in Figure 4, the HPGPC profiles suggested that the Se-POP-11 had a single, narrow and symmetrical peak, which indicated that the Se-POP-11 was homogeneous polysaccharides, the average molecular weight was estimated about 9.43 kDa by universal calibration curve and the retention time of Se-POP-11, Ye et al. [33] reported that MW of Se-EPS by *P. seudomonas* PT-8 was 7.3 kD [33], both of the results suggested that the molecular weight of selenium-polysaccharide became lower after selenizing by biological method.

Antioxidant activities assay

DPPH free radical can be reduced by accepting an electron or hydrogen in the presence of an antioxidant that has been used widely to estimate the free radical scavenging of various antioxidant samples. The scavenging effect of various concentration (1-6 mg/mL) of samples on DPPH radical was determined and the results were shown in Figure 5, in the test dosage range, the sample exhibited increasing scavenging activity on DPPH radical with increasing concentration, the scavenging activity was 93.58% after the concentration of the samples got to 6 mg/mL, which was nearly to the V_C. This demonstrated that Se affected the antioxidant activity and selenium-polysaccharides showed greater antioxidant activity.

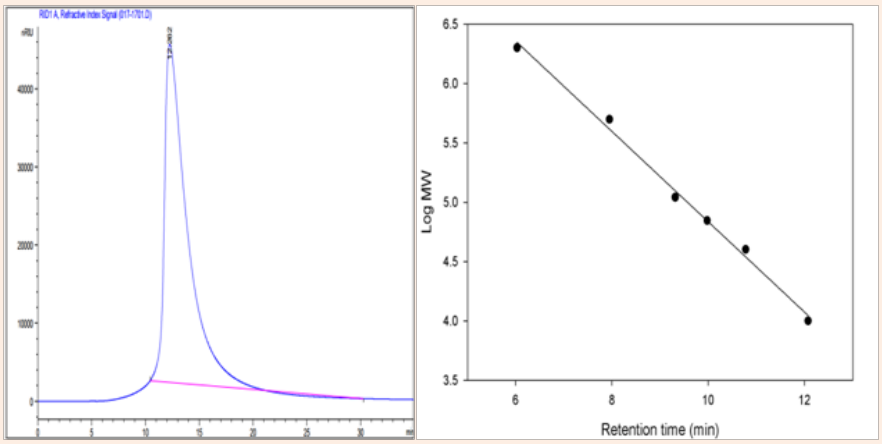

Figure 4: HPGPC of Se-POP-11 (1) and standard curve (2).

As shown in Figure 5, the hydroxyl radical scavenging activity of Se-POP-11 was presented dosage dependence relations at the test dosage range, the change of scavenging ability for hydroxyl radical was almost similar to the DPPH scavenging effect, the highest scavenging rate was 94.62% and 99.56% for Se-POP-11 and V_C, respectively.

The results of scavenging capacity of Se-POP-11 on ABTS radical were presented in Figure 5. The scavenging activity toward ABTS radical of Se-POP-11 in a concentration-dependent fashion, their highest scavenging effect at the concentration of 6 mg/mL was 96.45% and 99.56%, respectively.

The scavenging ability of sample on superoxide radical was presented in Figure 5, the inhibitory ability of Se-POP-11 and V_C on superoxide radical was directly related to their concentrations. At the concentration of 6 mg/mL, the scavenging activity was 97.49% and 99.84% for Se-POP-11 and V_C, respectively. It was reported that polysaccharide enriched with selenium displayed stronger scavenging activity of superoxide radical [34], mechanism may be that the three-dimensional structure of polysaccharide has been changed by the selenyl group (SeH) or seleno-acid ester, and increasing hydroxyl groups would emerge, which affected the hydrogen atom-donating capacity and antioxidant ability [34,35]. Otherwise, selenium itself has stronger antioxidant activity.

The reducing power of a compound served as a significant indicator of a potential antioxidant activity, Figure 5 showed that the reductive potential of Se-POP-11 and V_C was generally enhanced with their increasing concentrations in the test dosage, there was a correlation between the OD 700nm values and concentration of Se-POP-11 (R^2=0.871), when the concentration reached to 6 mg/mL, the value was 1.772 and 2.59 for Se-POP-11 and V_C, respectively.

