Catalytic Synthesis and Antioxidant Activity of Sulfated Polysaccharide from *Momordica charantia* L.

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ABSTRACT:

Sulfated derivatives of polysaccharide from Momordica charantia L. (MCPS) with different degree of sulfation (DS) were synthesized by chlorosulfonic acid method with ionic liquids as solvent. Fourier transform infrared spectra and ^{13}C nuclear magnetic resonance spectra indicated that C-6 substitution was predominant in MCPS compared with the C-2 position. Compared with the native polysaccharide from Momordica charantia L. (MCP), MCPS exhibited more excellent antioxidant activities in vitro, which indicated that sulfated modification could enhance antioxidant activities of MCP. Furthermore, high DS and moderate molecular weight could improve the antioxidant activities of polysaccharide.

Keywords: Momordica charantia L.; sulfated derivatives; polysaccharide; antioxidant

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INTRODUCTION

Oxidation is essential biological process to many organisms for the production of energy.[1] However, reactive oxygen species (ROS), in the forms of superoxide anion ($\cdot O^{2-}$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2), are generated in the process, and they can induce a wide variety of pathological effects, such as atherosclerosis, carcinogenesis and DNA damage as well as in degenerative processes associated with aging.[2,3] Antioxidants may have a positive effect on human health. They are substances that delay or prevent the oxidation of cellular oxidizable substrates, and protect human body against damage by ROS. Therefore, it is essential to develop and utilize effective antioxidants so that they can scavenge free radicals in the human bodies.[4]

In recent years, it has become an important branch to find natural antioxidants since the synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are suspected of being responsible for liver damage and carcinogenesis.[5] Plant polysaccharide displays excellent scavenging activity against superoxide radical and hydroxyl radical. Furthermore, some studies have confirmed that antioxidant activities of polysaccharide can be improved by the molecular modification and structure improvement.[6–8] For example, the addition of sulfation on the polysaccharide could not only enhance the water solubility but also change the chain conformation, resulting in alteration of their antioxidant activities.[9,10] The antioxidant capacity of sulfated polysaccharides depended on several structural parameters such as the degree of sulfation (DS), the molecular weight (M_w) and chain conformations.[9,11,12]

In the previous study, the sulfated modification of polysaccharide was most accomplished in organic reagents. However, the solubility of polysaccharide was very poor in organic solvents, which was not beneficial to achieving the reaction quickly and leading to lower DS. Furthermore, in strongly acidic conditions polysaccharide would be depolymerized severely due to the long reaction time, which led to loss original biological activities of polysaccharides. To solve these problems, ionic liquids (ILs) and catalyst were used in the reaction system. First, ILs had miscibility with water and organic solvents, which could offer good solubility for polysaccharide. If ILs was used as reaction solvent, it was a better choice for sulfated modification. Second, catalyst was added into the reaction system for decreasing the reaction time and the depolymerization of polysaccharide.

Momordica charantia L. (MC), a climber belonging to family Cucurbitaceae, was a multipurpose herb widely cultivated in many tropical and subtropical regions of the world. The fruits of MC were used as a food and medicine in different parts of the world.[13,14] Polysaccharide obtained from MC (MCP) was the main effective ingredient with a wide array of biological activities, such as antihyperglycemic, antiulcer, antiviral, antioxidant.[15,16] Cheng reported that MCP was composed of glucose, galactose, arabinose, rhamnose, and mannose with molar ratio of 24.84:27.94:16.47:24.03:6.72. The main glycosidic bond configuration was β- configuration.[17]

In this study, a series of the sulfated derivatives of MCP (MCPS) were prepared by chlorosulfonic acid (CSA)/pyridine method with ILs 1-butyl-3-methylimidazolium chloride ([C4mim]Cl) as solvent and 4-dimethylaminopyridine (DMAP) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl) as catalyst. The structure of MCPS was analyzed by elemental analysis, FT-IR and ^{13}C NMR spectroscopy. Their antioxidant activities in vitro were investigated, including superoxide, hydroxyl, DPPH radicals-scavenging effects and reducing power.

