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Carboxymethylation of a polysaccharide extracted from *Ganoderma lucidum* enhances its antioxidant activities *in vitro*

Jing Xu [1], Wei Liu [1], Wenbing Yao *, Xiubing Pang, Dengke Yin, Xiangdong Gao *

School of Life Science and Technology, China Pharmaceutical University, Nanjing 210009, PR China

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ABSTRACT

The chemical carboxylmethylated polysaccharide (C-GLP), which derived from water-insoluble crude *Ganoderma lucidum* polysaccharide (GLP), was prepared. Water solubility, chemical characterization, and antioxidant activities *in vitro* of C-GLP were determined. The solubility of C-GLP in distilled water reached 100 mg/ml, which was much higher than the solubility of GLP. Chemical analysis indicated that C-GLP was composed of Glc:Man:Gal = 33.0:1.0:3.4 with a molecular weight of 1.8×10^6 Da and a carboxymethyl content of 11.07%. The signals of carboxymethyl were found in IR and ^{13}C NMR spectra. Moreover, a high antioxidant activity of C-GLP was observed, especially in scavenging of hydroxyl radical (83.7% at 5 mg/ml) and hydrogen peroxide (51.6% at 10 mg/ml). This study indicates the effects of carboxymethylation on water-insoluble polysaccharide and explores a potential antioxidant in food industry and pharmaceuticals.

1. Introduction

In forms of superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2), reactive oxygen species (ROS) is generated by the normal metabolic processes or from exogenous factors and agents. ROS over-production is associated with many diseases, such as asthma, rheumatoid arthritis, cardiovascular diseases and cancer. Accumulation of excessive ROS results in oxidative damage to DNA, proteins and other macromolecules (Lee & Lee, 2006). However, most synthesized antioxidants are suspected of being responsible for liver damage and carcinogenesis (Qi et al., 2005). Thus, it is essential to explore new natural antioxidants to protect human from free radicals and abate the progression of many chronic diseases. Polysaccharides, which widely distributed in animals, plants and microorganisms, have been demonstrated to play an important role as free radical scavengers in the prevention of oxidative damage in living organism (Tsiapali et al., 2001). Bioactivity of polysaccharide primarily depends on its water solubility. Previous publications indicated that most polysaccharides with various biological activities were water-soluble while the water-insoluble fractions were usually regarded as industrial wastes.

Chemical modifications can improve water solubility of those poorly water-soluble fractions and increase their bioactivities (Nie, Shi, Ding, & Tao, 2006). Hence, modification of water-insoluble polysaccharide is favorable and necessary. It has been widely accepted that carboxymethylation could enhance the water solubility of polysaccharides. Different sources of various polysaccharides such as cellulose, chitin, scleroglucan, and schizophyllan can be applied as starting materials for carboxymethylation (Thomas & Andreas, 2005). Carboxylmethylated β-glucan (CMPTR), isolated from the sclerotia of *Pleurotus tuberregium*, has been found to have strong anti-proliferation activity (Zhang, Cheung, Chiu, Wong, & Ooi, 2006). Carboxymethylated *Achyrnthes bidentata* polysaccharide not only inhibits the tumor cell growth, but also increases NK cell activities (Shi, Zhou, Zhang, & Tian, 2006).

Ganoderma lucidum (Fr.) Karst, an edible herbal medicine, has been used for more than 2000 years in China and other oriental countries; it is also a popular dietary supplement in Western countries now. Since polysaccharides are the main bioactive components in *G. lucidum,* researches direct their focus on *G. lucidum* polysaccharides (Hao & Li, 2004). In our earlier study, we obtained a *G. lucidum* polysaccharide preparation (GLPP) with potential antitumor activity. During the preparation procedure, another crude *G. lucidum* polysaccharide (GLP), with a high molecular weight, was isolated (Pang et al., 2007). This crude polysaccharide GLP, which had poor water solubility showed a dark to brownish color and was difficult to be purified; it was thrown away as a waste product.

For further research and full utilization of resources and waste products, we optimized the carboxylmethylation procedure of GLP by orthogonal array design, and analyzed the physicochemical properties and structural characteristics of carboxylmethylated

* Corresponding authors. Tel./fax: +86 25 83271218 (W. Yao); tel.: +86 25 83271298; fax: +86 25 83271249 (X. Gao).
E-mail addresses: wbyao@cpu.edu.cn (W. Yao), xiangdong_gao@yahoo.com.cn (X. Gao).
[1] These authors contributed equally to this work.

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GLP (C-GLP). We also compared the *in vitro* antioxidant activities between GLP and C-GLP, including superoxide anion scavenging, hydroxyl radical scavenging, and hydrogen peroxide scavenging activities, metal chelating ability, and reducing power. The purpose of the current investigation was to evaluate the carboxymethylation and explore applications of water-insoluble polysaccharides.

2. Materials and methods

2.1. Materials

The fruiting bodies of *G. lucidum* were obtained from the Jingde Huangshan Lingzhi Industry Co. Ltd. Anhui, China and confirmed for identity and deposited by the Inst. of Microbiology, Chinese Academy of Sciences. All the chemicals and reagents were of analytical grade. Monochloroacetic acid (MCA), sodium hydroxide, 2-propanol, and hydrochloric acid were purchased from Nanjing chemical reagent Co. Ltd. 30% hydrogen peroxide was purchased from Sinopharm chemical reagent Co. Ltd. Sodium salicylate, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), ascorbic acid, chloride ferric, trichloracetic acid and deuterated dimethyysulfoxide (DMSO-d_6) were purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Preparation of GLP and its derivative C-GLP

2.2.1. Preparation of GLP

The crude polysaccharide was obtained during the procedure of a bioactive component named GLPP in our earlier study (Pang et al., 2007). The air-dried fruiting bodies of *G. lucidum* (1 kg) were ground and extracted with 20 L of hot water for 14 h. The solid residues were extracted with hot water for another two times. All filtrates were combined and concentrated to a final volume of 10 L. The fraction named GLP (22.48 g) was obtained through precipitation with 15 L of ethanol and vacuum dehydration. GLP was subjected to carboxymethylation for further research.

