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Extraction, characterization of the polysaccharide extracts from Se-enriched *G. lucidum* (Se-GLP) and its inhibition against oxidative damage in ischemic reperfusion mice

Wei-Lin Shi [a,1], Hui Han [b,1], Geng-Zhen Chen [b], Xi Chen [c], Ying-Kai Hong [d], Ling-Kong Chen [e], Dan Chen [f], Zhen Lu [a,*]

[a] Department of Cardiology, Taizhou Hospital of Zhejiang Province (Luqiao Branch), Taizhou, Zhejiang Province, PR China
[b] Department of General Surgery, The Second Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province, PR China
[c] Emergency Department, Taizhou Hospital of Zhejiang Province (Luqiao Branch), Taizhou, Zhejiang Province, PR China
[d] Department of Urology, The First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province, PR China
[e] Pharmaceutical Preparation Section, Luqiao Hospital of Traditional Chinese Medicine, Taizhou, Zhejiang Province, PR China
[f] Department of General Surgery, The Second People's Hospital of Luqiao, Taizhou, Zhejiang Province, PR China

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ABSTRACT

By analyses using high-performance liquid chromatography (HPLC), the polysaccharide extracts from Se-enriched *Ganoderma lucidum* (Se-GLP) was comprised mainly of glucose, xylose, arabose and mannose in the molar ratio of 1:4.3:0.7:0.18. the infrared (IR) spectra of the Se-GLP displayed a broad stretching intense characteristic peak at around 3402 and 3105 cm^{-1} for the hydroxyl group, and one weak C–H stretching bands at 2927 and 2702 cm^{-1}. The peaks at 1163, 947 and 890 cm^{-1} were characteristic of xylose, arabose and β-D-glucose, respectively. The results indicated that glucose and xylose were the major monoses constructing the backbones of Se-GLP. Then, the protective effects of the polysaccharide extracts from Se-enriched *G. lucidum* (Se-GLP) against oxidative damage were evaluated in heart and liver of ischemic reperfusion mice. The results showed that the treatment of Se-GLP significantly lowered the ischemic reperfusion-induced serum levels of malondialdehyde (MDA) and intercellular adhesion molecule-1 (ICAM-1). The heart and serum content of GSH, and activities of Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and total antioxidant capacity (TAOC) that were reduced by ischemic reperfusion were brought back to control levels by the supplement of Se-GLP. Therefore, the results of this study suggest that Se-GLP could protect heart against the ischemic reperfusion-induced oxidative damage in mice.

1. Introduction

Ischemia-reperfusion promotes both local injury and systemic inflammation (Carden & Granger, 2000). Increased vascular permeability and vascular resistance are manifestations of endothelial damage by ischemia-reperfusion injury in the pulmonary vascular bed (Davenpeck, Guo, & Lefer, 1993). A specific mechanism uniformly responsible for ischemia-reperfusion injury is unlikely since energy degradation during ischemia, generation of reactive oxygen species (ROS) during reperfusion, "no-reflow" phenomenon, and calcium overload reperfusion all have been reported to contribute to injury from ischemia-reperfusion (Hawaleshka & Jacobsohn, 1998; Lu, Chen, & Liu, 2002; Yellon, Alkhulaifi, Browne, & Pugsley, 1998).

Selenium (Se), an essential micronutrient with antioxidant properties, has received considerable attention for potential roles in cancer prevention for both human beings and animals (Berry, 2005). It is therefore suggested to find proper sources of dietary selenium supplement. Some authors suggest that organic Se is an ideal additive because animals and humans absorb and retain it more than inorganic Se. The main source of organic Se is seafood and fish, whereas foods like cereals, meat, nuts, mushrooms and eggs can also increase the dietary selenium intake (Muniz-Naveiro et al., 2005). Recently, more researches showed that Se-enriched yeast and some plant resources such as Se-enriched onion, garlic, fungi and tea were considered as effective organic selenium supplement (Whanger, Ip, Polan, Uden, & Welbaum, 2000). The oriental fungus, *Ganoderma lucidum* (Leyss. ex Fr.) Karst. (Lingzhi), has been used for centuries by Asian people to promote health and increase longevity. *G. lucidum* has been shown to prevent chronic diseases in clinical practice (Lu et al., 2004; Eo, Kim, Lee, & Han, 1999; Lin, Lin, Chen, Ujiie, & Takada, 1995). Increasing lines of evidence

* Corresponding author. Tel.: +86 13750633498.
E-mail address: zhenlu05722@yahoo.com.cn (Z. Lu).
[1] Both authors contributed equally to this work.

