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Antioxidant activity and potential hepatoprotective effect of polysaccharides from *Cyclina sinensis*

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ABSTRACT

In the present study, we investigated the preliminary structure, *in vitro* antioxidant and *in vivo* hepatoprotective activities of polysaccharides from *Cyclina sinensis* (CSPS). The analytic results of Fourier transform-infrared spectroscopy indicated the presence of α-type glycosidic linkages in CSPS-1 or CSPS-2, and the average molecular weights for CSPS-1, CSPS-2 and CSPS-3 were 69, 81 and 101 kDa, respectively. For antioxidant activities *in vitro*, crude CSPS, CSPS-1, CSPS-2 and CSPS-3 showed moderate H_2O_2 scavenging activity, lipid peroxidation inhibition effect and strong Fe^{2+} chelating activity. For hepatoprotective activity *in vivo*, the administration of CSPS significantly decreased serum levels of alanine aminotransferase and aspartate aminotransferase, inhibited the formation of malondialdehyde and enhanced the activities of liver superoxide dismutase and glutathione peroxidase in carbon tetrachloride-induced liver injury mice. These results suggested that CSPS had potent antioxidant and hepatoprotective activities.

1. Introduction

Cyclina sinensis, a well-known bivalve mollusk in the family of Veneridae, is widely distributed along the coastal waters of China, Korea, and south eastern Asia (Zhao, Li, Kong, & Mao, 2009). It has been reported that *C. sinensis* can be used for the treatment of inflammation, asthma and dental ulcer in traditional Chinese medicine (Wang et al., 2007). In addition, it has been demonstrated that it is rich in protein, amino acid, lipid and polysaccharides that may contribute to the biological functions, such as anti-tumor, anti-inflammation and immune-regulation (Abdulkadir & Tsuchiya, 2008; Gu, Yu, & Cai, 2006; Liu, Zhang, Dou, Lu, & Guo, 1997). Recently, we reported the extraction, purification, antioxidant and antitumor activities *in vitro* of polysaccharides from *C. sinensis* (CSPS) (Jiang, Wang, Liu, Gan, & Zeng, 2011). We found that the third purified fraction (CSPS-3) had higher amounts of protein, uronic acid and sulfuric radical and more complicated monosaccharide composition than the other two fractions (CSPS-1 and CSPS-2). In addition, crude CSPS and its purified fractions exhibited high superoxide radical and hydroxyl radical scavenging activities. However, little information on the antioxidant property *in vivo* of CSPS and potential relationship of structure–function is available. Therefore, we report here the preliminary characterization, antioxidant *in vitro* and potential hepatoprotective effect of CSPS. Firstly, the preliminary structures of crude CSPS and its purified fractions were investigated by ultraviolet-visible (UV) spectroscopy, high performance liquid chromatography (HPLC) and Fourier transform-infrared spectroscopy (FT-IR). Then, the antioxidant activities *in vitro* of CSPS including reducing ability, hydrogen peroxide (H_2O_2) scavenging activity, ferrous ion (Fe^{2+}) chelating activity and lipid peroxidation inhibition effect were investigated. Finally, the hepatoprotective effect of crude CSPS was investigated by using the carbon tetrachloride (CCl_4)-induced hepatocyte toxicity model in mice.

2. Materials and methods

2.1. Materials

Crude CSPS and its purified fractions of CSPS-1, CSPS-2 and CSPS-3 were prepared from *C. sinensis* according to our reported method (Jiang et al., 2011). Assay kits for protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were the products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The male Kunming mice were purchased from the Experiment Animal Center of Academy of Military Medical Sciences (Beijing, China). All other reagents were of analytical grade.

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2.2. Preliminary characterization of CSPS

2.2.1. Determination of molecular weight

The molecular weight determination of CSPS was performed using a size-exclusion HPLC chromatography instrument (Agilent, 1100, USA) equipped with a refractive index detector (RID). All samples (10.0 mg) were dissolved in distilled water (1.0 ml), passed through a 0.45 μm filter and applied to a gel-filtration chromatographic column of TSK-GEL G3000SW$_{xl}$ (7.5 mm × 300 mm, Tosoh Corp., Japan). The column was maintained at a temperature of 25 °C and eluted with 0.1 M Na_2SO_4 solution in PBS buffer (0.01 M, pH 6.8) at a flow rate of 0.8 ml/min. For preparing molecular weight calibration curve, P-5, P-10, P-20, P-100, P-200, P-400 and P-800 (Showa Denko K.K., Japan) were used as the molecular weight references.

2.2.2. UV and FT-IR spectrometric analysis

UV spectrum of CSPS was recorded with a UV-2450 Spectrophotometer (Shimadzu Co., Kyoto, Japan). FT-IR spectrum of CSPS was recorded with a Nicolet 6700 FT-IR Spectrometer (Thermo Co., USA) using KBr disks method (Wang et al., 2004). Briefly, samples were dried at 35–44 °C in vacuum over P_2O_5 for 48 h, ground with potassium bromide (KBr) powder and then pressed into pellet for FT-IR spectral measurement in the frequency range of 4000–400 cm^{-1}.