MATERIALS AND METHODS

Materials and Reagents

The crude MCP from *Momordica charantia* L. was obtained from Shaanxi Lixin Biotechnology Co. (China) and purified further in our laboratory.

Sephadex G-100 was from Pharmacia (Sweden). Papain was purchased from Beijing Huamei Biotechnology (China). 1-Butyl-3-methylimidazolium chloride [C4mim]Cl was purchased from Lanzhou Institute of chemical physics (China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl) and 4-dimethylamino pyridine (DMAP) were supplied from Aladdin Chemistry (Beijing, China). Chlorosulfonic acid (CSA), *N*,*N*-dimethylformamide (DMF) and pyridine were analytical grade and obtained from Gansu Yinguang Chemical Industry (China). Ascorbic acid (V_C), butylated hydroxyanisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), thiobarbituric acid (TBA) and deoxyribose were purchased from Sigma Chemical. Nicotinamide adenine dinucleotide (NADH), ethylene diamine tetra-acetic acid (EDTA), phenazine methosulfate (PMS), H_2O_2, potassium ferricyanide and nitroblue tetrazolium (NBT) were purchased from Tianjin Guangfu Chemical Research institute (Tianjin, China).

Purification of MCP

The protein in the crude MCP was removed by the Sevage and neutral enzyme (papain) method three times. After centrifugation, the supernatant was dialyzed with distilled water for 48 h. Through SephadexG-100, the purified MCP was obtained. The contents of MCP were measured by Vitriol–Phenol.[18] M_w of MCP was 8.5 KDa.

Sulfated Modification of MCP

CSA was dripped in anhydrous pyridine in ice water bath as sulfated reagent. 1000 mg of [C4mim]Cl was heated to 80°C in an oil bath. Then 100 mg of MCP was added into the system and stirred for 1 h. After completing dissolution, the sulfated reagent was added into the above solution. Then 30 mg of EDC.HCl and 30 mg of DMAP as catalyst were added and the mixture was stirred at 70°C for 1 h. After the reaction, the mixture was cooled to room temperature and the pH value was adjusted to 7–8 with 2.5 mol/L NaOH solution. To remove pyridine, salt and DMAP, the mixtures were dialyzed with distilled water for 24 h. After lyophilizing, a series of MCPS (MCPS1–MCPS4) with different DS were obtained. MCPS5 was obtained by chlorosulfonic acid–pyridine method and used as negative control.

Characterization

Mw of MCPS was determined by high performance gel-filtration chromatography (HPGFC) on a Waters 2695 instrument. The mobile phase was 0.05 mol/L Na_2SO_4, and a flow rate was 0.7 mL/min.

The sulfur contents of MCPS were determined by an elemental analyzer (Vario EL, Elementar, Germany). DS was calculated according to the equation:

$$DS = \frac{162 \times S\%}{32 - 102 \times S\%}$$

FT-IR (Nicolet NEXUS-670) was used to recognize the functional groups of MCPS and ^{13}C NMR spectra was recorded to get the information of MCPS structure by a Bruker AVANCE 600 MHz spectrometer (Rheinstetten, Germany) in D_2O.

Assay for Antioxidant Activities

Hydroxyl Radical-Scavenging Activity. Hydroxyl radicals were generated using a modified method of Ghiselli et al.[19] The reaction mixture (0.6 mL) consisted of 25 m*M* phosphate buffer (pH 7.4, PBS), 2.6 m*M* deoxyribose, 0.10 m*M* EDTA and 0.1 mL different concentration samples (0.1–2.0 mg/mL). Then 0.2 mL of 0.3 m*M* ferrous sulfate, 0.05 mL of 15 m*M* H_2O_2 and 0.05 mL of 1.5 m*M* Vc were added to the reaction solution. After incubation at 37°C for 20 min, 1.0 mL of 2.0% TCA and 1.0 mL of 1.0% TBA were added and heated in boiling water bath for 15 min. The mixture was cooled and measured at

532 nm against blank. V_C was used as positive control. Scavenging activity of hydroxyl radical was calculated with the following equation:

$$\text{Scavenging effect}(\%) = (1 - A/A_0) \times 100\%$$

where A_0 was the absorbance of mixture solution without sample; A was the absorbance of the sample mixed with reaction solution.

