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Tumoricidal effects of a selenium (Se)-polysaccharide from Ziyang green tea on human osteosarcoma U-2 OS cells

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ABSTRACT

Selenium(Se)-enriched green tea consumption in human diets is well-known to reduce the risk of a variety of diseases. Here, we isolated a Se-polysaccharide (Se-ZYTP) from Se-enriched Ziyang green tea and investigated its antitumor activity on human osteosarcoma U-2 OS cells in vitro and in vivo. Se-ZYTP contained 94.5% of carbohydrate and 2.1% of uronic acid, as well as 2.14 μg/g Se, revealing that Se-ZYTP was an acidic Se-conjugated polysaccharide. Monosaccharide composition analysis indicated that Se-ZYTP consisted of mannose, rhamnose and fucose in molar ratios of 2.4:1.5:1.2:0.2:0.1:0.3:0.3. In vitro, both MTT and LTH assays proved that Se-ZYTP (25, 50, 100 and 200 μg/ml) could significantly inhibit the proliferation of human osteosarcoma U-2 OS cells in a concentration-dependent fashion ($P < 0.05$ or $P < 0.01$). In U-2 OS cancer xenograft model in BALB/c athymic mice, Se-ZYTP oral administration at three doses of 100, 200 and 400 mg/kg body weight (B.W.) daily for 28 days resulted in obvious tumor regression as compared to model control ($P < 0.05$ or $P < 0.01$). In addition, body weights of mice in control or Se-ZYTP treated groups did not differ significantly and no mice died during experiment, suggesting the safety of Se-ZYTP. Therefore, we postulate that Se-ZYTP might have cancer-preventive and cancer-therapeutic benefit for human osteosarcoma.

1. Introduction

Osteosarcoma is the most common type of malignant bone tumor, predominantly occurring in adolescents and young adults (Mirabello, Troisi, & Savage, 2009). It is an extremely aggressive malignancy that arises mostly in the long bones (DuBois & Demetri, 2007). The current therapeutic modalities for osteosarcoma include surgery, radiotherapy, and chemotherapy but are still unsatisfying (Lee et al., 2008; Longhi, Errani, De Paolis, Mercuri, & Bacci, 2006; Marina, Gebhardt, Teot, & Gorlick, 2004). Although new therapies have been used in chemotherapy, the induction of drug-resistant and unwanted side effects are involved in chemotherapy which remain serious problems (de Saint Aubain Somerhausen & Fletcher, 1999). The introduction of adjuvant chemotherapy has increased the 5-year survival rate of patients with osteosarcoma by greater than 50% compared with surgery alone, In contrast, the patients who present with metastases at diagnosis continue to have a poor prognosis with 5-year survival rates of 20–30%. (Liang, Zhang, Liu, Shen, & Tao, 2012; Longhi et al., 2006; Marina et al., 2004). Therefore, there is a need for discovering novel agents with increased efficiency and reduced toxicity for their potential in improving the prognosis and therapy of patients with osteosarcoma.

Selenium (Se) is an essential element for human body and can only be obtained from food or other source of supplementation (Martinez & Charlet, 2009; Sanmartín, Plano, Sharma, & Palop, 2012). Unfortunately, in many regions of the world the selenium content in the soil and hence selenium intake, are very low. Selenium-enriched food supplements are the major source of Se to compensate this problem and its bioavailability comes mainly from Se organic forms generally more than 80% (Navarro-Alarcon & Cabrera-Vique, 2008). Inorganic selenite can be transformed into organic forms via binding with proteins and polysaccharides in various organisms, which are generally considered as more effective and safer than inorganic selenium as a dietary supplement (Fang, Wu, & Hu, 2003; Rayman, 2000; Sanmartín et al., 2012; Schrauzer, 2001). Se compounds have also been found to inhibit tumor formation in a variety of animal models, and recent studies have indicated that Se supplementation in human diets may reduce cancer risk (Combs & Gray, 1998). For example, selenium-enriched green tea inhibited the proliferation of human cervical adenocarcinoma HeLa cell and significantly inhibit the growth of lung carcinoma A549 and hepatoma HepG2 (Li et al., 2008). These were