2.2.2. Preparation of carboxylmethylated derivative C-GLP

Orthogonal array design was used for optimizing experimental parameters of carboxymethylation of GLP. A four-factor at three level orthogonal array experimental design $L_9(3^4)$ was adopted. Four controllable variables, including temperature (A), reaction time (B), volume of 20% NaOH solution (C), and volume of 4 mol/L MCA (D), were selected for optimization. The selected factors and levels are listed in Table 1.The process of carboxymethylation was achieved by a suspension of 500 mg GLP in 20 ml 2-propanol being vigorously stirred for 15 min at room temperature. Then 20% aqueous NaOH solution was added by dropwise. After stirred at room temperature for 1 h, MCA (4 mol/L) was added. The flask was immersed in a thermostatic oil bath to keep at a specific temperature for the desired duration time. The reaction products were cooled to room temperature in an ice bath, neutralized with HCl and dialyzed against distilled water for 72 h. The dialyzates were concentrated and precipitated with 95% ethanol then washed sequentially with ethanol, acetone and aether. An optimal condition was obtained by comparing yield and water solubility of the products using the orthogonal test and mathematical analysis.

Table 1
Assignment of the levels to factors.

Level	Temperature (°C) (A)	Reaction time (h) (B)	4 mol/L MCA (ml) (C)	20% NaOH (ml) (D)
1	20	2	1.5	3.0
2	40	3	2.0	5.0
3	60	4	2.5	7.0

Solubility test was conducted according to Pharmacopoeia of China (2005 edition). Certain amount of water was added into 100 mg sample at room temperature. The formed solution was shaken vigorously every 5 min and was set still for 30 min till the sample was completely dissolved. Ion-exchange chromatography was used for the purification of the derivative prepared under optimal condition. A portion of the derivative was then loaded onto a column (2.6 × 30 cm) of DEAE-Cellulose-32, followed by eluting with water and a gradient of 0–2.0 mol/L NaCl solution. The main fraction from the eluted NaCl solution which was determined by phenol–sulfuric acid method was collected, dialyzed, and lyophilized to give a carboxymethyl polysaccharide coded as C-GLP.

2.3. Characterization of C-GLP

2.3.1. Purity and molecular weight determination

The purity and molecular weight of C-GLP was determined using a size-exclusion HPLC chromatography instrument (Agilent 1100, USA). A 10 mg sample was dissolved in 1ml distilled water and passed through a 0.45 μm filter, applied to a gel-filtration chromatographic column of Shodex KS-805 (SHOWA DENKO K.K, Japan) at 35 °C, eluted with the distilled water at a flow rate of 1.0 ml/min and detected by a refractive index detector. Standard dextrans T-2000, T-500, T-70, T-40, T-10 and glucose were passed through the column. Then a standard curve was plotted according to the retention time and the logarithm of their respective molecular weights. The molecular weight of C-GLP was determined in comparison to the standard curve.

2.3.2. Determination of carboxymethyl content

Carboxymethyl content was determined by the method of neutralization titration with a few modifications (Regina, Heatley, & Budd, 1998). Briefly, C-GLP solution interacted with Amberlite IR-120H$^+$ to change the salt form into acid. The acid C-GLP solution was then titrated with 0.02 M HCl after addition of known amount of NaOH.

2.3.3. Monosaccharide composition analysis

Monosaccharide composition was analyzed according to the following procedure (Susumu, Shigeo, Kazuaki, Akiko, & Tsuneo, 1981): in order to turn into monosaccharide components, polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 8 h. Its corresponding alditol acetates were analyzed by gas chromatography (GC) on a Hewlett-Packard model 6890 instrument equipped with a capillary column of HP-5.5% phenyl methyl siloxane (30 × 0.25 × 0.25 μm) and a flame-ionization detector, and programmed from 150–220 °C to 280 °C at 30 °C/min.

2.3.4. Infrared spectral analysis

The IR spectra were recorded with KBr pellets on a Nicolet-170X spectrophotometer between 400 and 4000 cm^{-1}.

2.3.5. ^{13}C NMR

^{13}C NMR spectra were recorded at 500 MHz using a Brucker DRX-400 NMR Spectrometer. GLP was dissolved in DMSO-d_6 and C-GLP was dissolved in D_2O and examined at 30 °C, respectively.

2.4. Antioxidant activity

2.4.1. Scavenging activity of hydroxyl radical

The hydroxyl radical scavenging activity was analyzed as described previously (Smirnoff & Cumbes, 1989). The reaction mix-

ture (3 ml) contained 1 ml of $FeSO_4$ (1.5 mM), 0.7 ml of H_2O_2 (6 mM), 0.3 ml of sodium salicylate (20 mM) and samples (0–10 mg/ml). After incubation for 1 h at 37 °C, the hydroxyl radical was detected by monitoring absorbance at 562 nm. For the control, sample was substituted with antiscorbic acid. The percentage of scavenging effect was calculated as

$$\text{Scavenging rate} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample, A_2 was the absorbance without sodium salicylate.

2.4.2. Scavenging activity of superoxide anion radical

Superoxide radical was generated in the PMS-NADH system which contained 10 M PMS, 100 μM NADH and 600 μM NBT in 0.1 M PBS at pH 7.8 as described previously (Robak & Gryglewski, 1988). The prepared mixture was incubated at room temperature for 5 min and the absorbance was measured at 560 nm against the blank. Samples with different concentrations (0–10 mg/ml) were added to the test tubes before adding PMS. For the control, sample was substituted with Tris–HCl buffer. The capability of scavenging to superoxide radical was calculated as

$$\text{Scavenging rate} = [1 - A_1/A_0] \times 100\%$$

where A_0 was the absorbance of the control (without sample) and A_1 was the absorbance in the presence of the sample.