Protective effect against cell oxidation induced by H_2O_2

Apoptosis of PC12 cells was assayed through flow cytometry with Annexin V-FITC/PI double staining, the results were shown in Figure 6, cells in the lower left quadrant were recognized as the viable cells, the early and late apoptotic cells appeared in the lower right quadrant and the upper right quadrant, respectively [36]. The AV+/PI + cell population in upper left quadrant has been described as advanced apoptotic or necrotic. In early and late apoptosis, the percentage of apoptotic cells were only 1.89% and 2.16% for the control group, while these were significantly increased to 8.31% and 15.38% after H_2O_2 treated alone (P<0.05), however, viable ratios was increased and hence apoptosis was significantly reduced to 5.72% and 7.89% after preincubation with Se-POP-11 at concentration of 400μg/mL. The results demonstrated that cell apoptosis induced by H_2O_2

could be significantly alleviated after pretreatment with Se-POP-11 (P<0.05), also it was suggested that the Se-POP-11 could effectively mediate oxidative damage from H_2O_2.

Severe oxidative stress usually contributed to DNA damage which can be monitored by Flow cytometry with PI-staining, the sub-G1 peak was traditional considered as one of the important apoptosis characteristics [25]. As shown in Figure 7, while the cells were exposed to H_2O_2, a distinct increasing DNA content in the sub-G1 peak (18.82%) was discovered compared with control (0.21%), but a decreased percentage (12.36%) in G0/G1 was obtained when the cells were incubated with Se-POP-11 (400 μg/mL) for 24h before treating with H_2O_2 for 2h, therefore, it was indicated the Se-POP-11 could decrease the ratio of cells in the G0/G1 phase, promote DNA synthesis and cell proliferation in H_2O_2-induced PC12 cells.