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have shown that *G. lucidum* has immunoregulatory (Chen et al., 2004) and anti-tumor activities (Lakshmi, Ajith, Jose, & Janardhanan, 2006) and antioxidant activities (Jia et al., 2009).

In this study, we investigated the antioxidative activity and potential protective effects of the Se-GLP in the oxidative damage in ischemia-reperfusion rats.

2. Materials and methods

2.1. Materials

The *Ganoderma lucidum* mycelium comes from the Fungal Institute of our university (Sichuan province, China).

2.2. Culture conditions

During the experimental work, the isolates were kept on Petri dishes on Potato Dextrose Agar at 30 °C and were re-inoculated every 3 weeks to maintain their viability and activity. Isolates were grown in a liquid medium containing: 50 g l^{-1} of glucose, 2.0 g l^{-1} of polypeptone, 2.0 g l^{-1} of yeast extract, 5.0 g l^{-1} of KH_2PO_4, 2.5 g l^{-1} of $MgSO_4$, 10 g l^{-1} of maltose, and 21.90 g l^{-1} of Na_2SeO_3, pH 5.7. Mycelia were cultivated on a rotary shaker at 26 °C for 28 days at 110 rpm. The 28-days period of cultivation was chosen on the basis of preliminary experiments which had shown that after this time the nutrient medium became clear.

2.3. Extraction of G. lucidum polysaccharides

After cultivation, the biomass was filtered through a filter paper on a Buchner funnel under reduced pressure, washed carefully with 250 mL of distilled water, and dried at 40 °C. Mycelium was then pulverized and dried.

Se-GLP was extracted from the spores of *G. lucidum* using a method similar to the standard methodology of (Ma, Guan, Yang, Liu, & Guo, 2008). Briefly, the spores of *G. lucidum* (300 g) were defatted with 95% alcohol and then refluxed with 70 volumes of water. The aqeous extract was fractionated into a polysaccharide fraction (alcohol insoluble) and nonpolysaccharide fraction (alcohol soluble), and further treated with trichloroacetic acid to remove proteins, and dialyzed against tap water for 2 days and distilled water for 1 day (molecular weight cut-off 3000–5000). The retentate was washed sequentially with EtOH and acetone and concentrated under vacuum and freeze dried to obtain the polysaccharides as a yellow-white powder (3.6 g). Se-GLP was dissolved in RPMI-1640 medium, filtered through a 0.22 μm filter and stored at −20 °C before use in biological study.

2.4. Determination of selenium

Selenium analysis was performed using a Model AAS5 atomic absorption spectrometer (Analytik Jena AG, Jena, Germany) with deuterium background correction, equipped with a transversely heated graphite tube atomizer. The analysis was carried out at 196.1 nm with an operating lamp current of 5 mA and a 2.0 nm slit width. A solution of 5.0 mg/mL $Ni(NO_3)_2$ (Fluka) as a modifier in 1% GR grade HNO_3 was used as the blank. Approximately 10.00 mg of the sample was mineralized with GR grade 65% HNO_3 in a teflon crucible placed in a microwave mineralizator. After mineralization, the sample was transferred to a 25 mL volumetric flask and made up to the volume with blank. All measurements of Se content were performed in triplicate. The calibration curve was obtained using different concentrations of selenous acid standard solution (Sigma–Aldrich) correspond to 40.0, 100.0, and 200.0 ng/mL of Se.

2.5. HPLC

The HPLC system used was a Waters liquid chromatography system (Model 2690), a cooled autosampler (4 °C) and a Waters 996 diode-array detector. The chromatography method is based on the method by Cardoso et al. (2009). The reversed phase column was a 150 × 3.9 mm Waters DeltaPak C18 column (17% carbon load, fully endcapped, particle size 5 μm) protected by a guard column (Waters Nova-Pak, C18, particle size 5 μm). The column was maintained at a constant temperature of 28 °C using a column thermostat (Waters Column heater Model 2690). The flow rate was 0.8 mL min^{-1} and the injection volume was 50 μL. The chromatographic separation was carried out at 25 °C. Atlas 2001 R1 software (Thermo LabSystems, Cheshire, UK) was used to collect and process the chromatographic data.