Hydroxyl Radical-Scavenging Activity. The superoxide radical scavenging ability of MCP and MCPS was assessed by the method of Nishimiki et al.[20] The reaction mixture, containing 0.5 mL of NBT (74 μ*M*) solution, 0.5 mL of NADH (397 μ*M*) solution and different concentrations samples (0.1–2.0 mg/mL), was incubated in 4.5 mL of Tris–HCl buffer solution (16 m*M*, pH 8.0). The reaction was started with adding 0.5 mL of PMS (45 μ*M*) solution. After incubation at 25°C for 5 min in the dark, the absorbance was read at 560 nm against the blank. The superoxide radical-scavenging rate (%) was calculated by the following equation:

$$\text{Scavenging effect}(\%) = (1 - A/A_0) \times 100\%$$

where A_0 was the absorbance of mixture solution without sample; A was the absorbance of the sample mixed with reaction solution.

DPPH Radical Scavenging Assay. The free-radical scavenging capacity of MCP and MCPS was analyzed using the DPPH radical according to the method of Shimada[21] with some modifications. 2 mL of sample solution at different concentrations (0.1–2.0 mg/mL) was added to 2 mL 0.2 mmol/L 60% ethanol solution of DPPH, the reaction mixture was shaken vigorously and incubated for 20 min at room temperature. Then the absorbance was read at 517 nm against a blank. BHA was used as positive control. The radical scavenging activity was calculated using the following equation:

$$\text{Scavenging effect}(\%) = (1 - A/A_0) \times 100\%$$

where A_0 was the absorbance of DPPH solution without sample; A was the absorbance of the sample mixed with DPPH solution.

Reducing Power Assay. The reducing power of all samples was investigated according to the method reported by Yen and Chen.[22] 2.5 mL of samples in different concentrations (0.1–2.0 mg/mL) was mixed with 2.5 mL of PBS (100 m*M*, pH 6.6) and 2.5 mL of potassium ferricyanide (1%), and incubated at 50°C for 20 min. Then, TCA (10%, w/v) was added to the mixture to terminate the reaction. Finally, the solution was mixed with ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm.

RESULTS AND DISCUSSION

Chemical Analysis of MCPS

Four sulfated derivatives of MCP were obtained by varying the ratio of CSA to pyridine in the sulfating reagent. M_w and DS of MCPS were presented in Table I. The results showed that Mw of MCPS varied from 5.42 to 7.07 KDa, DS of MCPS increased from 0.72 to 1.13. MCPS4 had the highest DS of 1.13. It indicated that hydroxyl groups on the polysaccharide were substituted efficiently by sulfate groups. Compared with MCPS5, the molecular mass of samples had not been decreased obviously. It might be that the reaction could achieve dynamic equilibrium quickly after the addition of catalyst, which decreased the depolymerization of polysaccharide. MCP was dissolved in ILs, the reaction would be carried out in a homogenous system, which would be beneficial to increasing DS of polysaccharide.

Table I Chemical Analysis of the Sulfated MCP

MCPS[a]	CSA:PD[b]	Time (h)	M_w (kDa)	*S* (%)	DS
MCPS1	1:4	1.0	7.07	9.79	0.72
MCPS2	1:3	1.0	6.76	10.24	0.77
MCPS3	1:2	1.0	6.29	11.89	0.97
MCPS4	1:1	1.0	5.42	13.01	1.13
MCPS5	1:4	3.0	4.33	11.66	0.93

[a] Sulfation of polysaccharide from *Momordica charantia* L.
[b] The ratio of chlorosulfonic acid to pyridine in sulfated reagent.

