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Sulfated modification and antioxidant activity of exopolysaccahrides produced by *Enterobacter cloacae* Z0206

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ABSTRACT

Nine modification conditions were designed to sulfate exopolysaccharides (EPS) produced by *Enterobacter cloacae* Z0206 by chlorosulfonic acid–pyridine (CSA–Pyr) method according to the orthogonal test and focusing on three affecting factors such as the ratio of CSA to Pyr, reaction temperature and reaction time. And nine sulfated derivatives with various degrees of substitution (DS) were obtained. Their antioxidant activities were evaluated *in vitro*, by scavenging abilities on superoxide radical and hydroxyl radical. The results indicated that sulfated derivatives of EPS showed noticeable effects on scavenging superoxide radical and hydroxyl radical compared with native one, and sulfated derivative with moderate DS of 0.60 showed highest antioxidant activities. The optimum sulfated conditions of EPS were the ratio of CSA to Pyr of 1:2, the reaction time of 2 h and reaction temperature of 70 °C.

1. Introduction

Polysaccharide is a species of macromolecular substance existing widely in organism [1], and they have been widely studied in the biochemical and medical areas due to specific biological activities such as immunostimulating, antioxidant, antiviral, antitumor [2]. The biological activities of polysaccharide mainly depend on its molecular structure, such as sugar unit and glycosidic bond of the backbone, the type and polymerization degree of the branch, the flexibility and spatial configuration of the chains [3]. Therefore, structural improvement and chemical modification of polysaccharides provide opportunities to obtain new pharmacological agents with possible therapeutic uses [4,5].

Sulfated polysaccharide, including natural sulfated polysaccharides extracted from plants, heparin or synthesized derivatives from neuter polysaccharide, is a kind of ones whose hydroxyls are partially replaced by sulfated group [1,6]. Many studies have demonstrated that the biological activities of polysaccharides could be obviously improved by the sulfated method [7–10]. The sulfation of many natural polysaccharides could not only enhance water solubility but also change the chain conformation, resulting in alteration of their biological activities [11,12]. Therefore, sulfated modification may be used to improve the biological activities of some polysaccharides [3].

Enterobacter cloacae Z0206, a bacterial strain, can produce large amounts of exopolysaccharides. In our previous study [2], we have extracted and purified the major exopolysaccharide (EPS) produced by *E. cloacae* Z0206. The composition of EPS is glucose, mannose and galactose (molar ratio of 6.860:1.180:0.455) with the average molecular weight of 2.39×10^4 Da. The administration of EPS at the dose of 200 mg/kg body weight to cyclophosohamide-exposed mice resulted in significant increase and recovery of B lymphocyte proliferation, tumor necrosis factor α production and activities of antioxidant enzymes (superoxide dismutase and glutathione peroxidase). However, to the best of our knowledge, there is limited literature on the sulfated modification of EPS. Meanwhile, antioxidant activities of sulfated derivatives of EPS have never been studied.

In the present research, nine sulfated derivatives of EPS produced by *E. cloacae* Z0206 with different degrees of substitution (DS) were prepared by chlorosulfonic acid–pyridine (CSA–Pyr) method using the orthogonal array design. Their antioxidant activities were also evaluated *in vitro*, by scavenging abilities on superoxide radical and hydroxyl radical. The purpose of this study was to validate the probability of improving the antioxidant activity of EPS through the sulfated modification and screen out the optimum reaction conditions. In addition, it may provide a basic understanding of the relationship between DS and bioactivities.

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2. Materials and methods

2.1. Materials and chemicals

E. cloacae Z0206, the exopolysaccharide-producing bacterial strain, was identified and kept in our laboratory, and it has been collected by China General Microbiological Culture Collection Center (CGMCC).

Phenazine methosulfate (PMS) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nitroblue tetrazolium (NBT) and dihydromicotineamidadenine dinucleotide (NADH) were from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). All other reagents used were of analytical grade available. DEAE cellulose dialysis bags were produced by Spectrum Co. (USA), and molecular weight was cut off at 8000 Da.