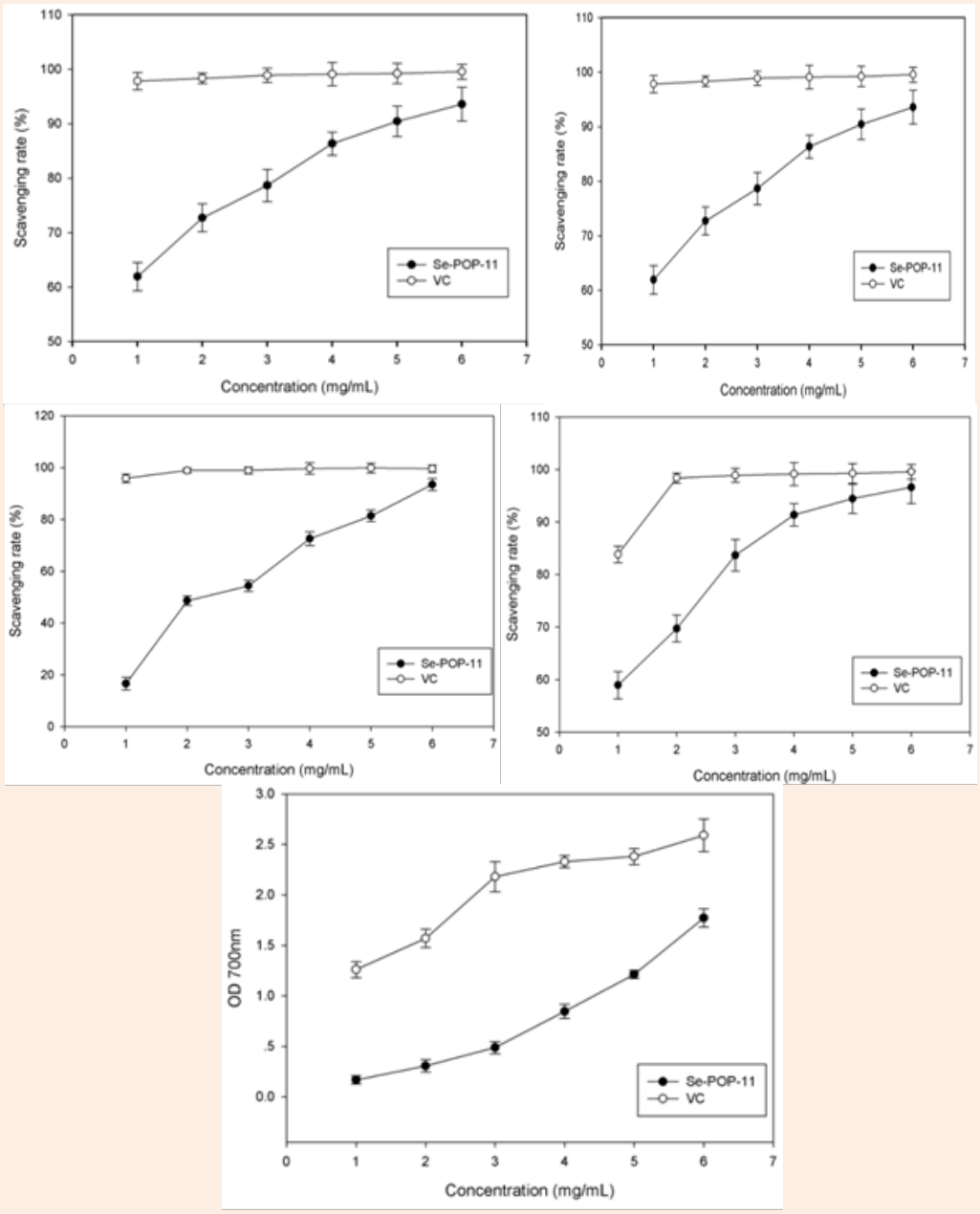

Figure 5: Antioxidant activities of Se-POP-11 at different concentrations on DPPH radical (1) hydroxyl radical (2) ABTS radical (3) superoxide anion radical (4) and reducing power (5).

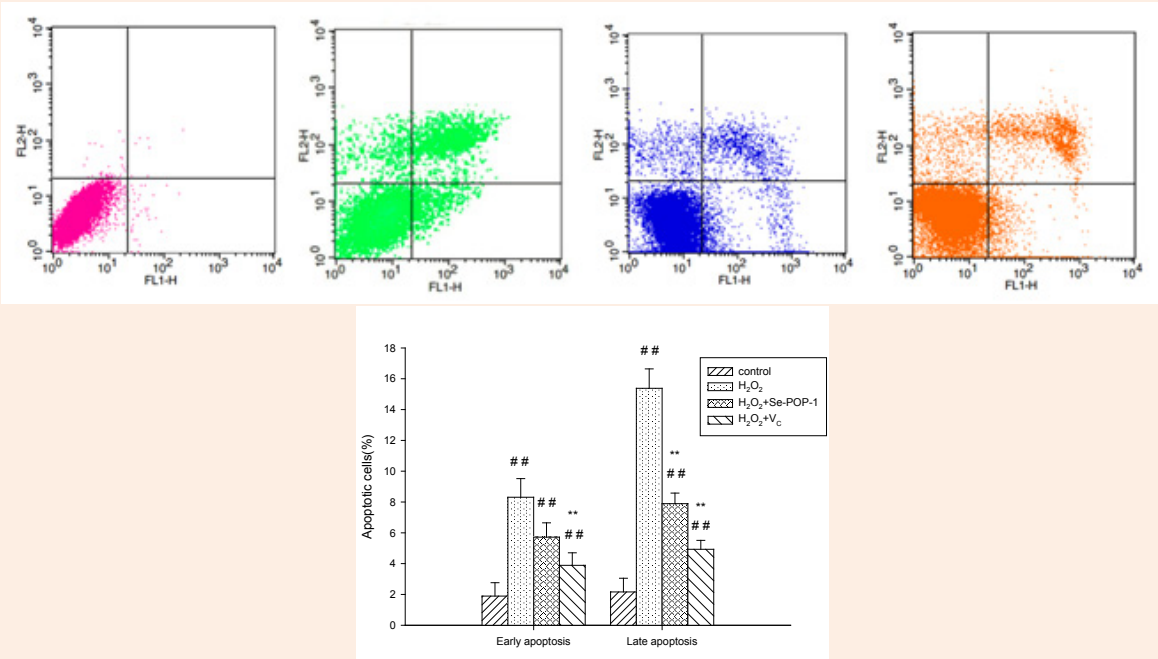

Figure 6: Effects of Se-POP-11 on cell apoptosis of H_2O_2-treated PC12 cells. Control (1) H_2O_2 treated (2) Se-POP-11+ H_2O_2 treated (3) V_C+ H_2O_2 treated (4).
##p < 0.05 versus the control group; **p<0.05 versus the model group