2.4.3. Scavenging activity of hydrogen peroxide

The scavenging capacity of GLP and C-GLP on hydrogen peroxide was measured according to a literature procedure (Zhao, Xiang, Ye, Yuan, & Guo, 2006) with a minor modification. H_2O_2 (1.0 ml, 0.1 mM) and 1.0 ml of various concentrations of samples were mixed, followed by 100 μl 3% ammonium molybdate, 10 ml H_2SO_4 (2 M) and 7.0 ml KI (1.8 M). The mixed solution was titrated by $Na_2S_2O_3$ (5 mM) until the yellow color disappeared. The scavenging effect was calculated as

$$\text{Scavenging rate} = [(V_0 - V_1)/V_0] \times 100\%$$

where V_0 was the volume of $Na_2S_2O_3$ solution used to titrate the control in the presence of hydrogen peroxide (without sample), V_1 was the volume of $Na_2S_2O_3$ solution used in the presence of the sample.

2.4.4. Reducing power assay

The reducing power was quantified by the method described earlier (Barry, 1987) with a few modifications. Briefly, 2.5 ml of reaction mixture, containing sample (0–10 mg/ml) in phosphate buffer (0.2 M, pH 6.6), was incubated with 2.5 ml potassium ferricyanide (1%, w/v) at 50 °C for 20 min. The reaction was terminated by 2.5 ml TCA solution (10%, w/v). Then 5 ml distilled water and 1 ml ferric chloride (0.1%, w/v) were added to the reaction mixture. The absorbance was measured at 700 nm. Ascorbic acid was used as the control. Increased absorbance of the reaction mixture indicates increased reducing power of the sample.

2.4.5. Chelating effect on ferrous ions

The method used to determine the chelating effect of GLP and C-GLP was based on the previous literature (Decker & Welch, 1990). Samples in different concentrations (0–10 mg/ml) were mixed with 50 μl ferrous chloride (0.05 ml, 2 mM) and 0.2 ml ferrozine (5 mM), shaken well and stayed still for 10 min at room temperature, and then the absorbance of the mixture was determined at 562 nm. In the control, sample was substituted with EDTA. The ion-chelating activity was calculated as

$$\text{Chelating rate} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control (without sample) and A_1 was the absorbance in the presence of the sample, A_2 was the absorbance without ferrozine.

2.5. Statistical analysis

Each assay of all experiments was performed in triplicate. Data were presented as mean ± standard deviation of mean (SDM) within significance $p < 0.05$ after passing Duncan's multiple-range test, and processed with Excel and Statistica (2003).

3. Results and discussion

3.1. Preparation of GLP and C-GLP

Crude polysaccharide GLP with poor water solubility isolated from *G. lucidum* fruiting bodies formed a dark to brownish in color. One hundred milligrams of GLP could barely dissolve in 1 L distilled water. The poor solubility of GLP severely limits its application, purification and further research.

The procedure of carboxymethylation reaction included two steps. Firstly, sodium hydroxide reacted with the hydroxyl groups of the GLP to produce alkoxides groups. Secondly, the carboxymethyl groups formed between the GLP alkoxide and MCA through a SN_2 reaction (Puja, Vineet, & Pradeep, 2007). Orthogonal array design is used for optimizing experimental parameters of carboxymethylation. In the orthogonal experiment, nine tests were performed. The analytical results including calculated *K* and *R* values were listed in Table 2. The influential order of four factors on yield of carboxymethylation was D > C > B > A by comparing *R* values (Table 2). The result indicated that the amount of NaOH solution (D factor) exerted most potent impact on the yield of carboxymethylation, which is supported by previous studies (Durcilene et al., 2004). Moreover, water solubility of the derivatives was greatly improved except test number1 with a solubility of 34 mg/ml. According to the K value, the optimal combination of experimental parameters was $A_3B_3C_2D_3$, which meant carboxymethylation of GLP were performed at 60 °C (temperature) for 4 h (reaction time) and with 2.0 ml (volume of 4 mol/L MCA) and 7.0 ml (volume of 20% NaOH). The results demonstrated that longer reaction time ensured that the swelling of GLP as well as the diffusion and adsorption of the reactants were adequate. To avoid a side reaction, such as formation of glycolate or degradation, the reaction temperature was strictly controlled less than 70 °C. After carboxymethylation, a purification process was preceded by loading the sample onto the DEAE-Cellulose-32 column. The water elution fraction was the neutral polysaccharides without carboxymethyl. By eluting with a NaCl gradient, the polysaccharides showed as a single peak, indicating that the purified carboxylmethylated polysaccharide C-GLP was close to homogeneity.

3.2. Characteristics of C-GLP

A single and symmetrical peak on HPLC suggested a high purity of carboxylmethylated polysaccharide C-GLP (Fig. 1). The average molecular mass of C-GLP was determined to be 1.8×10^6 Da and the carboxymethyl content of C-GLP was 11.07%. Quantitative determination of monosaccharide composition pattern by GC analysis suggested that both GLP and C-GLP were mainly composed of glucose with minor amounts of mannose and galactose. The monosaccharide composition ratio of C-GLP and GLP was Glc:Man:Gal = 33.0:1.0:3.4 and Glc:Man:Gal = 37.1:1.0:6.6, respectively. There might be several reasons resulted in the variance of proportion: (1) GLP itself was a crude polysaccharide while C-GLP was a homogeneous one; (2) some degradation occurred in company

Table 2
Experimental arrangement and test result.