2.3. Antioxidant activity in vitro of CSPS

2.3.1. Assay of reducing ability

Reducing ability of CSPS was assayed according to the reported method with some modifications (Liu, Luo, Ye, & Zeng, 2012). One milliliter of phosphate buffer (0.2 M, pH 6.6), 1.0 ml of 1% $K_3Fe(CN)_6$ solution and 0.5 ml of sample at different concentrations (0, 0.4, 0.8, 2.0, 3.2 and 4.0 mg/ml) were incubated at 50 °C for 20 min. Then, 1.0 ml of trichloroacetic acid (10%, w/v) was added to the mixture, and the mixture was centrifuged for 10 min at 2500 × *g*. The upper layer of solution (1.5 ml) was mixed with water (1.5 ml) and $FeCl_3$ (0.3 ml, 0.1%, w/v), and the absorbance was measured at 700 nm.

2.3.2. Assay of H_2O_2 scavenging activity

The H_2O_2 scavenging activity of CSPS was determined according to the method reported by Liu et al. (2010) with some modifications. One milliliter of sample with different concentration (0, 0.4, 0.8, 1.6, 3.2 and 4.0 mg/ml) was mixed with 2.4 ml of phosphate buffer (0.1 M, pH 7.4) and 0.6 ml of H_2O_2 solution (40 mM). The mixture was shaken vigorously and incubated at room temperature for 10 min. Then, the absorbance of the reaction mixture was determined at 230 nm. Ascorbic acid was used as positive control in the present study. The H_2O_2 scavenging activity was calculated according to the formula below:

$$H_2O_2 \quad \text{scavenging activity} \quad (\%) = 100 \times \frac{A_0 - A_1 + A_2}{A_0}$$

where A_0 is the absorbance of control sample (water instead of sample), A_1 is the absorbance in the presence of tested sample, and A_2 is the absorbance of the sample only (water instead of H_2O_2 solution).

2.3.3. Assay of Fe^{2+} chelating activity

The Fe^{2+} chelating activity of CSPS was determined according to the reported method (Liu, Wang, Xu, & Wang, 2007). One milliliter polysaccharide sample at different concentration (0, 0.4, 0.8, 1.6, 3.2 and 4.0 mg/ml) was added to 0.05 ml of ferrous chloride ($FeCl_2$) solution (2 mM), 0.2 ml of ferrozine solution (5 mM) and 2.75 ml of water. The mixture was shaken well and incubated for 10 min at room temperature, and then the absorbance of the mixture was determined at 562 nm. The Fe^{2+} chelating activity was calculated by the following formula:

$$Fe^{2+} \quad \text{chelating activity} \quad (\%) = 100 \times \frac{A_0 - A_1 + A_2}{A_0}$$

where A_0 is the absorbance of control sample (water instead of sample), A_1 is the absorbance in the presence of tested sample, and A_2 is the absorbance of the sample only (water instead of $FeCl_2$ solution). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was used as positive control in the present study.

2.3.4. Assay of lipid peroxidation inhibition effect

The lipid peroxidation inhibition effect of CSPS was determined by thiobarbituric acid-reactive-substances (TBARS) assay using mouse liver homogenate as the lipid rich media with some modification (Yen & Hsieh, 1998). Briefly, 1.0 ml of 1% (w/v) mouse liver homogenate was mixed with 1.0 ml sample solution with different concentrations (0, 0.4, 0.8, 2.0, 3.2 and 4.0 mg/ml), then 0.05 ml of $FeCl_2$ (0.5 mM) and H_2O_2 (0.5 mM) were added to initiate lipid peroxidation, which was carried out in a 37 °C water bath for 30 min. Reaction was terminated by adding 1.5 ml of trichloroacetic acid (TCA, 20%, w/v) and 1.5 ml of thiobarbituric acid solution (0.8%, w/v). The resulting mixture was vortexed and heated in a boiling water bath for 10 min. After centrifugation at 4500 × *g* for 10 min, the TCA–TBA phase was removed and the absorbance of the upper layer was recorded at 532 nm. Ascorbic acid was used as positive control. The inhibition effect on lipid peroxidation was calculated according to the formula below:

$$\text{Lipid peroxidation inhibition effect} \quad (\%) = 100 \times \frac{A_0 - A_1 + A_2}{A_0}$$

where A_0 is the absorbance of control sample (water instead of sample), A_1 is the absorbance in the presence of tested sample, and A_2 is the absorbance of the sample only (water instead of liver homogenate).