Figure 1 showed the FT-IR spectra of MCP and MCPS4. Compared with MCP, new band at 1248 cm^{-1} described an

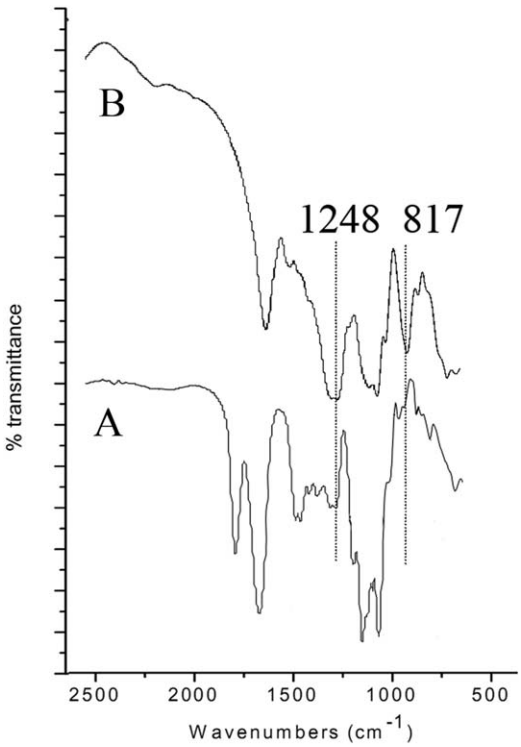

FIGURE 1 FTIR spectra of MCP and its sulfated derivatives. (A) MCP, (B) MCPS4.

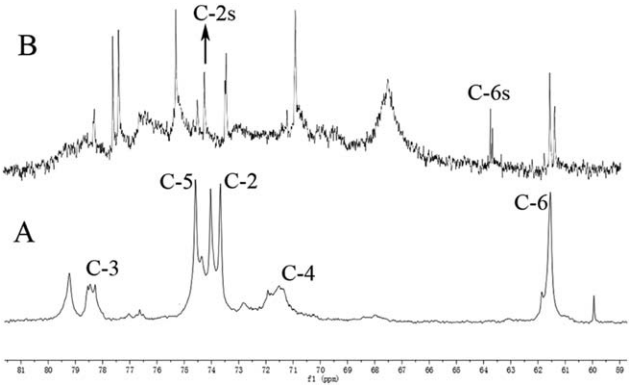

FIGURE 2 ^{13}C NMR spectra of MCP and its sulfated derivatives. (A) MCP; (B) MCPS4.

asymmetrical S═O stretching vibration, and a moderate intensity band at 817 cm^{-1} indicated a symmetrical C—O—S vibration, which were attributed to the sulfate substitution in MCPS4.

The sulfated position on the polysaccharide was usually determined by ^{13}C NMR spectrum. The ^{13}C NMR spectra of MCP and MCP4 were showed in Figure 2. As shown in Figure2A, the signals of MCP at 78.5, 75.0, 73.9, 71.5, and 61.5 ppm were assigned to C-3, C-5, C-2, C-4, and C-6, respectively. It was found that the ^{13}C NMR spectra became more complicated after sulfation because the carbon directly attached to an electron-withdrawing sulfate group would shift to a lower field position, while the carbon indirectly attached to sulfate group would shift to higher field position.[23] As shown in Figure2B, the new peak of MCPS4 at 63.4 ppm was assigned to the signal of C-6s; the peak at 74.6 ppm was assigned to the signal of C-2s. At 61.5 ppm the peak was weakened obviously, which indicated that C-6 had been primarily substituted by the sulfate group, but C-2 had been partially substituted. The intensity of the signals denoted that C-6 substitution was predominant in MCPS4 compared with other positions at C-2, probably due to steric hindrance.