Test number	Temperature (°C)	Reaction time (h)	4 mol/L MCA (ml)	20% NaOH (ml)	Yield (%)	Solubility (mg/ml)
1	20	2	1.5	3.0	38.98	34
2	20	3	2.0	5.0	76.12	67
3	20	4	2.5	7.0	100.46	87
4	40	2	2.0	7.0	93.82	105
5	40	3	2.5	3.0	50.88	102
6	40	4	1.5	5.0	76.06	61
7	60	2	2.5	5.0	87.88	110
8	60	3	1.5	7.0	72.34	106
9	60	4	2.0	3.0	74.70	104
K1	71.85	73.56	62.46	54.85		
K2	73.59	66.45	81.55	80.02		
K3	78.31	83.74	79.74	88.87		
R	6.46	17.29	19.09	34.02		

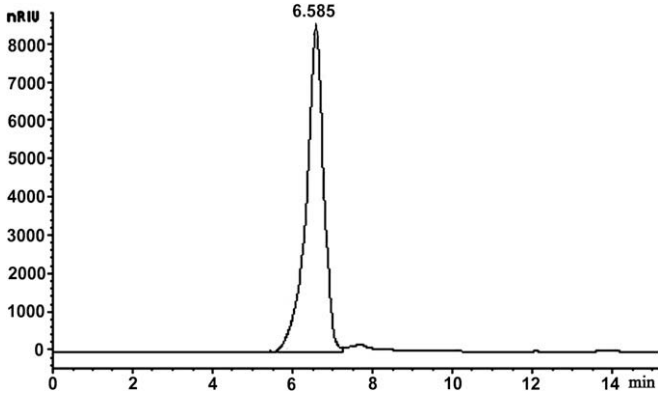

Fig. 1. Profile of C-GLP in HPGPC, eluted with H_2O at 1.0 ml/min.

with the process of derivatization reaction (Ren, Sun, & Peng, 2008); (3) the introduce of carboxymethyl, Sun et al. (2009) had also reported an interesting fact that the carbohydrate content decrease with the increase in the degree of substitution. But the derivant still retained the main component of the original polysaccharide.

The IR spectra of GLP and C-GLP in 4000–500 cm^{-1} were similar (Fig. 2). Carboxyl of C-GLP was confirmed by two new strong absorption bands in the 1601 region [$\gamma_{sym}(COO^-)$] and 1421 cm^{-1} [$\gamma_{as}(COO^-)$]. The weak absorption band at 1720 cm^{-1} which belonged to carboxylic acid groups indicated that the C-GLP was predominantly in salt form. The strong absorption band at about 3400 cm^{-1} in both spectra was assigned to OH stretching vibrations and the peak at 2920 cm^{-1} was assigned to CH stretching of the CH_2 groups. The band due to ring stretching of glucose appeared at 1641 cm^{-1} (Regina et al., 1998). And the bands in the region 1350–1450 cm^{-1} were corresponded to symmetrical deformations of CH_2 and COH groups. The bands attributed to primary alcoholic -CH_2OH stretching mode and C–O–C stretching vibrations appeared at about 1075 cm^{-1}.

^{13}C NMR spectra of GLP and C-GLP provided more detailed structure information. In the spectrum of GLP (Fig. 3A), signal at around δ 39 ppm was assigned to the solvent of DMSO-d_6 (Ana et al., 2008). According to a comparison with β-D-glucan (Debabrata et al., 2008; Storseth, Hansen, & Skjerrrno, 2004), the peaks at around 103.1 (C-1), 72.9 (C-2), 86.2 (C-3), 68.5 (C-4), 76.8 (C-5), 61.0 (C-6) ppm might attribute to the signals of the backbone chain. The signal at 87.1 ppm was attributed to substitution at O-3, while that at 85.9 ppm arose from 3,6-di-O-substitutions units. The shift of C-6 from 61.0 to 69.5 ppm indicated that there might be existence of (1→6)-linked glycosidic bond which was in agreement with Ana et al. (2008) and Chakraborty, Mondal, Pramanik, Rout, and Islam (2004). Thus, GLP probably had a backbone chain mainly composed of β-(1→3), β-(1→6)-linked glycosidic bond. After carboxymethylation, better solubility was achieved due to the introduced hydrophilic carboxymethyl groups, while legible signals of aqueous C-GLP solution in the

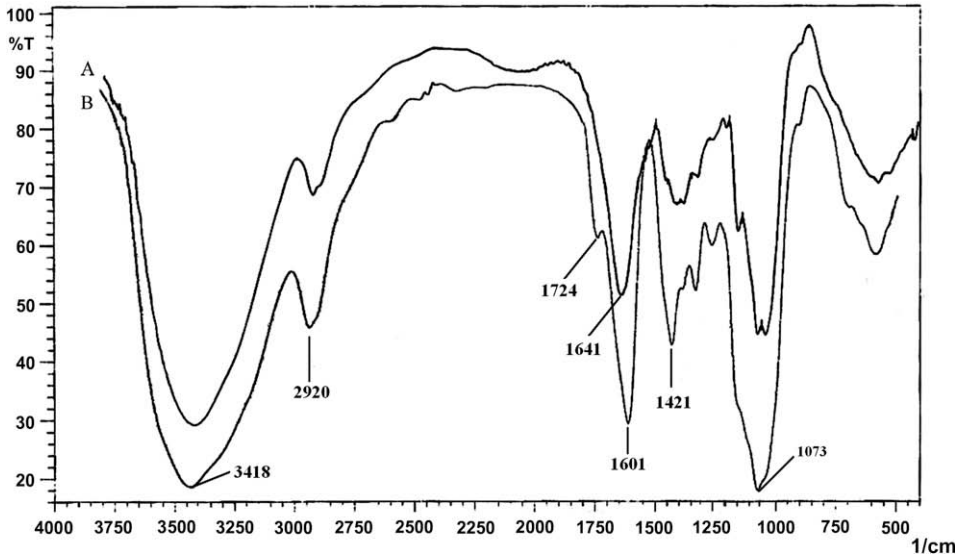

Fig. 2. IR spectra of (A) GLP, (B) C-GLP.