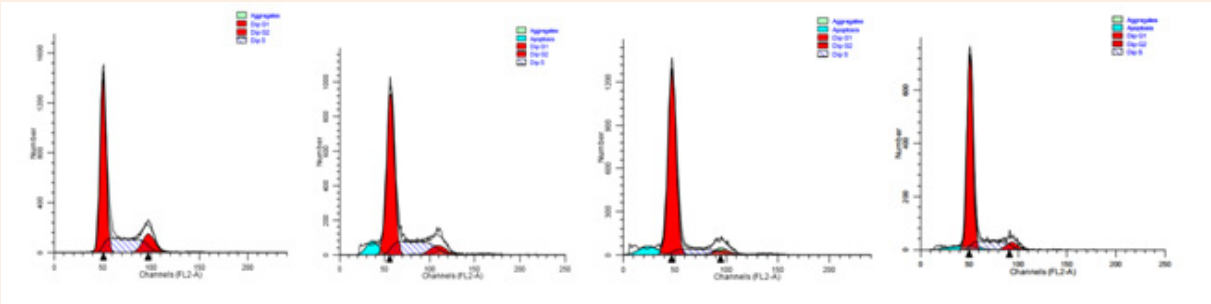

Figure 7: Effects of Se-POP-11 on cell cycle of H_2O_2-treated PC12 cells. Control (1) H_2O_2 treated (2) Se-POP-11+ H_2O_2 treated (3) V_C+ H_2O_2 treated (4).

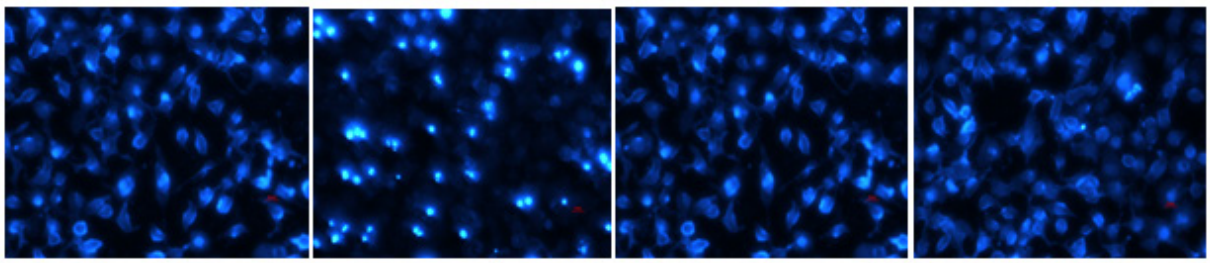

Figure 8: Effects of Se-POP-11 on cell nuclear morphological changes of H_2O_2-treated PC12 cells. Control (1) H_2O_2 treated (2) Se-POP-11+ H_2O_2 treated (3) V_C+ H_2O_2 treated (4).

In order to further understand the protective effects of Se-POP-11 on DNA and nuclear structure in PC12 cells, nuclear morphology changes were examined with DAPI staining. As presented in Figure 8, cells treated with H_2O_2 alone showed a lot of small bright blue dots and chromatin condensation or nuclear fragmentation, which was an important typical characteristic of

apoptosis. Meanwhile, H_2O_2-induced cells pretreated with Se-POP-11 (400 μg/mL) and V_C (200 μg/mL) significantly decreased the apoptotic cells, most of cells nuclei were close to their normal shape and size. The phenomenon indicated that pretreatment of cells with Se-POP-11 could inhibit the nucleic morphological changes and significantly alleviate the oxidative damage in H_2O_2-induced PC12 cells.

Conclusion

In summary, a relatively purified Se-POP-11 with the selenium content of 3.21 μg/g from *Pleurotus ostreatus* fortified with sodium selenite was obtained after purified by DEAE-52 and G-100, it was mainly composed of glucose, mannose, galactose, arabinose and xylose, with the molar ratio of 5.30:1.55:2.14:0.29:0.63, respectively, average molecular weight was approximately 9.43 kDa. The Se-POP-11 exhibited stronger scavenging activity toward superoxide anion radical, hydroxyl radical, ATBS radical and DPPH radical, it also showed the inhibition of severe oxidative damage for PC12 cells induced by H_2O_2. All above results suggested that Se-POP-11 has potent antioxidant properties *in vitro* and should be explored as a functional food ingredient or a kind of medicine with organic Se supplements. The more detailed research should be carried out about the binding form of selenium with polysaccharide, the anticancer and other biology function should also be performed in subsequent research.

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Conflict of Interest

No conflict of interest associated with this work.

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