2.2. Preparation of EPS

Preparation and purification of EPS were carried out according to the previous report [2]. Briefly, the exopolysaccharides production was performed in a 10 dm^3 bioreactor (Shanghai Biotech Ltd., China). After cultivation, the broth was centrifuged to remove the mycelia. The supernatant was collected and evaporated under reduced pressure, then precipitated upon addition of 3 vol. of cold 95% EtOH. The resulting precipitates were collected, dissolved in distilled water, and deproteinized by a combination of papain and trypsin enzymolysis and the Sevag method [13]. The solution was dialysed in a DEAE cellulose bag against distilled water for 3 days to remove the low-molecular-weight materials. Then the solution was evaporated and precipitated with 3 vol. of cold 95% EtOH at 4 °C overnight. The precipitates were collected by centrifugation, and then lyophilized to get a white powder.

2.3. Sulfated modification of EPS

Nine reacting conditions were designed according to orthogonal test as $L_9(3^3)$ to investigate the effect of three factors, such as the ratio of CSA to Pyr, the reaction temperature and reaction time, on sulfation of EPS [14]. Three levels per factor were used with the ratio of CSA to Pyr of 1:2, 1:4 and 1:6, the reaction temperature of 50 °C, 70 °C and 90 °C, and the reaction time of 1, 2 and 3 h, respectively (Table 1).

2.3.1. Preparation of sulfating reagent

CSA was dropped one by one in anhydrous Pyr (12 ml) filled in three-necked flask, under continuous stirring and cooling in ice water bath [14]. The ratio of CSA to Pyr referred to Table 1. All determinations were completed in 40 min and nine kinds of sulfating reagents were obtained.

2.3.2. Sulfation reaction

EPS powder (500 mg) was suspended in anhydrous formamide (20 mL) and the mixture was stirred for 30 min at room temperature. Then the sulfating reagents were added, the mixture was stirred for various durations and/or temperatures (Table 1). After the reaction, the mixture was cooled to room temperature and neutralized with 2.0 M NaOH solution. The nine obtained polymers were precipitated with 3 vol. of 95% EtOH, washed, redissolved in water, and then dialyzed (molecular weight cut off 8000 Da) against tap water for 24 h and distilled water for 48 h to remove pyridine, salt and potential degradation products. Nine sulfated polysaccharides named SEPS-1, SEPS-2, SEPS-3, SEPS-4, SEPS-5, SEPS-6, SEPS-7, SEPS-8, and SEPS-9 with different DS were collected after lyophilizing and kept in dryness box.

2.4. Analysis of SEPS

The content of polysaccharide was determined by the phenol–sulphuric acid method [15], and protein in the polysaccharide was quantified according to Bradford' method [16] using bovine serum albumin as the standard. The sulfur contents of SESP were determined by Wang's method [14]. A calibration curve was constructed with sodium sulfate as standard. The DS was calculated according to the equation:

$$\mathrm{DS} = \frac{1.62 \times \mathrm{S}\%}{32 - 1.02 \times \mathrm{S}\%}$$

The chemical structure of EPS and its sulfated derivatives SEPS were determined by a BECKMAN DU640 Ultraviolet (UV) spectrophotometer and a Fourier transform infrared spectra (FT-IR, AVATAR 370, Thermo Nicolet, USA) using KBr-disk method.

2.5. Determination of antioxidant activities

2.5.1. Scavenging ability on superoxide radical

The superoxide radical scavenging activity was determined according to the methods of Chang [17] with a minor modification. 50 μl of sample solution at gradient concentrations (0.125–8 mg/ml) was mixed with 100 μl of 150 μM NBT solution and 100 μl of 470 μM β-NADH solution. After mixing thoroughly, 20 μl of 60 μM PMS solution was added and shaken for a few seconds, followed by standing at room temperature for 5 min and measuring the absorbance at 560 nm. Vitamin C (VC) was used as a positive control. The superoxide radical scavenging effect was determined as follows:

$$\text{Scavenging ability}\,(\%) = \frac{A_0 - A_1}{A_0} \times 100\%$$

where A_0 was the absorbance of mixture solution without sample and A_1 was the absorbance of the text sample mixed with reaction solution.