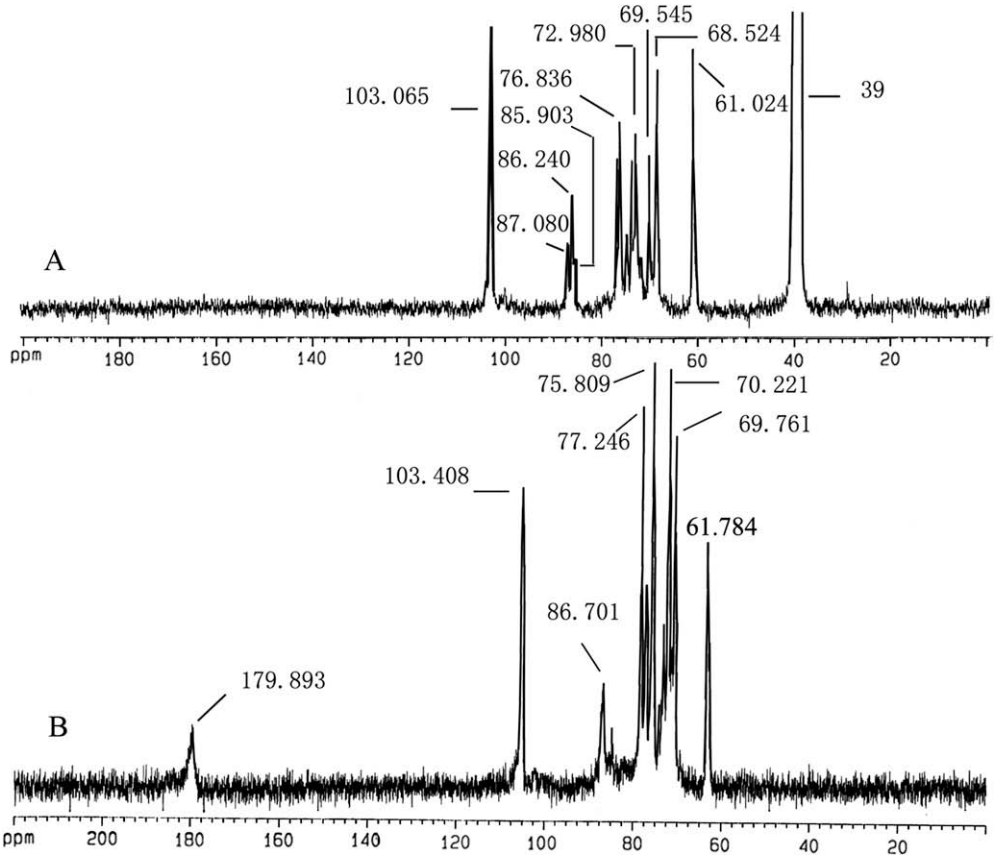

Fig. 3. ^{13}C NMR spectra of (A) GLP, (B) C-GLP.

NMR spectra shown in Fig. 3B also confirmed this result. The signals of the backbone chain of GLP mentioned above still existed, which indicated that the main structure of GLP in C-GLP was reserved. A new peak at about 179.9 ppm belonged to the carbonyl group, which also could be seen as an evidence of carboxymethyl substitution in agreement with IR results. And the signals at 71.4 and 73.2 ppm were assigned to the methylene carbon atoms of the carboxymethyl substituents (Ren et al., 2008). The increased signal at 70.2 ppm and the decreased signal at 61.8 ppm revealed the substitution occurred at C-6 position (Durcilene et al., 2004). Moreover, the enhancement in peak intensities between 85 and 70 ppm was possibly due to the substitution of -CH_2COOH at C-2 or C-4 position. Based on the above information, we proposed that the substitutions occurred at C-6, C-4 or C-2. NMR spectroscopy is a nondestructive technique for giving valuable structural information to carboxymethylated polysaccharides. Through NMR spectra, we confirmed the successful replacement of carboxymethyl and gained some useful information about the location of substitution. The subtle structural analysis on the sample is underway and will be reported in the following study.

3.3. Enhanced antioxidant activity of C-GLP

3.3.1. Scavenging activity of hydroxyl radical

Fig. 4A showed that the inhibitory effects of C-GLP and GLP (0.15–10 mg/mL) on hydroxyl radicals were evident and dose-dependent. At 5 mg/ml, C-GLP exhibited strong ability to quench hydroxyl radical (83.7%), which was similar to antiscorbic acid, whereas the maximal scavenging activity of GLP was only 42.9% at 10 mg/ml. Previous studies reported two types of antioxidant mechanism (Qi et al., 2005): the suppression against hydroxyl radical generation, and the clearance of the generated hydroxyl radical. In the former, the antioxidant activity was related to the transition of metal ions. Without the transition of metal ions, hydrogen peroxide was fairly stable. However, hydroxyl radicals acted in superoxidation with metal ions, usually ferrous or copper. Weak ion chelating effect by C-GLP as described in Fig. 4E explained that either the combination of metal ions might not be the main reason for the inhibition of oxidation or both of the two mechanisms might be responsible for the inhibition of oxidation. Therefore, the mechanism of C-GLP on cleaning hydroxyl radicals needs to be further investigated.