2.4. Evaluation of protective effect of CSPS against CCl_4-induced hepatotoxicity in mice

2.4.1. Animal grouping and experimental design

The protective effect of crude CSPS against CCl_4-induced hepatotoxicity was evaluated according to the reported method (Simile et al., 2001) with some modifications. Briefly, male Kunming mice (8-week old, 20 ± 2 g) were used in the present study. All animals were housed under standard environmental conditions (22 ± 0.5 °C, 55 ± 5% humidity and a 12 h light/12 h dark cycle) and maintained with free access to standard laboratory pellet diet and water. All procedures involving animals throughout the experiments were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals. After a 7-day acclimation period, these mice were randomly divided into six groups (eight for each) including normal control group, model control group, silymarin group (positive control) and CSPS group. Mice in normal control group and model control group were given physiological saline (25 ml/kg BW) once daily by gastric gavage. Mice in silymarin group were treated with silymarin (100 mg/kg BW) by gastric gavage. Mice in CSPS groups were fed with crude CSPS in three different doses (100, 200 and 400 mg/kg BW/day, respectively) by gastric gavage. On the 15th day of treatment, all groups were given CCl_4 (50% CCl_4 in corn oil, 150 μl/100 g BW) by intraperitoneal injection to induce hepatic injury. Then all the animals were fasted for 16 h and were subsequently used for the following biochemical analysis.

2.4.2. Biochemical assay

After overnight fasting following the last drug administration, the mice were killed by decapitation. Blood samples were collected immediately and centrifuged at 3000 × *g* at 4 °C for 10 min to afford the serums. The liver was excised, weighed and homogenized immediately in 0.1 g/ml wet weight of ice-cold physiological saline. The suspension was centrifuged and the supernatant was collected for further analysis. All above treatments were done at 4 °C.

Protein content, activities SOD and GSH-Px, levels of MDA, ALT and AST were determined by using commercially available kits according to the instructions. In brief, the protein content was measured according to the Bradford method using bovine serum albumin as the standard. Activities of SOD and GSH-Px were determined according to the methods of xanthine oxidase-xanthine reaction system and reduced glutathione (GSH)–H_2O_2 reaction system, respectively. These enzymatic activities were expressed as unit per milligram of protein (U/mg protein). MDA level was measured by the TBARS method and expressed as nmol/milligram of protein (nmol/mg protein). In addition, both ALT and AST were measured using the ketoglutarate reaction and expressed as units per liter (IU/l).

2.5. Statistical analysis

The data were expressed as mean ± standard deviation (SD) and evaluated by one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range tests. *P*-values of less than 0.05 were regarded as significant. All statistical analyses were carried out by using SPSS for Windows, Version 13.0 (SPSS, Chicago, IL).

3. Results and discussion

3.1. Preliminary characterization of CSPS

3.1.1. Molecular weight of CSPS

For determination of homogeneity and molecular weight of CSPS-1, CSPS-2 and CSPS-3, an Agilent 1100 HPLC system was used. As shown in Fig. 1, the HPLC profile of each purified fraction had a single and symmetrical narrow peak, which indicated that they were homogeneous polysaccharides. In addition, the average molecular weights of CSPS-1, CSPS-2 and CSPS-3 were estimated to be 69, 81 and 101 kDa, respectively.

3.1.2. UV and FT-IR spectrometric characterization of CSPS

The UV spectra of CSPS are shown in Fig. 2a. Notably, significant absorption at 260–280 nm was observed in the UV spectrum of CSPS-3, indicating the possible presence of protein. However, no corresponding absorptions were found for CSPS-1 and CSPS-2. The results are in accordance with the analytic result of protein content (Jiang et al., 2011).

An FT-IR spectroscopy is used to investigate the vibrations of molecules and polar bonds between the different atoms. It is possible to analyze the structures of polysaccharides such as monosaccharide types, glucosidic bonds and functional groups using an FT-IR spectroscopy (Yang & Zhang, 2009). Crude CSPS, CSPS-1, CSPS-2 and CSPS-3 were characterized by FT-IR spectroscopy (Fig. 2b). A strong and broad absorption peak at 3400 cm^{-1} for O—H stretching vibrations, a peak at 2930 cm^{-1} for C—H stretching vibrations, and a strong extensive absorption in the region of 900–1200 cm^{-1} for coupled C—O and C—C stretching and C—OH bending vibrations were observed in crude CSPS, CSPS-1, CSPS-2 and CSPS-3, indicating the characteristic absorptions of polysaccharides (Liu et al., 2008). Furthermore, the bands at 1654 cm^{-1} and 1339 cm^{-1} were characteristic signals for the deprotonated carboxylic group (COO^-), indicating CSPS-2 and CSPS-3 being acidic polysaccharide (Gan, Ma, Jiang, Xu, & Zeng, 2011), which is in good accordance with the analytical results for CSPS-2 and CSPS-3 (Jiang et al., 2011). In addition, the absorption peak at 1238 cm^{-1} was assigned to the asymmetric stretching vibrations of SO, an evidence of sulfate ester, confirming directly that CSPS-2 and CSPS-3 were sulfated polysaccharides (Qiao et al., 2009). Finally, there was an absorption peak at about 850 cm^{-1} in the FT-IR spectrum of CSPS-1 or CSPS-2, indicating the presence of α-type glycosidic linkages in CSPS-1 or CSPS-2 (Li et al., 2008). The glycosidic linkage type of CSPS was similar to that of polysaccharide from *Hyriopsis cumingii* (Qiao et al., 2010).