2.5.2. Scavenging ability on hydroxyl radical

Hydroxyl radical scavenging activity was measured according to Fenton method [18] with a minor modification. The hydroxyl radicals were generated in an H_2O_2–$FeSO_4$ system by oxidation of $FeSO_4$ and were assayed by the color change of salicylic acid [19]. 160 μl of sample solution at gradient concentrations (0.125–8 mg/ml) was incubated with 9.0 mM $FeSO_4$ (40 μl), 0.03% H_2O_2 (40 μl) in 20 μl salicylic acid–ethanol solution (9.0 mM) for 30 min at 37 °C. The change in absorbance caused by salicylic acid was measured at 510 nm. VC was used as a positive control. The hydroxyl radical scavenging effect was calculated as follows:

$$\text{Scavenging ability}\,(\%) = \frac{A_0 - A_1}{A_0} \times 100\%$$

where A_0 was the absorbance of mixture solution without sample and A_1 was the absorbance of the text sample mixed with reaction solution.

2.6. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's *t*-test. Results were presented as mean ± SEM. Differences between groups were considered statistically significant at the 5% ($p < 0.05$) level.

Table 1
Sulfation of EPS with CSA–Pyr method.

Samples	CSA:Pyr	Temperature (°C)	Time (h)	Yield (mg)	Carbohydrate (%)	S (%)	DS
EPS	–	–	–	–	97.24	nd	nd
SEPS-1	1:2	50	1	962.0	32.70	9.40	0.68
SEPS-2	1:2	70	2	916.1	27.67	8.63	0.60
SEPS-3	1:2	90	3	881.1	31.62	8.31	0.57
SEPS-4	1:4	50	2	644.8	55.89	3.09	0.17
SEPS-5	1:4	70	3	574.2	55.66	2.08	0.11
SEPS-6	1:4	90	1	751.6	51.26	8.13	0.56
SEPS-7	1:6	50	3	591.8	57.93	2.38	0.13
SEPS-8	1:6	70	1	861.7	35.46	8.22	0.56
SEPS-9	1:6	90	2	542.9	45.50	1.88	0.10

nd: not detected.

3. Results and discussion

3.1. Effects of various reaction conditions on DS

There are many methods to sulfate polysaccharides, such as sulfuric acid, sulfur trioxide (SO_3)–Pyr, CSA–Pyr, and SO_3–dimethylacetamide [9]. CSA–Pyr method is the most popular one due to advantages such as high DS, high yield and convenient production reclamation [3,20]. In this study, nine sulfated derivatives of EPS were prepared with CSA–Pyr method by varying sulfation conditions, which are the important effective factors such as the ratio of CSA–Pyr, reaction temperature and reaction time. Polysaccharides performed at different conditions result in sulfated polysaccharides with various DS and bioactivities [9]. The yield, DS and carbohydrate content of nine polymers are listed in Table 1. The results indicated that SEPS-1 had the highest yield of 962.0 mg, while SEPS-9 had the lowest yield of 542.9 mg. The DS of nine sulfated derivatives of EPS were listed in a decreasing order as follows: $SEPS_1 > SEPS_2 > SEPS_3 > SEPS_8 > SEPS_6 > SEPS_4 > SEPS_7 > SEPS_5 > SEPS_9$ and were 0.68, 0.60, 0.57, 0.56, 0.56, 0.17, 0.13, 0.11 and 0.10, respectively. The carbohydrate content in sulfated derivatives of EPS varied from 27.67% to 57.93%, and it was in accordance with the previous studies that the carbohydrate content of some polysaccharides decreased after sulfated modification [21].

Analyses on the orthogonal array design indicated that the extent of the impact of variables on DS followed the order: variable A (molar ratio of CSA to Pyr) > C (reaction time) > B (reaction temperature). The molar ratio of CSA to Pyr of reaction was the major factor affecting DS. And it suggested that controlling the reagent amount was better than controlling the reaction temperature to get sulfated polysaccharide derivatives with high DS [22]. Many studies indicated that the enhancement of the molar ratio of CSA to Pyr, the increase of reaction temperature, and the prolongation of reaction time may contribute to high DS [14]. In the present study, with the increase of the molar ratio of CSA to Pyr and reaction temperature from 50 °C to 70 °C, DS of product increased very rapidly. However, the DS decreased when the temperature up to 90 °C. This was in accordance with the previous studies that reaction should be in the relatively mild condition [14]. It was also found that the increased reaction time from 1 h to 3 h caused the decrease of DS. This was in accordance with the previous study [23], which indicated that about 85% of the possible substitution occurs within the first hour, and the rate of reaction was higher in the primary stage.