2.6. Fourier-transform infrared spectra

The FT-IR spectra of the extracts were obtained according to the manufacturer's recommendations. Measurements were carried out in a room with stabilisation and control of temperature, which was fixed at 24 °C. During an experiment spectra were recorded continuously at spectral resolution of 8 cm^{-1}, by co-adding 32 scans for each spectrum at a scanning velocity of 100 kHz (HeNe frequency). The spectral range recorded extended from 950 to 1650 cm^{-1} covering the major absorption bands of the sugars used in this work. The sample was filled with cyclohexane and the single-beam spectrum collected used as reference (background). A single-beam spectrum was collected against that of the reference (background) and converted to absorbance. Triple measurement of each extract was made.

2.7. Animals and treatment

Kunming mice (22 ± 2 g) were randomly divided into four groups with each consisting of 10 mice. Group I served as normal control. Group II served as model control. Groups III and IV were administered orally the Se-GLP dissolved in distilled water at doses of 100 and 200 mg/kg, respectively, daily for a period of 50 days. At the end of the experiment, animals were sacrificed by cervical dislocation. Blood was collected into heparinized tubes (50 U/mL). Heart samples were dissected out and washed immediately with ice cold saline to remove as much blood as possible.

2.8. Biochemical assays

Reduced glutathione was measured according to the method of Ellman (1959). Lipid peroxides were determined as MDA by the TBARS method (Zhang, Cheung, Zhang, Chiu, & Ooi, (2004); Yuan et al., 2009). SOD was estimated in heart cytosol according to the method of Marklund and Marklund (1974). The determination of glutathione peroxidase (GSH-Px) activity was performed according to the method of Wu, Duan, Liu, & Cen, (2010). Catalase activity was determined according to the literature (Aebi & Verlag Chemic Weinheim, 1974). Total antioxidant capacity was measured in serum by means of a commercial kit.

2.9. Statistical analysis

Statistical analyses were carried out by SPSS 10 program for Windows (SPSS, Chicago, IL). Data are presented as mean ± SEM. For statistical analysis, Kruskal–Wallis test was used, followed by the Mann–Whitney *U* test. A level of $p < 0.05$ was accepted as statistically significant.

3. Result

3.1. Selenium content in polysaccharides

Selenium analysis indicated that selenium content in Se-GLP was 0.834 ± 0.039 mg/g.

3.2. HPLC of Se-GLP

The chromatograms of Se-GLP, and the available reference compounds glucose, xylose, arabose and mannose monitored at 320 nm are shown. The bioactive candidates of peaks 1 and 2 of Se-GLP were unequivocally identified as glucose, and xylose by comparison of retention times of reference compounds, while peaks 3 and 4 were tentatively assigned as arabose and mannose by the comparison of their standard sugar samples. Their mole ratios were 1:4.3:0.7:0.18 (Table 1). The results indicated that glucose and xylose were the major monoses constructing the backbones of Se-GLP.

3.3. FT-IR spectroscopy

The FT-IR spectra of carbohydrates are used for determination of their structural features. As shown in Fig. 1, the IR spectra of the Se-GLP displayed a broad stretching intense characteristic peak at around 3402 and 3105 cm^{-1} for the hydroxyl group, and one weak C–H stretching bands at 2927 and 2702 cm^{-1} (Qian, Chen, Zhang, & Zhang, 2009). The wave number between 400 and 1300 cm^{-1} is often called the fingerprint of molecules because it allows the identification of major chemical groups in polysaccharides: the position and intensity of the bands that are specific for each polysaccharide. Since monosaccharide analysis revealed that Se-GLP was mainly composed of xylose, FT-IR spectra of Se-GLP were compared against the commercial xylan standard. It was found that the FT-IR spectra of Se-GLP exhibited similarities in absorption pattern to xylan, confirming the preliminary conclusion derived from chemical composition analysis that the polysaccharide is a xylan. The broader band of absorption between 4000 and 3000 cm^{-1} was due to O–H stretching whereas an intense ring and (COH) side group band at 1047 cm^{-1} dominated the spectrum of xylan with $\beta(1 \rightarrow 4)$ backbone (xylan standards), whilst in the $\beta(1 \rightarrow 3)$ linked xylan two partially overlapping bands at 1051 and 998 cm^{-1} were found instead. The absorbance of polysaccharides in the range 950–1200 cm^{-1} were where the C–O–C and C–O–H link band positions were found (Jafari, Nateghi, & Rabbani, 2010). Two stretching peaks, at 1632 and 605 cm^{-1} in the IR spectra of Se-GLP, suggested the presence of C–O bonds.

Table 1
Chemical composition of Se-GLP.