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attributed to its effect on a variety of molecular targets and cellular processes (El-Sayed, Aboul-Fadl, Lamb, Roberts, & Franklin, 2006). For this reason, the selenium content in natural foods is drawing the attention of researchers and consumers, and selenium consumption is very popular in dietary supplements and functional foods. Selenium-polysaccharides, including natural selenium polysaccharides extracted from plants or their synthesized derivatives, are kinds of organic selenium-conjugated biological macromolecules and possesses more or stronger biological activities in comparison with selenium-free polysaccharide, such as the immunomodulation, hypoglycaemic, hypolipidemic, antitumor and antibacterial effects and so on (Fan et al., 2006). More importantly, such substances might serve as a source of selenium in dietary supplements or as an ingredient for the formulation of nutraceuticals. Therefore, discovering Se-containing polysaccharides with beneficial potential to human health has become a hot spot in polysaccharides research field.

Tea from the plant *Camellia sinensis* L. is the second most common beverage consumed worldwide next to water (Kao, Chang, Lee, & Chen, 2006) and is particularly popular in Asian countries (Lee et al., 2006). It contains many compounds including polyphenols, polysaccharides, amino acids, and vitamins, and it reduces the risk of a variety of diseases (Crespy & Williamson, 2004). Traditionally, most of the polyphenols from green tea are recognized as the main ingredients responsible for its multiple pharmacological actions (Sharangi, 2009). In recent decades, much attention has been paid to tea polysaccharide and its conjugate due to their immunological, anti-radiation, antiblood coagulation, anti-cancer, anti-HIV antioxidant and hypoglycaemic activities (Chen & Xie, 2001; Isiguki, Takakuwa, & Takeo, 1991; Zhou, Ding, Wang, & Xie, 1997). Ziyang green tea, a specific variety of selenium-enriched *C. sinensis* L. is widely distributed in the second seleniferous region (Ziyang County, Shaanxi) in China. However there is no available information regarding the characteristics and antitumor activity of purified Se-polysaccharides from Se-enriched Ziyang green tea. Therefore, in this paper, we first isolated and characterized the purified Se-polysaccharide from Se-enriched Ziyang green tea, and then investigated its tumoricidal effect on human osteosarcoma U-2 OS cells in vitro and in vivo.

2. Materials and methods

2.1. Materials and chemicals

Ziyang tea (Grade one) was purchased from Zijian Natural Selenium-enriched Tea Industry Co., Ltd. (Ziyang, Shaaxi Province, China).

2.2. Isolation and purification of polysaccharide Se-ZYTP

The small pieces of dried tea were pretreated by refluxing with 95% EtOH at 70 °C to remove lipophilic compounds, and then successively extracted with 5000 ml of distilled water at 100 °C for three times and each time for 3 h. After cooling down to room temperature, the whole extract was combined, filtered, concentrated and centrifuged under reduced pressure, and then the supernatant was precipitated with 3 volumes of 95% ethanol at 4 °C overnight. After centrifugation (2000 × *g* at 4 °C for 15 min), the precipitate was dissolved in water to sufficiently mix with Sevag reagent for removing protein (Staub, 1965), followed by exhaustive dialysis with water for 48 h. The supernatant was gathered, condensed and freeze-dried to obtain polysaccharide of Ziyang tea (ZYTP).

The crude polysaccharide ZYTP was fractionated on a DEAE Sepharose Fast Flow column (Cl^- form, 40 cm × 2.6 cm), and eluted with 0.15 M NaCl. The tubes containing polysaccharide were monitored and combined by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). One resulting main fraction was concentrated, dialyzed and lyophilized, and then purified on a Sephacryl S-400/HR column (2.6 cm × 100 cm) eluting with 0.15 M NaCl. The main fraction was collected and then dialyzed. Finally, a purified polysaccharide named Se-ZYTP was obtained by lyophilization for further analysis.