Sulfated derivatives of EPS had a negative response to the Bradford' test and no absorption at 280 nm or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. In comparison with EPS, two characteristic absorption bands appeared in the FT-IR spectra of SEPS. Fig. 1 shows the FT-IR spectra of EPS, SEPS-2 (DS = 0.60) and SEPS-4 (DS = 0.17). Two characteristic absorption bands appeared in the spectrum of sulfated derivatives compared with EPS. New band around 1256 cm^{-1} was attributed to an asymmetrical S=O stretching vibration and the other around 830 cm^{-1} represented a symmetrical C–O–S vibration associated with a C–O–SO_3 group, indicating incorporation of the sulfating group [12]. These results indicated that sulfation of EPS were successfully obtained. Meanwhile, with the increase of DS, the absorbance of these bands increased. And intensities of these absorptions were in coincidence with the degrees of substitution.

3.2. Scavenging activity of superoxide radical

Superoxide anion is considered as an initial free radical, formed from mitochondrial electron transport systems, to create other cell-damaging free radicals, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems [24], and increase local oxidative stress and initiate cellular damage or lipid peroxidation and pathological incidents [25]. Therefore, the scavenging ability of superoxide anion radicals is extremely important to anti-oxidation work [5]. In the present study, the superoxide radical scavenging effects of EPS and its sulfated derivatives were tested in a PMS/NADH system for being assayed in the reduction of NBT [26].

As shown in Fig. 2, all the tested samples were found to have the ability to scavenge superoxide radicals in a dose dependent manner. A significant increase of the scavenging activity was observed at the concentration range from 0.125 to 1.0 mg/ml of SEPS-1, SEPS-2, SEPS-3, SEPS-8, SEPS-6, and SEPS-4, and from 0.25 to 4.0 mg/ml of SEPS-5, SEPS-7, SEPS-9 and EPS. The EC_{50} values of EPS, SEPS-1, SEPS-2, SEPS-3, SEPS-4, SEPS-5, SEPS-6, SEPS-7, SEPS-8 and SEPS-9 were 4.02, 0.08, 0.05, 0.20, 1.13, 2.68, 0.38, 2.30, 0.35 and 3.27 mg/ml, respectively. All sulfated samples showed stronger scavenging effect than EPS. At the concentration of 0–0.25 mg/ml, the scavenging activity for superoxide radical of SEPS-1 and SEPS-2 was a little weaker than that of VC. However, with continually increasing concentration from 0.25 to 8.0 mg/ml, the scavenging effects of above two polymers were close to or even stronger than that of VC. At the concentration of 2.0 mg/ml, the scavenging effect of SEPS-1 and SEPS-2 was 85.80% and 89.06%, while at the same concentration, the scavenging effect of VC was only 80.66%.

Apparently the sulfated derivatives with high DS such as SEPS-1, SEPS-2, SEPS-3, SEPS-8 and SEPS-6 were more efficient in scavenging superoxide anion radicals than the other ones. This demonstrated that DS affected the antioxidant activity, which was in accordance with the previous reports that the presence of the sulfated group could increase the scavenging activity of radicals due to the addition of electron-withdrawing groups [27], and higher sulfate contents showed greater scavenging effect of superoxide radical [28,29]. It has been reported that polysaccharide may possess the capacity to donate hydrogen to superoxide anion because of weak dissociation energy of O–H bond [30]. Theoretically, the more the electron-withdrawing groups such as sulfate group and carboxyl group in polysaccharide, the weaker the dissociation energy of O–H bond [17].