3.3.2. Scavenging activity of superoxide anion radical

Although superoxide is a relatively weak oxidant, it could trigger lipid peroxidation and induce pathological incidents such as arthritis and Alzheimer's disease (Wang, Gao, Zhou, Cai, & Yao, 2008). Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals and initiates lipid peroxidation indirectly. Apart from that, superoxide anion can magnify the cellular damage because it produces other kinds of free-radicals and oxidizing agents (Athukorala, Kim, & Jeon, 2006). In the present study, as shown in Fig. 4B, comparing with the scavenging ability of original GLP, the scavenging activity of C-GLP against superoxide anion moderately improved. C-GLP showed good superoxide anion-scavenging activities (56%) at the concentration of 10 mg/ml, while GLP was only 3% at the same concentration. Polysaccharides with a scavenging effect on the superoxide anion radical had the same structural feature in that all of them had one or more alcohol or phenolic hydroxyl groups. Scavenging ability of polysaccharides was related to the number of active hydroxyl groups in the molecule (Guo et al., 2005). Tsiapali et al. (2001) found that greater antioxidant ability of phosphated and sulphated glucan may be attributed to polyelectrolytes. In other words, the addition of electron-donating substituents probably increased radical scavenging

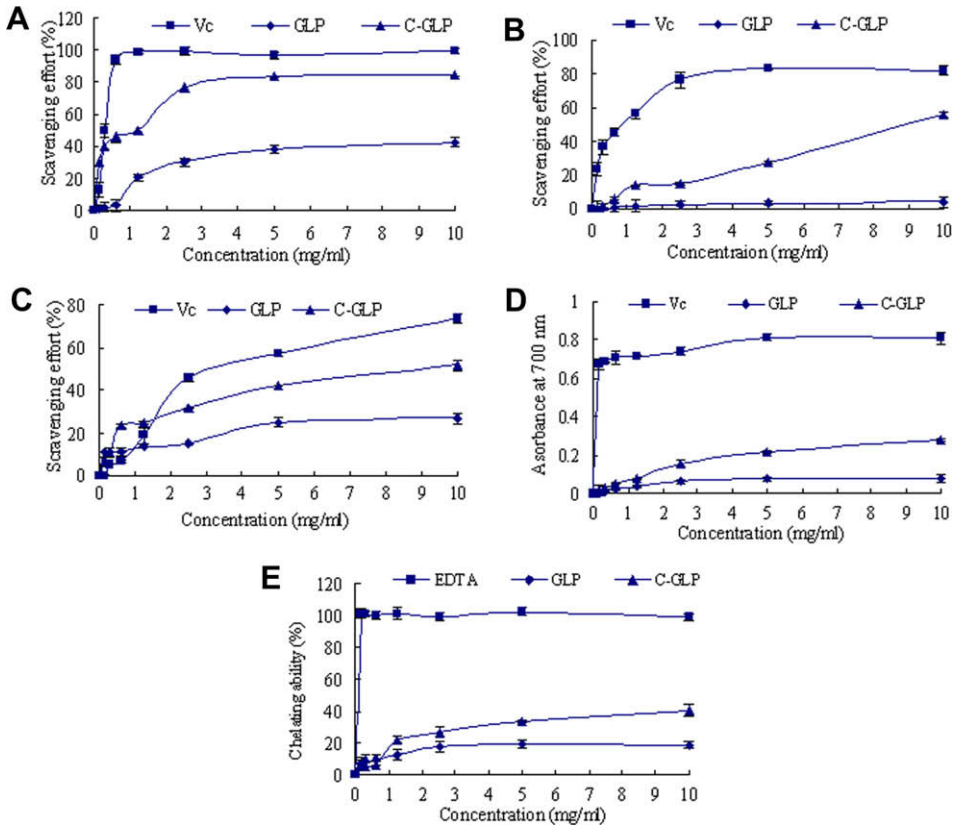

Fig. 4. Antioxidant activity analysis of GLP and C-GLP with various methods: (A) the hydroxyl radical scavenging activities of GLP and C-GLP; (B) the superoxide anion radical scavenging activities of GLP and C-GLP; (C) the hydrogen peroxide scavenging activities of GLP and C-GLP; (D) reducing power of GLP and C-GLP; (E) chelating abilities of GLP and C-GLP to ferrous ions; data are presented as mean values ($n = 3$).

activity as a result of increasing electron density on the heterocyclic ring of the carbons. Low activity of GLP may be associated with the formation of strong intermolecular and intramolecular hydrogen bonds, leading to inhibition of the reactivity of hydroxyl in the polymer chains. After modification, carboxylic groups were introduced into the polysaccharide's chains, which rendered C-GLP with strong electron-withstanding ability and enhanced the superoxide scavenging activity.

3.3.3. Scavenging activity of hydrogen peroxide

Hydrogen peroxide played a radical forming role as an intermediate in the production of more ROS molecules, such as hydroxyl radical. High penetrability of cellular membrane leads to hydroxyl radical formation when hydrogen peroxide reacts with ferrous ion or with the superoxide anion radical in cells (Wang H. et al., 2008). Hydrogen peroxide is one of main inducers of cellular aging and would attack many cellular energy-producing systems. Fig. 4C showed that the hydrogen peroxide scavenging activities of all the samples were relied on their concentration. The scavenging ability of C-GLP was weaker than ascorbic acid but was much stronger than GLP. At 10 mg/ml, scavenging activity of C-GLP was around 52%, while GLP was only 24% at the same concentration.

3.3.4. Reducing power

Analysis of reducing power measures the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. The reducing capacity of a compound would possibly serve as a significant indicator of its potential antioxidant activity. In this study, the reducing power of ascorbic acid was 0.73, which was the same as previous study (Xing et al., 2005). The reducing power of C-GLP was greatly improved in comparison with GLP. As shown in Fig. 4D, the reducing power of C-GLP was 0.28 at 10 mg/ml while original GLP was 0.082 at the same concentration. Interestingly, it was reported that a sulfated polysaccharide fractions extracted from *Laminaria japonica* had the similar reducing power as C-GLP (Wang, Zhang, Zhang, & Li, 2008). The activities of antioxidants have been attributed to various mechanisms, such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Zou et al., 2008). And the reduction was generally associated with reductones, a strong antioxidant, which can break free-radical chain by donating a hydrogen atom. The result obtained in our study could possibly relate to the introduction of carboxymethyl group which enhanced the electron cloud density of active hydroxyl groups. Thus the electron-donating activity increased and the reducing power improved. But the result of the reducing power of C-GLP was not strongly improved, possibly due to the steric conformation of C-GLP prevented some active carboxymethyl binding to the metal ion.