Glucose	Xylose	Arabose	Mannose
15.7%	69.8%	11.6%	2.9%

3.4. Effect of Se-GLP on ICAM-1 level in heart

Table 2 shows that ischemic reperfusion treatment induced a significant increase of ICAM-1 level in heart of model mice (II). In the heart of model mice (II), level of ICAM-1 was higher than in control ($P < 0.01$) mice. However, the level of ICAM-1 in heart of Se-GLP-treated mice was lower than in heart of model animals ($P < 0.01$), indicating well-known organ dependence of Se effects.

3.5. Effect of polysaccharides on GSH and MDA levels in blood and heart

In the model group (II), the GSH levels in blood and hearts were significantly ($P < 0.01$) lower than control group (I), while MDA

Table 2
Effect of Se-GLP on ICAM-1 level in heart.

Group (mg/ml)	ICAM-1 (mg/ml)
I	17.93 ± 1.84
II	35.52 ± 3.25
III	28.37 ± 1.94
IV	19.62 ± 1.49

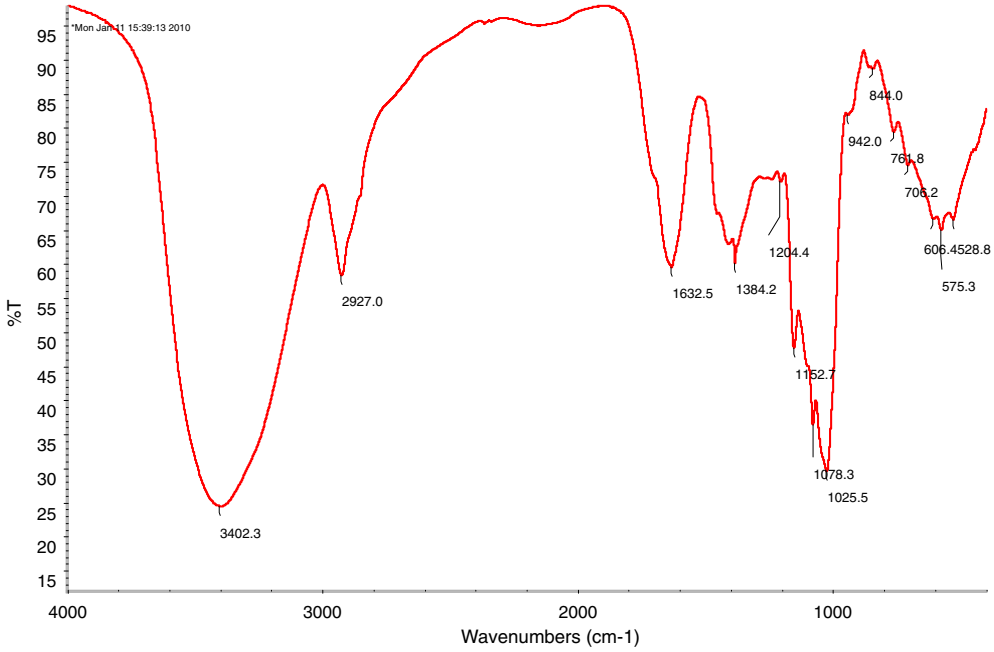

Fig. 1. FT-IR analysis of Se-GLP.

levels was found to be significantly ($P < 0.01$) increased when compared to control group (Table 3). In the Se-GLP-treated groups (III and IV), the MDA levels in blood and hearts was significantly ($P < 0.01$) lower (Table 3) and GSH levels was found to be significantly ($P < 0.01$) increased (Table 3) in response to Se-GLP treatment. MDA and GSH levels were returned near to the control levels after Se-GLP treatment in blood and hearts (Table 3).

3.6. Effect of Se-GLP on SOD, CAT, GSH-Px and TAOC activities in blood

Data on SOD, CAT, GSH-Px and TAOC activities in blood are presented in Table 4. As can be seen, significant changes of SOD, CAT, GSH-Px and TAOC activities were observed. In model mice (II), activity of blood SOD, CAT, GSH-Px and TAOC was lower ($P < 0.01$) with respect to controls (I). On treatment with Se-GLP (group III and group IV), the activities of blood SOD, CAT, GSH-Px and TAOC were significantly restored to near normal. No adverse effect was observed in Se-GLP-treated groups (III and IV).

3.7. Effect of Se-GLP on SOD, CAT, GSH-Px and TAOC activities in liver

Data on SOD, CAT, GSH-Px and TAOC activities in heart are presented in Table 5. In model mice (II), activity of heart SOD, CAT, GSH-Px and TAOC was lower ($P < 0.01$) with respect to controls (I). Activity of heart SOD, CAT, GSH-Px and TAOC in group III and IV mice were significantly increased with the treatment of Se-GLP in a dose dependent manner. This indicated that Se-GLP treatment effectively brought back the activities of heart antioxidant enzymes to near control.