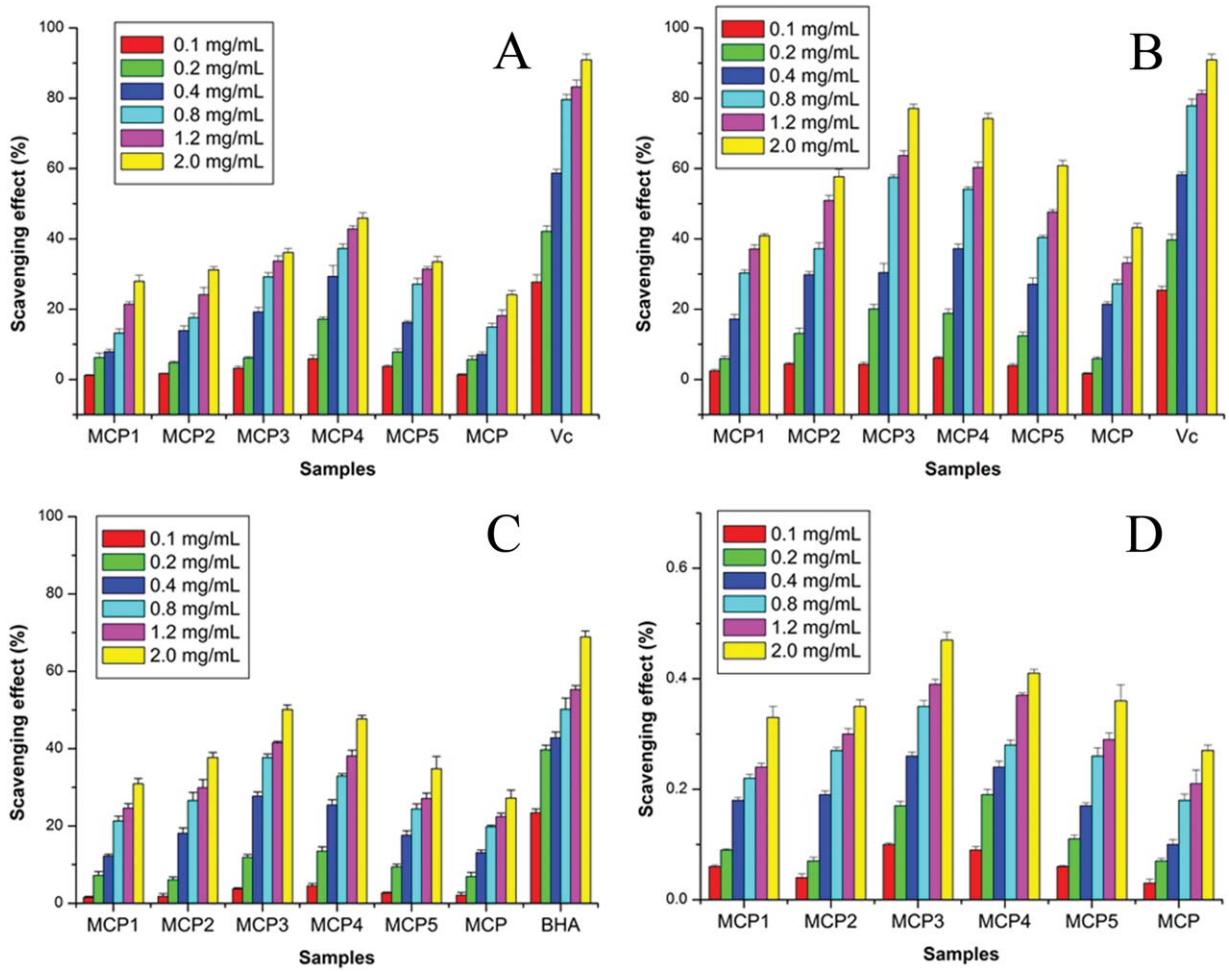

FIGURE 3 Antioxidant effect of MCP and MCPS: (A) scavenging activity of hydroxyl radicals; (B) scavenging activity of superoxide radicals; (C) scavenging activity of DPPH radicals; and (D) reducing power.