2.3. Monosaccharide composition and properties of Se-ZYTP

The polysaccharide was determined by phenol-sulfuric acid reaction using glucose as standard (Dubois et al., 1956). The protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard. The content of uronic acid was determined according to the method of Blumenkrantz and Asboe-Hansen (1973) using D-glucuronic acid as the standard. The selenium (Se) content was given by the Se concentration detected by atomic fluorescence spectroscopy (AFS, WFX-210, Rayleigh, China) over the exact weight of lyophilized samples as described by Zhao et al. (2008).

Gas chromatography (GC) was used for identification and quantification of the monosaccharide in polysaccharide, as described by Lobas et al. (1994). Briefly, Se-ZYTP (10 mg) was hydrolyzed with 2 M TFA at 120 °C for 6 h, and then hydrolyzed products were conventionally converted into the alditol acetates according to conventional procedures (Jones & Albersheim, 1972; Oades, 1967). The resulting alditol acetates were examined by GC on a 6890 N instrument (Angilent, USA) with a DB-35 capillary column (30 m × 0.32 mm × 0.25 μm) and a flame-ionization detector (FID) detector.

2.4. Cell culture and drug treatment

The human osteosarcoma cell line (U-2 OS) was obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, where they were tested and authenticated. U-2 OS were cultured in McCoy's 5a medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 Units/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere (5% CO_2, 95% air) (Huang et al., 2010).

2.5. MTT assay of cell proliferation

The MTT assay was performed to assess osteosarcoma cell viability. Briefly, cells were seeded in 96-well plates at 2×10^5 cells/well in 200 μl medium and cultured for 24 h. After the cells were treated with Se-ZYTP (25, 50, 100 and 200 μg/ml) for 24, 48 and 72 h, 20 μl of 5 mg/ml MTT solution was added to each well and incubated for 4 h at 37 °C. Finally, 150 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals with shaking. Optical density was measured at 570 nm (reference wavelength 630 nm) in a microplate reader (Bio-Tek, ELX 800, USA). The absorbance of the untreated control cells was set at 100%. Cell viability was expressed as the relative formazan formation in treated samples compared with control cells.

2.6. Assay for lactate dehydrogenase (LDH)

A LDH kit (Gaochuang Chemical Technology Co., Ltd. Shanghai, China) was used to determine cellular membrane damage in response to Se-ZYTP treatments according to manufacturer's instructions, with minor modifications. Briefly, after U-2 OS cells were treated with various concentrations of polysaccharide for indicated time, 20 μl of culture medium was collected for extracellular LDH analysis. The absorbance was read at a wavelength

Table 1
Chemical properties of Se-ZYTP.

Sample	Total carbohydrate (%)	Protein (%)	Uronic acid (%)	Selenium content (μg/g)
Se-ZYTP	94.5	–	2.1	2.14

of 450 nm and the results were expressed as the enzymatic activity (U/L) present in culture media of Se-GTPs-treated cells versus control cells.

2.7. Xenograft model

Male athymic (BALB/c nu/nu) nude mice at 4–6 weeks of age were used for the U-2 OS xenograft model. The mice were maintained under specific pathogen-free conditions and were fed commercial diet and water ad libitum. Approximately 1×10^7 U-2 OS cells (suspended in 0.2 ml PBS) were subcutaneously injected into the flanks of each nude mouse under aseptic conditions and incubated for 21 days to form xenofraft tumors when it reached to 100 mm^3. Thereafter tumor-bearing mice were randomly divided into four groups ($n = 8$ mice per group) as follows: one model control group and three Se-ZYTP-treated groups. The mice inoculated with U-2 OS cells were oral administrated with Se-ZYTP in saline at the doses of 100, 200 and 400 mg/kg body weight for 28 days and the model control mice received the same volume of saline. Twenty-four hours after last drug administration, mice were weighed and sacrificed by cervical dislocation. The tumor was removed, weighted and measured in two dimensions using digital vernier calipers as previously described (Xin et al., 2012). All animal (used in this experiment) handling procedures were performed in strict accordance with the PR China legislation the use and care of laboratory animals, with the guidelines established by Institute for Experimental Animals of the Fourth Military Medical University, and were approved by the College Committee for Animal Experiments.