3.2. Antioxidant activities in vitro of CSPS

3.2.1. Reducing ability of CSPS

The antioxidant activity has been reported to have a direct, positive correlation with the reducing ability (Osman, Nasarudin, & Lee, 2004). The reducing ability is generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Song, Zhang, Zhang, & Wang, 2010). The reducing ability assay measures the electron-donating ability of reductones using the potassium ferricyanide reduction method. In the assay, the presence of antioxidants in the tested samples results in reducing Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}), and the yellow color of test solution changes into various shades of green and blue colors depending on the reducing ability of antioxidant samples (Zhang et al., 2010).

Fig. 3a shows the reducing abilities of crude CSPS, CSPS-1, CSPS-2, CSPS-3 and ascorbic acid. The reducing abilities of all samples and ascorbic acid increased with the increase of concentrations. Notably the reducing ability of CSPS-3 was higher than that of crude CSPS, CSPS-1 or CSPS-2. At a concentration of 4.0 mg/ml, the reducing abilities were 41.6, 42.9, 43.4 and 59.6% for crude CSPS, CSPS-1, CSPS-2 and CSPS-3, respectively. However, ascorbic acid showed higher reducing ability than CSPS-3. The results demonstrated that crude CSPS, CSPS-1, CSPS-2 and CSPS-3 possessed moderate reducing ability.

3.2.2. Scavenging activity on H_2O_2 of CSPS

H_2O_2 is produced by 2-electron reduction of molecular oxygen or by dismutation of the superoxide radical (Ozyurek, Bektasoglu, Guclu, Gungor, & Apak, 2010). It induces the oxidative degradation of most biological macromolecules such as lipids, proteins or enzymes, carbohydrates and nucleic acids through generation of hydroxyl radicals (Halliwell & Gutteridge, 1984). Thus, removing H_2O_2 is very important for life being away from damage.

Fig. 3b shows the scavenging activities on H_2O_2 of crude CSPS, CSPS-1, CSPS-2, CSPS-3 and ascorbic acid. The H_2O_2 scavenging effects of crude CSPS, CSPS-1, CSPS-2, CSPS-3 and ascorbic acid were increased with the increase of concentration up to 4.0 mg/ml. The scavenging effects of crude CSPS and CSPS-3 were higher than that of CSPS-1 or CSPS-2. At a concentration of 4.0 mg/ml, the H_2O_2 scavenging activities were 47.8, 33.0, 39.6 and 45.6% for crude CSPS, CSPS-1, CSPS-2 and CSPS-3, respectively. However, ascorbic acid showed higher H_2O_2 scavenging activity than crude CSPS or CSPS-3. The results demonstrated that crude CSPS and CSPS-3 possessed moderate H_2O_2 scavenging effect.

3.2.3. Fe^{2+} chelating activity of CSPS

It has been reported that some transition metals (Fe^{2+}, Cu^+, Pb^{2+}, Co^{2+} and so on) can trigger the production of free radicals and magnify the cellular damage. Among these transition metals, Fe^{2+} is known as the most powerful prooxidant due to its high reactivity. And it can stimulate lipid peroxidation by generating hydroxyl radicals through Fenton reaction (Sun & Kennedy, 2010). It has been