4. Discussion

In this study, Se-GLP was shown to effectively reduce ischemic reperfusion-induced heart damage in mice. Plasma and heart biochemical parameters were assayed. Plasma activities of ICAM-1 are the most commonly used biochemical markers of heart and liver injury (Meyer et al., 1998). In the present examination of the progress of heart injury by ischemic reperfusion, activities of both plasmas ICAM-1 markedly increased. Se-GLP could clearly reduce the increase in plasma ICAM-1 caused by ischemic reperfusion; thus, it showed significant action in reducing the heart damages induced by ischemic reperfusion.

The reactive oxygen species are important as direct and indirect initiators as well as promoters of heart damage. They also increase the lipid peroxidation, which in turn alter the integrity of membrane bound enzymes (Cheeseman & Slater, 1993). The free radical scavenging efficiency of Se-GLP thus might be playing an important role in inhibiting heart damage. The treatment of animals with ischemic reperfusion caused a significant increase in lipid peroxidation and decrease in activities of antioxidant enzymes. The present investigations revealed that the Se-GLP was able to decrease the levels of MDA and increase the activities of antioxidant enzymes. Se-GLP reduced heart damage by acting on antioxidant response elements and thereby increasing the synthesis of enzymes involved in detoxification. The prevention of heart damage of Se-GLP might be mediated by the changes in the GSH content, which is important for reducing mammalian susceptibility to the effects of toxins. The significant increase of superoxide dismutase in the extract treated group of animals appears to facilitate removal of superoxide anions, and H_2O_2 formed in the process by GSH-Px and CAT which are also increased by the treatment of extract. It has been established that reactive oxygen species are involved in inflammation (Godin, Ko, Qayumi, & Jamieson, 1989), and the protective action of Se-GLP extract against ischemic reperfusion-induced heart damage could involve mechanisms related to scavenging activity.

In conclusion, we have shown that ischemic reperfusion elevates the levels of the heart ICAM-1, and decreases the activities of serum and heart antioxidant enzymes. Treatment with Se-GLP could reduce damage induced by ischemic reperfusion. The mechanisms of protection include the inhibition of lipid peroxidation, increasing the content of GSH, elevating the expression of antioxidant enzymes. Thus, the present study reveals that Se-GLP extract has antioxidant activity and a protective effect on ischemic reperfusion-induced oxidative damage in heart and liver.

Table 3
Effect of Se-GLP on GSH and MDA levels in blood and heart.

Group	Blood		Heart	
	GSH (mg/l)	MDA (nmol/l)	GSH (mg/mg)	MDA (nmol/mg)
I	427.5 ± 30.6	13.57 ± 1.09	631.2 ± 41.3	24.83 ± 1.73
II	215.2 ± 13.2	35.28 ± 1.53	301.5 ± 17.4	57.38 ± 4.28
III	327.4 ± 24.8	23.04 ± 1.11	499.27 ± 33.2	41.03 ± 2.11
IV	441.3 ± 37.1	15.22 ± 1.42	621.4 ± 73.1	25.29 ± 1.72

Table 4
Effect of Se-GLP on SOD, CAT, GSH-Px and TAOC activities in blood.

Group	SOD (U/ml)	CAT (U/ml)	GSH-Px (U/ml)	TAOC (U/ml)
I	197.3 ± 13.2	17.31 ± 1.13	25.26 ± 1.48	11.21 ± 0.68
II	86.3 ± 7.3	8.36 ± 0.67	15.39 ± 1.31	6.38 ± 0.35
III	139.2 ± 11.4	13.82 ± 0.92	20.77 ± 1.72	9.37 ± 0.72
IV	188.1 ± 15.2	16.39 ± 1.22	26.16 ± 1.39	13.24 ± 1.22

Table 5
Effect of Se-GLP on SOD, CAT, GSH-Px and TAOC activities in heart.

Group	SOD (U/mg)	CAT (U/mg)	GSH-Px (U/mg)	TAOC (U/mg)
I	235.1 ± 19.7	21.52 ± 1.06	17.46 ± 1.32	16.31 ± 1.52
II	121.7 ± 11.0	13.27 ± 1.32	10.42 ± 0.83	8.39 ± 0.62
III	199.3 ± 14.3	18.69 ± 0.94	16.28 ± 1.42	14.61 ± 1.22
IV	228.3 ± 24.5	22.51 ± 1.66	20.55 ± 1.73	17.47 ± 1.53

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