2.8. Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical significance was assessed by the Student's *t*-test, carried out using the software GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA, USA). The significance was established at $P < 0.05$.

3. Results and discussion

3.1. Isolation and purification of polysaccharide Se-ZYTP

The crude polysaccharide ZYTP was isolated from Ziyang green tea by a series of purification procedures such as defatting, water extraction, ethanol sedimentation, deproteination and dialysis. The final yield of ZYTP was about 5.3% of the dried material. The crude ZYTP was firstly eluted with 0.15 M NaCl through an anion-exchange chromatography of DEAE Sepharose Fast Flow, affording one independent elution peaks (ZYTP-I) as detected by the phenol-sulfuric acid assay (Fig. 1A). Furthermore the obtained 0.15 M NaCl-eluting fraction was purified by gel filtration chromatography of Sephacryl S-400/HR, finally giving a single elution peak named as Se-ZYTP (Fig. 1B). The recovery rates of Se-ZYTP based on the amount of ZYTP were 36.4%.

3.2. Characterization of polysaccharide Se-ZYTP

The total carbohydrate content, uronic acid content, protein content and selenium content of Se-ZYTP were summarized in Table 1, and monosaccharide composition was recorded in Table 2.

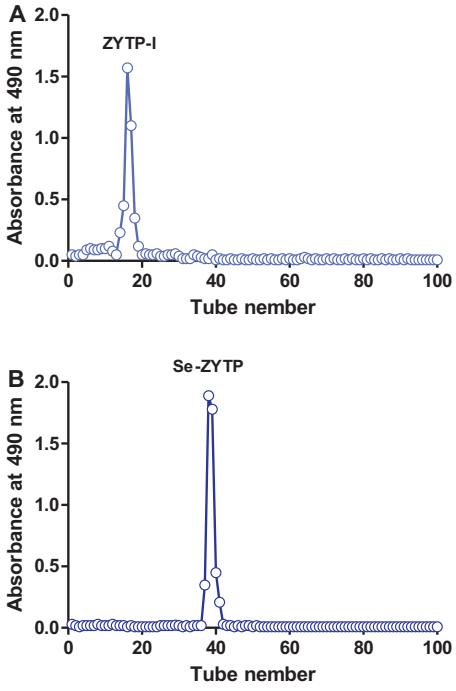

Fig. 1. (A) Elution profile of crude polysaccharide ZYTP extracted from Ziyang green tea on a DEAE Sepharose Fast Flow anion-exchange column with 0.15 M NaCl elute. (B) Elution profile of polysaccharide fraction ZYTP-I on a Sephacryl S-400/HR column with 0.15 M NaCl elute.

Table 2
Monosaccharide composition of Se-ZYTP.

Monosaccharide composition	Relative content (%)
Galactose	2.4
Glucose	1.5
Arabinose	1.2
Rhamnose	0.2
Fucose	0.1
Mannose	0.3
Glucuronic acid	0.3

The Se-ZYTP had a negative response to the Bradford's method and had no absorption in 280 nm, indicating the absence of protein. The total carbohydrate content and total uronic acid content in Se-ZYTP were 94.5% and 2.1%, respectively, and organic selenium content was 2.14 μg/g, revealing Se-ZYTP was a acidic selenium-containing polysaccharide. The high performance size-exclusion chromatography (HPSEC) profile also suggested that Se-ZYTP was a homogeneous polysaccharide (data not shown). The sugar composition determined by GC showed that Se-ZYTP was a typical heteropolysaccharide and was composed mainly of galactose, glucose, arabinose, rhamnose, fucose, mannose and glucuronic acid with molar ratios of 2.4:1.5:1.2:0.2:0.1:0.3:0.3.