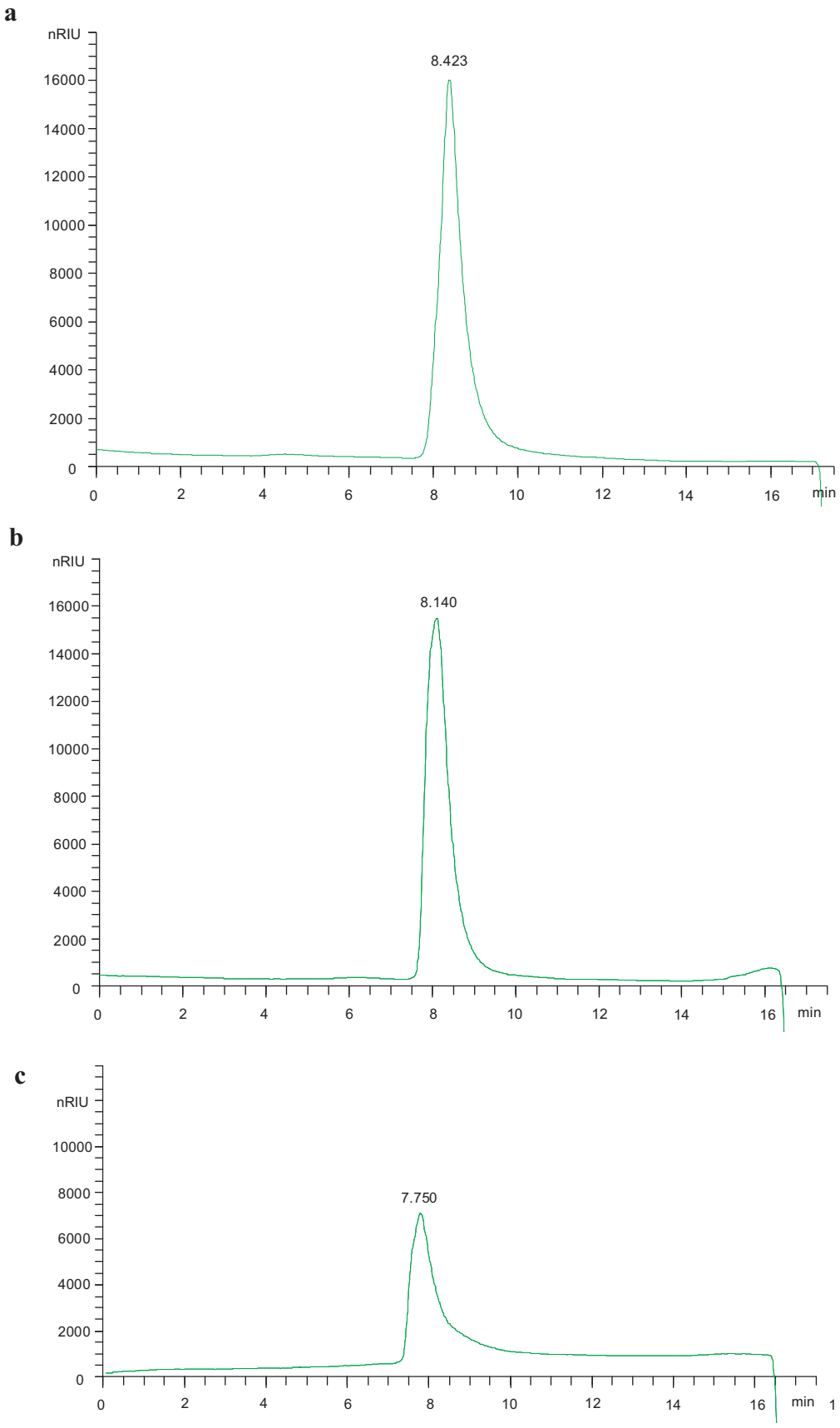

Fig. 1. HPLC profiles of CSPS-1 (a), CSPS-2 (b) and CSPS-3 (c).

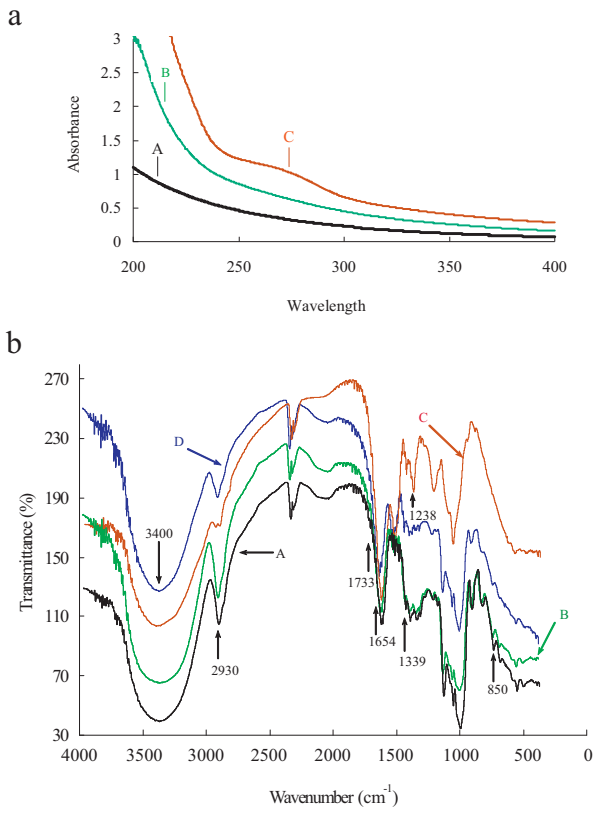

Fig. 2. (a) UV spectra of CSPS-1 (A), CSPS-2 (B) and CSPS-3 (C) and (b) FT-IR spectra of CSPS-1 (A), CSPS-2 (B), CSPS-3 (C) and crude CSPS (D).

recognized that mental ion chelating agents may inhibit lipid oxidation by stabilizing Fe^{2+}. In the Fe^{2+} chelating assay, the chelating activities of the antioxidants are assayed by inhibiting the formation of red-colored ferrozine–Fe^{2+} complex (Liu et al., 2010).