Antioxidant Activity Analysis

Scavenging Activity of Hydroxyl Radical. Hydroxyl radical, known to be generated through the Fenton reaction, can easily cross cell membranes, and readily react with biomolecules including carbohydrates, proteins, lipids, and DNA in cells, and cause tissue damage or cell death.[24] Figure 3A described the scavenging ability on hydroxyl radical of MCP and MCPS. For all the sulfated samples, scavenging activities on hydroxyl radical were in a concentration-dependent manner. Compared with the original MCP, MCPS showed higher scavenging effects on hydroxyl radical. Furthermore, MCPS4 showed the best scavenging ability, and the scavenging ability was 45.9% at the concentration of 2.0 mg/mL. This suggested that scavenging activity of polysaccharide on hydroxyl radical could be improved by sulfated modification.

It was reported that the scavenging activity of hydroxyl radical was due to the inhibition of hydroxyl radical generation by chelating ions such as Fe^{2+} and Cu^{2+}.[9] The sulfate group had chelating ability for Fe^{2+}, so, we believed that sulfated polysaccharide could reduce the generation of hydroxyl radicals by chelating the Fe^{2+}.

Scavenging Activity of Superoxide Radical. The superoxide radical ($\cdot O^{2-}$) was a highly toxic species that was generated in a PMS/NADH system for being assayed in the reduction of NBT. Figure3B depicted the concentration above 2.0 mg/mL, MCPS3 and MCPS4 showed the best scavenging ability, and the scavenging ability was 77.2 and 74.1%, respectively, while the original MCP and MCPS5 showed a low scavenging effect. In addition, scavenging activity of V_C for the superoxide radical was 90.9% at 2.0 mg/mL. This demonstrated that DS affected the antioxidant activity, and higher sulfate content showed greater scavenging effect of superoxide radical. It might be that sulfate groups could change the three-dimensional structure of polysaccharide, and expose more hydroxyl groups, which affected scavenging activity of superoxide radical.

Scavenging Activity of DPPH Radical. The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants. DPPH scavenging effects of all samples at varying concentrations were measured and the results were depicted in Figure3C. All the sulfated derivatives showed higher scavenging activity than the native MCP. Furthermore, at the concentration of 2.0 mg/mL, MCPS3 and MCPS4 with high DS showed the highest chelating abilities of 50.1 and 47.7%, respectively, which again demonstrated the importance of the sulfate ratio in antioxidant ability. However, the scavenging effects of MCP and MCPS on DPPH radical were all relatively lower than that of BHA at the same concentration. It was reported that the effect of antioxidant on DPPH radical scavenging was due to their hydrogen donating ability.[6] The sulfated derivatives showed excellent scavenging activity on DPPH radical, which might be attributable to its strong hydrogen-donating ability.

Reducing Power. The reducing power assay measures the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. Figure3D described the reducing power of MCP and MCPS. The reducing power of all samples correlated positively with increasing concentrations. At a concentration of 2.0 mg/mL, the reducing power values of MCPS1 to MCPS5 were 0.33, 0.35, 0.47, 0.41, and 0.36, which were higher than that of MCP. Furthermore, MCPS4 had the best reducing power. The results showed that MCPS had strong reducing capacity.

The reducing capacity of a compound might serve as a significant indicator of its potential antioxidant activity. The reducing properties were associated with the presence of reductones, which had been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[25] Our data on the reducing power of all samples, especially MCPS3 and MCPS4, suggested that it was likely to contribute toward the observed antioxidant effect.

CONCLUSION

Four sulfated derivatives of MCP from *Momordica charantia* L. were synthesized by chlorosulfonic acid method with ILs as solvent and EDC.HCl and DMAP as catalyst. ILs could be much better to dissolve polysaccharide, which would be beneficial to increasing DS of polysaccharide. Sulfated derivatives of MCP showed greater antioxidant activities compared to MCP. It was obvious that the antioxidant activity had certain relationship with DS. It was concluded that high DS and moderate Mw could improve the antioxidant activities of polysaccharide.

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