3.3. Antitumor activities of Se-ZYTP in vitro

The MTT assay was performed to evaluate the antiproliferative effects of Se-ZYTP on human osteosarcoma U-2 OS cells. As shown in Fig. 2A, the results of MTT revealed that U-2 OS cells are sensitive to Se-ZYTP at each concentration, and the inhibitory

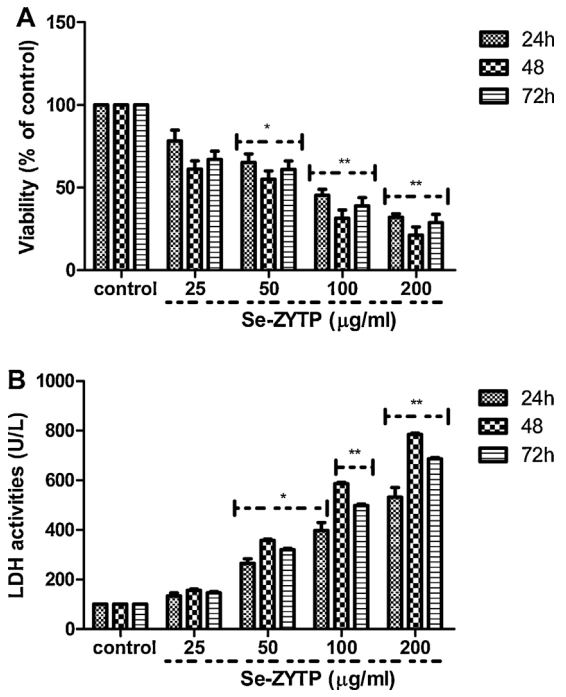

Fig. 2. Antiproliferative effect and cytotoxicity of Se-ZYTP on human osteosarcoma cancer U-2 OS cells estimated by MTT assay (A) and LDH leakage analysis (B). The data were expressed as mean ± SD obtained from triplicate samples.

effect was significant at the concentrations from 50 to 200 μg/ml as compared to the control group ($P < 0.05$ or $P < 0.01$). Especially after 48 h cultivation with Se-ZYTP, the cell viability was lower than 24 or 72 h treatment, with the value of 61.2% for 25 μg/ml, 55.0% for 50 μg/ml, 31.4% for 100 μg/ml and 21.2% for 200 μg/ml, respectively.

To further confirm whether Se-ZYTP is cytotoxic to human osteosarcoma U-2 OS cells, a LDH kit was used to measure LDH enzyme leakage from the cytosol of damaged cells into the medium, an indicator of irreversible cell death due to cell membrane damage (He, Yang, Jiao, Tian, & Zhao, 2012). As seen in Fig. 2B, treatment with Se-ZYTP for 48 h dose-dependently resulted in a more increase in LDH release from U-2 OS cells to medium than 24 or 72 h treatment, as reflected by the 1.56, 3.58, 5.87 and 7.85 fold increases for 25, 50, 100 and 200 μg/ml, respectively. It is worth noting that Se-ZYTP exhibited high cytotoxic effect on U-2 OS cells, similar to the observation assayed by MTT method.