As shown in Fig. 3c, the Fe^{2+} chelating activities of crude CSPS, CSPS-1, CSPS-2 and CSPS-3 were evident at all of the tested concentrations. In addition, their Fe^{2+} chelating activities were correlated well with the increase of concentration up to 4.0 mg/ml. At a concentration of 4.0 mg/ml, the Fe^{2+} chelating activities for crude CSPS, CSPS-1, CSPS-2 and CSPS-3 were 91.5, 57.7, 63.5 and 86.3%, respectively. Apparently, CSPS-3 had better Fe^{2+} chelating effect than CSPS-1 or CSPS-2. It has been reported that compounds with metal ion chelating activities usually contain two or more of the following functional groups: —OH, —SH, —COOH, $—PO_3H_2$, CO, $—NR_2$, —S— and —O— (Yuan, Bone, & Carrington, 2005). The chelating effect of CSPS-3 might be partly due to the strong Fe^{2+} chelating groups in the structure.

3.2.4. Lipid peroxidation inhibition effect of CSPS

Lipid peroxidation is a common consequence of free radical-mediated chain reactions. Its end-products can damage DNA directly or indirectly (Zhu et al., 2004). MDA, one of the products of lipid peroxidation, can react with TBA, yielding a pinkish red chromogen with the maximum absorbance at 532 nm. In the present study, $FeCl_2$–H_2O_2 system was used to induce lipid peroxidation in mouse liver homogenate (Liu et al., 2009).

The lipid peroxidation inhibition effects of crude CSPS, CSPS-1 CSPS-2 and ascorbic acid increased with the increase of sample concentrations (Fig. 3d). At a concentration of 4.0 mg/ml, the inhibitory rates of crude CSPS, CSPS-1, CSPS-2 and CSPS-3 on lipid peroxidation were 59.6, 45.1, 43.4 and 51.0%, respectively. The result showed that CSPS had noticeable effect on lipid peroxidation inhibition at high concentration. Moreover, crude CSPS and CSPS-3 had significantly higher effects on lipid peroxidation inhibition than CSPS-1 and CSPS-2. And the lipid peroxidation inhibition abilities of crude CSPS and CSPS-3 were similar to that of ascorbic acid at 4.0 mg/ml. These results suggested that crude CSPS and CSPS-3 were effective in inhibiting lipid peroxidation.

It has been demonstrated that the molecular weight of polysaccharides influence their antioxidant activities. A relatively low molecular weight of polysaccharide appears to increase the antioxidant activity (Chen, Zhang, Qu, & Xie, 2008). However, CSPS-3 with a relatively higher molecular weight was found to exhibit higher reducing ability, lipid peroxidation inhibition effect, Fe^{2+} chelating activity and H_2O_2 scavenging activity than CSPS-1 or CSPS-2. The inconsistent findings might be due to the presence of protein in CSPS-3 (protein content: 6.34%, Jiang et al., 2011). The existence of protein might affect the physicochemical properties of the polysaccharides and enhance their antioxidant activities.

Table 1
Effects of CSPS on serum ALT and AST in liver injury mice.

Group	ALT (U/l)	AST (U/l)
I (normal control group)	30 ± 9^{e}	61 ± 14^{d}
II (model control group)	241 ± 44^{a}	246 ± 73^{a}
III (positive control group)	129 ± 60^{d}	63 ± 12^{d}
IV (CSPS treatment group, 100 mg/kg)	222 ± 7^{b}	123 ± 13^{b}
V (CSPS treatment group, 200 mg/kg)	188 ± 35^{c}	115 ± 44^{b}
VI (CSPS treatment group, 400 mg/kg)	169 ± 22^{c}	103 ± 51^{c}

Data were presented as mean ± SD ($n = 8$) by one-way ANOVA followed by the Duncan's multiple-range tests, and values not sharing a common superscript letter donate significant difference ($P < 0.05$).

3.3. Protective effect of CSPS against CCl_4-induced hepatotoxicity in mice

CCl_4 is a well-known hepatotoxic chemical. It is accumulated in hepatic parenchyma cells and metabolized to CCl_3 radical by cytochrome P450-dependent monooxygenases in the liver (Recknagel, 1983). The CCl_3 radical reacts rapidly with oxygen to yield trichloromethyl peroxyl radical (Recknagel, Glende, Dolak, & Waller, 1989). The resulting trichloromethyl peroxyl radical is highly reactive and can attack the polyunsaturated fatty acids of the cellular membranes, resulting in the loss of membrane integrity and leakage of microsomal enzymes (Manibusan, Odin, & Eastmond, 2007). Silymarin, an antioxidant flavonoid complex derived from milk thistle (*Silybum marianum*), has long been used in the treatment of liver diseases. It has been proved to have a protective effect against experimental hepatotoxicity by regulating the actions of the ultrastructures of the liver cells and improving the activities of hepatocellular enzymes (Hagymasi, Kocsis, Lugasi, Feher, & Blazovics, 2002). In this study, therefore, mice treated with CCl_4 were used as hepatotoxicity animal model and silymarin was used as positive control medicine.