3.3.5. Chelating effect on ferrous ions

Iron can stimulate lipid peroxidation by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + {}^{-}OH$), and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals which can abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The main mechanism of ion-chelating activity is the ability to deactivate and/or chelate transition metals which can promote the Fenton reaction and hydroperoxide decomposition. The ferrous ion-chelating effect of C-GLP was concentration-dependent as shown in Fig. 4E. Chelating effect of C-GLP was low, especially compared to EDTA. Even at the maximal con-

centration, the chelating rate of C-GLP could not reach 50% while EDTA at the minimal concentration showed nearly 100% chelating rate. Although it was not significant, C-GLP still improved the ability of binding to ions. Previous studies showed that the chelating to ferrous was dependent on the numbers of hydroxyl, and the hydroxyl substitution in the *ortho* position was desirable (Wang H. et al., 2008). The low chelating effect of the derivative was probably due to the structure of GLP, which was not suitable for combining the metal ion. The same result was also found in the experiment of determining reducing power elsewhere. Further investigations had to be carried out to explain the relationship between structure and activity.

3.4. Correlation of structure to antioxidant activity

The bioactivity results *in vitro* showed that carboxymethyl modification of water-insoluble crude polysaccharide GLP from *G. lucidum* fruiting bodies led to antioxidant activity. The derivative with good antioxidant activity probably related to the introduction of substituting groups which changed the structure of GLP and decreased the intermolecular/intramolecular hydrogen bond. The nature of the substituting group had an important effect on the activities of derivative. Introduction of carboxymethyl groups significantly increased the original polysaccharide's water solubility so that the antioxidant activity was increased. It was reported that chemical modifications of water-insoluble β-D-glucan from *Poriacocos sclerotium* not only increased the water solubility, but also increased its bioactivity of antitumor (Wang, Zhang, Li, Hou, & Zeng, 2004). Therefore, water solubility is an important factor to facilitate polysaccharides to exhibit their bioactivity. The same conclusion was also obtained from the studies of a sulfated polysaccharide from *Pleurotus tuberregium* (Tao, Zhang, & Cheung, 2006).

High solubility is not the only reason to explain various activities of polysaccharides. Structural features including molecular weight, monosaccharide components and chain conformation are also influencing factors to the activities of polysaccharides. Molecular modification is an important way to study the structure–activity relationship of polysaccharides which should be focus on. After molecular modification, various derivatives of different types of structure and biological activities are gained. These lay the foundation for the structure–activity relationship analysis of polysaccharides. On the other hand, the results of structure–activity relationship research also guide the directions of molecular modification and provide with theoretical supports for drug design, research and development. Besides, research on conformation of polysaccharides is also very important. A previous study showed that relative extended chain conformation of carboxylmethylated derivatives was beneficial for enhancing the antitumor activity (Berit, Terje, Liv, & Bjorn, 2000). It is urgent for us to study the conformation of GLP and C-GLP.

4. Conclusions

The carboxymethyl derivative C-GLP, which derived from *Ganoderma lucidum* water-insoluble crude polysaccharide, was prepared using the MCA/NaOH method and purified by DEAE-cellulose-32 chromatography. C-GLP was mainly composed of glucose with minor amounts of galactose and mannose with an average molecular weight of 1.8×10^6 Da and a carboxymethyl content of 11.07%. Three characteristic absorption bands (1601, 1421, and 1720 cm^{-1}) appeared in IR spectrum and the signal at 179ppm in ^{13}C NMR spectrum indicated that the carboxymethyl reaction had actually occurred. Besides, C-GLP exhibited obvious strong abilities of scavenging hydroxyl radical and hydrogen peroxide, moderate ability of scavenging superoxide anion radical, while GLP only showed weakly in these abilities. The research on C-GLP is a further study of crude water-insoluble polysaccharide from *Ganoderma lucidum* and it also provides a method to fully utilize natural resources. It is noteworthy that C-GLP should be explored as a novel potential antioxidant after its further research on the safety for human consumption and antioxidant activity *in vivo*.

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References

Ana, F. D. V., Nilson, K. M., Robert, F. H. D., Aneli, M. B., Elaine, R. C., Joana, L. M. S., et al. (2008). Three exopolysaccharides of the β-(1→6)-D-glucan type and a β-(1→3;1→6)-D-glucan produced by strains of *Botryosphaeria rhodina* isolated from rotting tropical fruit. *Carbohydrate Research, 343*, 2481–2485.

Athukorala, Y., Kim, K. N., & Jeon, Y. J. (2006). Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*. *Food and Chemical Toxicology, 44*, 1065–1074.

Barry, H. (1987). Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. *Biochemistry, 165*, 215–219.

Berit, H. F., Terje, E., Liv, R., & Bjorn, T. S. (2000). The cytokine stimulating activity of (1→3)-β-D-glucans is dependent on the triple helix conformation. *Carbohydrate Research, 329*, 587–596.

Chakraborty, I., Mondal, S., Pramanik, M., Rout, D., & Islam, S. S. (2004). Structural investigation of a water-soluble glucan from an edible mushroom, *Astraeus hygrometricus*. *Carbohydrate Research, 339*, 2249–2254.

Debabrata, M., Krishnendu, C., Subhas, M., Arnab, K. O., Debsankar Das, S. K. R., et al. (2008). Isolation and characterization of a heteroglycan from the fruits of Astraeus hygrometricus. *Carbohydrate Research, 343*, 817–824.

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry, 38*, 674–677.

Durcilene, A. S., Regina, C. M. deP., Judith, P. A. F., Ana, C. F. deB., Jeanny, S. M., & Haroldo, C. B. P. (2004). Carboxymethylation of cashew tree exudate polysaccharide. *Carbohydrate Polymers, 58*, 163–171.

Guo, Z. Y., Xing, R. E., Liu, S., Yu, H. H., Wang, P. B., Li, C. P., et al. (2005). The synthesis and antioxidant activity of the Schiff bases of chitosan and carboxymethyl chitosan. *Bioorganic & Medicinal Chemistry Letters, 15*, 4600–4603.