3.4. Antitumor activities of Se-ZYTP in vivo

Finally, we examined the in vivo antitumor activity of Se-ZYTP in a mouse U-2 OS xenograft model. When xenograft tumor mass reached a volume of about 100 mm^3, mice were treated with Se-ZYTP at the dose of 100, 200 and 400 mg/kg or a vehicle by oral route daily for 28 days. At the end of the experiment, in average, Se-ZYTP reduced the tumor volume from 1267.05 mm^3 in vehicle-treated model group to 595.85 mm^3 ($P < 0.01$), 362.04 mm^3 ($P < 0.01$) and 222.20 mm^3 ($P < 0.01$) in 100, 200 and 400 mg/kg GA-fed group (Fig. 3A), respectively. Accordingly, Se-ZYTP treatment at the dose of 100, 200 and 400 mg/kg resulted in individual decrease of 21%, 25% and 34% in tumor weight (Fig. 3B). However, body weights did not differ significantly among the control and Se-ZYTP-fed groups during the 4 weeks of the experimental period (Fig. 3C). In addition, no mouse died the experimental period (data not shown).

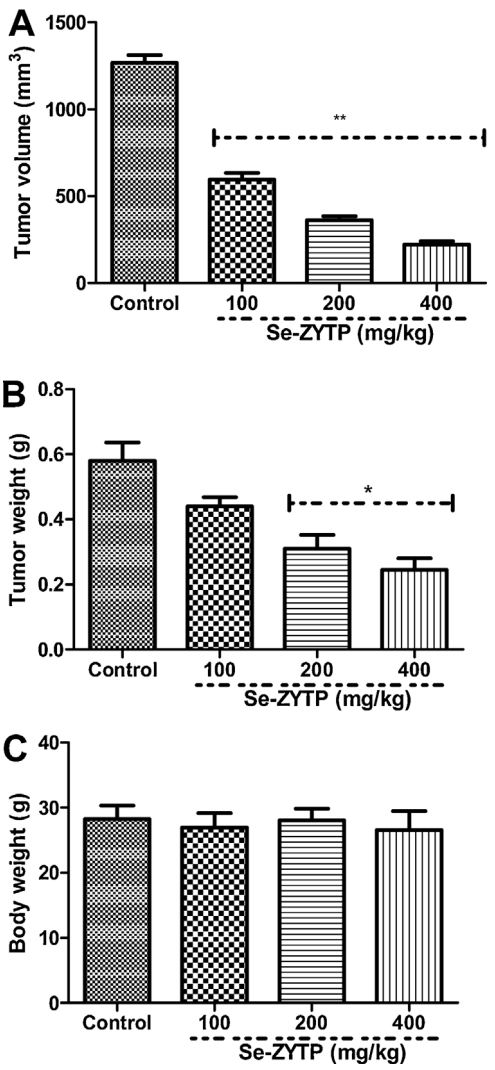

Fig. 3. Effects of Se-ZYTP on (A) tumor volume, (B) tumor weight, and (C) body weight in the xenograft animal model. The data were expressed as mean ± SD obtained from eight animals.

4. Conclusions

In summary, one homogeneous Se-polysaccharide, Se-ZYTP, was successfully purified from the Se-enriched Ziyang green tea by DEAE Sepharose Fast Flow and Sephacryl S-400/HR column chromatography. Se-ZYTP contained 94.5% of carbohydrate, 2.1% of uronic acid and 2.14 μg/g of Se, revealing Se-ZYTP was an acidic Se-conjugated polysaccharide. Monosaccharide analysis showed that Se-ZYTP was composed of mannose, rhamnose and fucose with a molar ratio of 2.4:1.5:1.2:0.2:0.1:0.3:0.3. In vitro, Se-ZYTP significantly inhibited the proliferation of human osteosarcoma U-2 OS cells in a concentration-dependent fashion as determined by MTT and LTH assays. In vivo, a remarkable decrease in tumor volume and tumor weight was also observed in mice bearing U-2 OS cancer cells. Furthermore, body weights of mice in control or Se-ZYTP treated groups did not differ significantly and no mice died during experiment. These observations offer the first evidence that Se-ZYTP possibly possesses anticancer activity in human osteosarcoma. However, the antitumor mechanisms of Se-ZYTP remain unclear, which must be studied further in subsequent research.

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