The serum levels of AST and ALT have been used as biochemical markers for the early acute hepatic damages (Rudnicki et al., 2007). Increased activities of AST and ALT in serum after CCl_4 intoxication compared with those of normal mice indicate hepatocellular damage and leakage of cytosolic contents into the systemic circulation (Kew, 2000). The effects of CSPS on serum AST and ALT levels are shown in Table 1. Apparently, significant increases in ALT and AST levels ($P < 0.05$) were observed in serums of the model control group compared with those of the normal control group, indicating that the hepatotoxicity mice model was well-established in the present study. Moreover, the elevated levels of serum AST and ALT were significantly reduced ($P < 0.05$) in the groups pretreated with CSPS (especially at a dose of 200 or 400 mg/kg BW). The results demonstrated that the administration of CSPS could decrease the levels of AST and ALT in serums of CCl_4-treated mice.

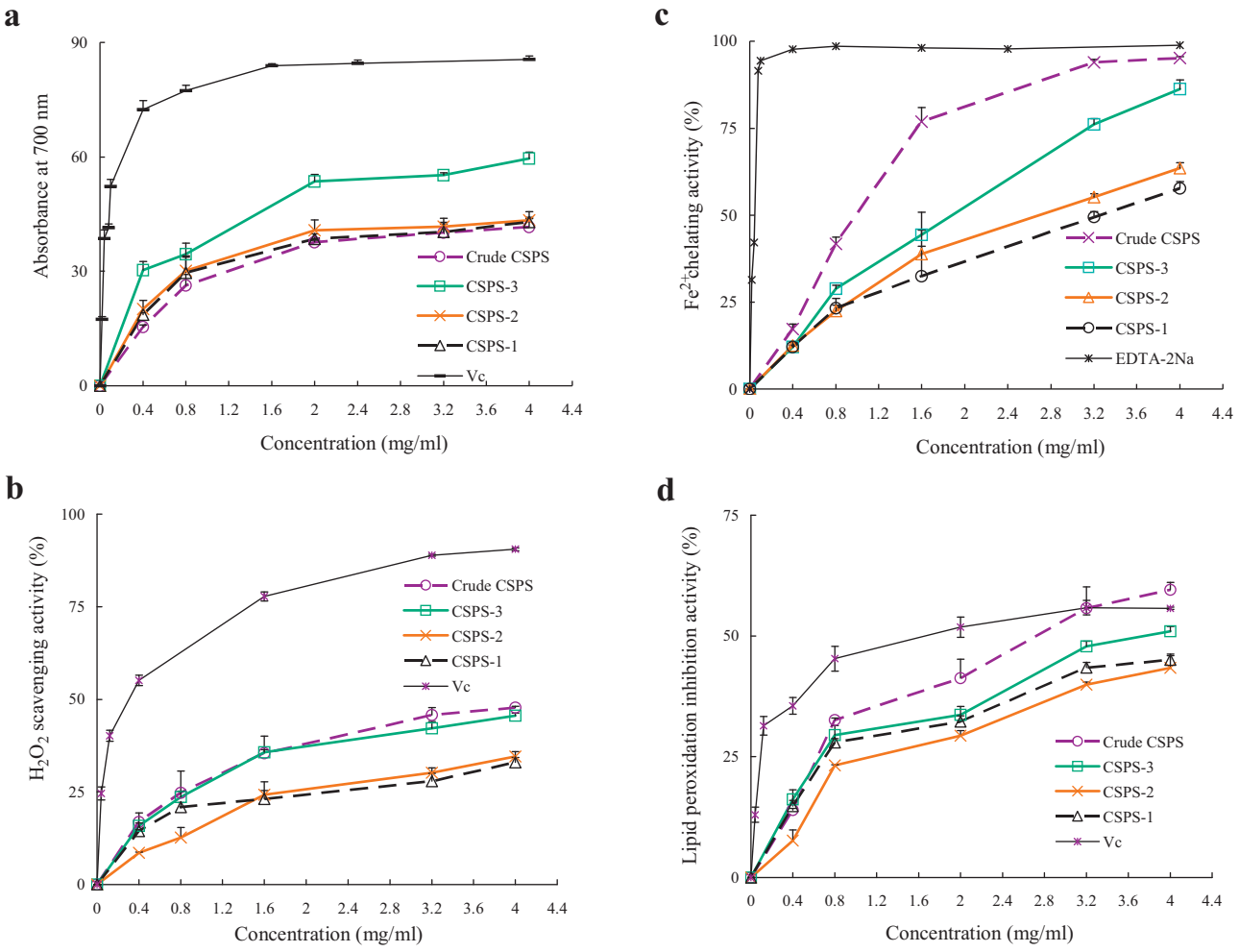

Fig. 3. Reducing ability (a), H_2O_2 scavenging activity (b), Fe^{2+} chelating activity (c) and lipid peroxidation inhibition effect (d) of crude CSPS, CSPS-1, CSPS-2 and CSPS-3.