Hao, R. F., & Li, R. C. (2004). Pharmacological and health effect of *Ganoderma lucidum* polysaccharide and its application prospect. *Acta Edulis Fungi, 11*(4), 57–62.

Lee, K. W., & Lee, H. J. (2006). Biphasic effects of dietary antioxidants on oxidative stress-mediated carcinogenesis. *Mechanisms of Ageing and Development, 127*(5), 424–431.

Nie, X. H., Shi, B. J., Ding, Y. T., & Tao, W. Y. (2006). Preparation of a chemically sulfated polysaccharide derived from *Grifola frondosa* and its potential biological activities. *International Journal of Biological Macromolecules, 39*, 228–233.

Pang, X., Chen, Z., Gao, X., Liu, W., Slavin, M., Yao, W., et al. (2007). Potential of a novel polysaccharide preparation (GLPP) from Anhui-grown *Ganoderma lucidum* in tumor treatment and immunostimulation. *Journal of Food Science, 72*(6), 435–442.

Puja, G., Vineet, K., & Pradeep, S. (2007). Carboxymethylation of Tamarind kernel powder. *Carbohydrate Polymers, 69*, 251–255.

Qi, H. M., Zhang, Q. B., Zhao, T. T., Chen, R., Zhang, H., & Niu, X. Z. (2005). Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) *in vitro*. *International Journal of Biological Macromolecules, 37*, 95–199.

Regina, C. M. deP., Heatley, F., & Budd, P. M. (1998). Characterization of Anacardium occidentale exsudate polysaccharide. *Polymer International, 45*, 27–35.

Ren, J. L., Sun, R. M., & Peng, F. (2008). Carboxymethylation of hemicelluloses isolated from sugarcane bagasse. *Polymer Degradation and Stability, 93*, 786–793.

Robak, J., & Gryglewski, R. (1988). Flavonoids are scavengers of superoxide anions. *Journal of Biochemical Pharmacology, 37*, 837–841.

Shi, C. J., Zhou, Y. D., Zhang, J. B., & Tian, G. Y. (2006). Research of polysaccharides from *Achynthes bidentata*. *Chinese Journal of New Drugs, 15*(16), 1330–1334.

Smirnoff, N., & Cumbes, Q. J. (1989). Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry, 28*(4), 1057–1060.

Storseth, T. R., Hansen, K., & Skjerrrno, J. (2004). Characterization of a β-D-(1→3)-glucan from the marine diatom Chaetoceros mulleri by high-resolution magic-angle spinning NMR spectroscopy on whole algal cells. *Carbohydrate Research, 339*, 421–424.

Sun, Y. X., Liang, H. T., Cai, G. Z., Guan, S. W., Tong, H. B., Yang, X. D., et al. (2009). Sulfated modification of the water-soluble polysaccharides from *Polyporus albicans* mycelia and its potential biological activities. *International Journal of Biological Macromolecule, 44*, 14–17.

Susumu, H., Shigeo, S., Kazuaki, K., Akiko, H., & Tsuneo, T. (1981). Analysis of the monosaccharide compositions of total non-dialyzable urinary glycoconjugates by the dithioacetal method. *Journal of Chromatography, 226*, 341–350.

Tao, Y., Zhang, L., & Cheung, P. C. (2006). Physicochemical properties and antitumor activities of water-soluble native and sulfated hyperbranched mushroom polysaccharides. *Carbohydrate Research, 341*(13), 2261–2269.

Thomas, H., & Andreas, K. (2005). Carboxymethyl ethers of cellulose and starch – a review. *Macromolecular Symposium, 223*, 13–39.

Tsiapali, E., Whaley, S., Kalbfleisch, J., Ensley, H. E., Browder, I. W., & Williams, D. L. (2001). Glucans exhibit weak antioxidant activity, but stimulate macrophage free radical activity. *Free Radical Biology and Medicine, 30*, 393–402.

Wang, H., Gao, X. D., Zhou, G. C., Cai, L., & Yao, W. B. (2008). *In vitro* and *in vivo* antioxidant activity of aqueous extract from *Choerospondias axillaris* fruit. *Food Chemistry, 106*, 888–895.

Wang, Y. F., Zhang, L. N., Li, Y. Q., Hou, X. H., & Zeng, F. B. (2004). Correlation of structure to antitumor activities of five derivatives of a β-glucan from *Poriacocos sclerotium*. *Carbohydrate Research, 339*, 2567–2574.

Wang, J., Zhang, Q. B., Zhang, Z. S., & Li, Z. E. (2008). Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. *International Journal of Biological Macromolecule, 42*, 127–132.

Xing, R. E., Liu, S., Yu, H. H., Guo, Zh. Y., Li, Zh. E., & Li, P. C. (2005). Preparation of high-molecular weight and high-sulfate content chitosans and their potential antioxidant activity *in vitro*. *Carbohydrate Polymers, 61*, 148–154.

Zhang, M., Cheung, P. C.-K., Chiu, L. C.-M., Wong, E. Y.-L., & Ooi, V. E.-C. (2006). Cell-cycle arrest and apoptosis induction in human breast carcinoma MCF-7 cells by carboxymethylated β-glucan from the mushroom sclerotia of *Pleurotus tuberregium*. *Carbohydrate Polymers, 66*, 455–464.

Zhao, G. R., Xiang, Z. J., Ye, T. X., Yuan, Y. J., & Guo, Z. X. (2006). Antioxidant activities of *Salvia miltiorrhiza* and *Panax notoginseng*. *Food Chemistry, 9*, 767–774.

Zou, C., Du, Y. M., Li, Y., Yang, J. H., Feng, T., Zhang, L., et al. (2008). Preparation of lacquer polysaccharide sulfates and their antioxidant activity *in vitro*. *Carbohydrate Polymers, 73*, 322–323.