Besides AST and ALT, the activities of SOD and GSH-Px and levels of MDA in livers of mice were determined. As shown in Table 2, the activities of SOD and GSH-Px in livers of model control group mice were significantly decreased compared with those of the normal control group mice ($P < 0.05$). On the contrary, the level of MDA significantly increased ($P < 0.05$). However, the activities of SOD and GSH-Px were significantly increased ($P < 0.05$) in the groups by pretreatment with CSPS (especially at a dose of 200 or 400 mg/kg BW), while the levels of MDA were markedly decreased ($P < 0.05$) in a dose-dependent manner.

It has been demonstrated that reactive oxygen species plays critical roles in the pathogenesis of various liver diseases (Albano, 2008; Nagata, Suzuki, & Sakaguchi, 2007). These reactive oxygen species generated by hepatocyte and neutrophils during detoxification and metabolic reaction attack most biological macromolecules such as DNA, lipids and proteins (Upur, Amat, Blazekovic, & Talip, 2009). The major antioxidant enzymes, including SOD and GSH-Px are regarded as the primary defense system against the reactive oxygen species (Inal, Kanbak, & Sunal, 2001). For example, SOD reduces superoxide radical (O_2^-) into H_2O_2 plus O_2, thus participating with other antioxidant enzymes in the enzymatic defense against oxygen toxicity. GSH-Px catalyzes the reduction of H_2O_2 to H_2O and O_2, thereby preventing the formation of hydroxyl radicals (Yao et al., 2005). The results in the present study showed that the activities of SOD and GSH-Px were dramatically decreased in mice by the administration of CCl_4. However, pretreatment with CSPS could improve markedly the activities of SOD and GSH-Px in livers of CCl_4-treated mice. The results suggested that the antioxidative system in liver tended to be normalized by the protective action of CSPS.

Table 2
Effects of CSPS on liver SOD, GSH-Px and MDA in liver injury mice.

Group	SOD (U/mg protein)	GSH-Px (U/mg protein)	MDA (nmol/mg protein)
I (normal control group)	797 ± 106^a	1336 ± 252^a	0.40 ± 0.07^d
II (model control group)	319 ± 24^c	376 ± 39^e	1.08 ± 0.16^a
III (positive control group)	746 ± 73^a	1145 ± 226^b	0.60 ± 0.15^c
IV (CSPS treatment group, 100 mg/kg)	330 ± 64^c	560 ± 96^d	1.02 ± 0.26^a
V (CSPS treatment group, 200 mg/kg)	405 ± 72^b	621 ± 87^d	0.88 ± 0.33^b
VI (CSPS treatment group, 400 mg/kg)	454 ± 75^b	782 ± 107^c	0.64 ± 0.28^c

Data were presented as mean ± SD ($n = 8$) by one-way ANOVA followed by the Duncan's multiple-range tests, and values not sharing a common superscript letter donate significant difference ($P < 0.05$).

MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acid (Vaca, Wilhelm, & Harms-Ringdahl, 1988). It is an indicator of lipid peroxidation. And lower MDA level suggests that there is less lipid peroxidation and weaker oxidant stress (Bagchi, Bagchi, Hassoun, & Stohs, 1995). In the present study, CCl_4-induced toxicity caused an increase in the liver tissue MDA level as compared to the normal control group. Pretreatment with CSPS significantly reversed these changes. The administration of CSPS caused a significant decrease in MDA level as compared to the model control group. The results suggested that polysaccharides from *C. sinensis* had a significant protective effect against CCl_4-induced acute hepatotoxicity in mice. The hepatoprotective effect of CSPS might be partly due to the enhancement of endogenous antioxidant enzymatic activities (SOD and GSH-Px) and decrease of lipid peroxidation product (MDA) level.

4. Conclusions

In the present study, the preliminary characterization, antioxidant *in vitro* and hepatoprotective activity *in vivo* of polysaccharides from *C. sinensis* were investigated. Preliminary structural analysis indicated the presence of α-type glycosidic linkages in CSPS-1 or CSPS-2, and the average molecular weights for CSPS-1, CSPS-2 and CSPS-3 were 69, 81 and 101 kDa, respectively. The assay of the antioxidant activity *in vitro* demonstrated that crude CSPS, CSPS-1, CSPS-2 and CSPS-3 showed moderate H_2O_2 scavenging activity, lipid peroxidation inhibition effect and strong Fe^{2+} chelating activity. Furthermore, the assay of hepatoprotective activity *in vivo* demonstrated that the administration of CSPS significantly decreased serum ALT and AST levels, inhibited MDA formation and enhanced the activities of liver SOD and GSH-Px in CCl_4-induced liver injury mice. These results suggested that CSPS had potent antioxidant and hepatoprotective activities.

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