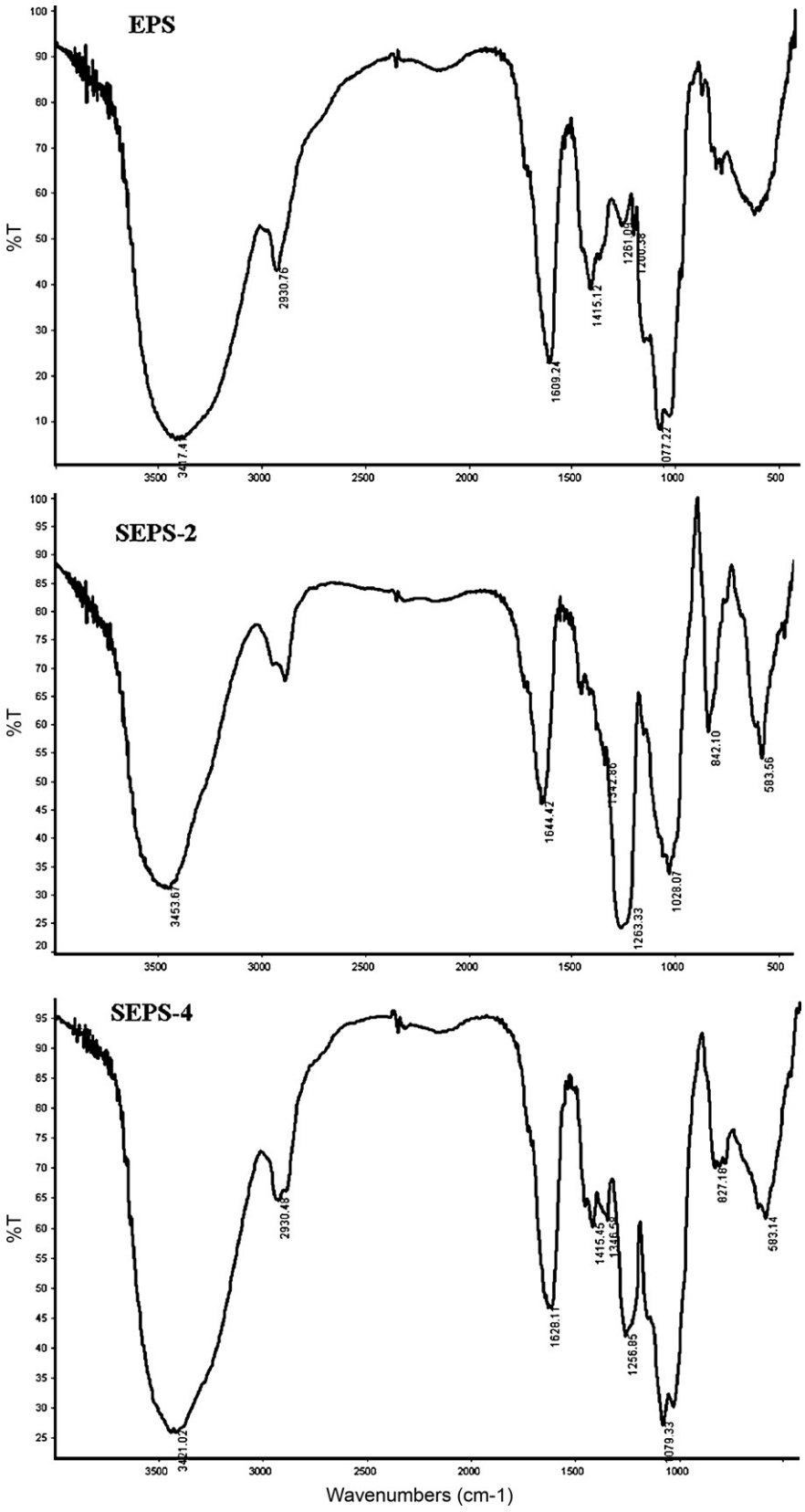

Fig. 1. FT-IR spectra of EPS and its sulfated derivatives SEPS-2 and SEPS-4.

3.3. Scavenging activity of hydroxyl radical

Hydroxyl radicals, which are well known to be the most reactive of all the reduced forms of dioxygen, can react with most biomacromolecules functioning in living cells and induce severe damage to the adjacent biomolecules in the form of abstracting hydrogen atoms, addition reaction and electron transportation [31]. Thus, removing hydroxyl radicals is important for antioxidant defense

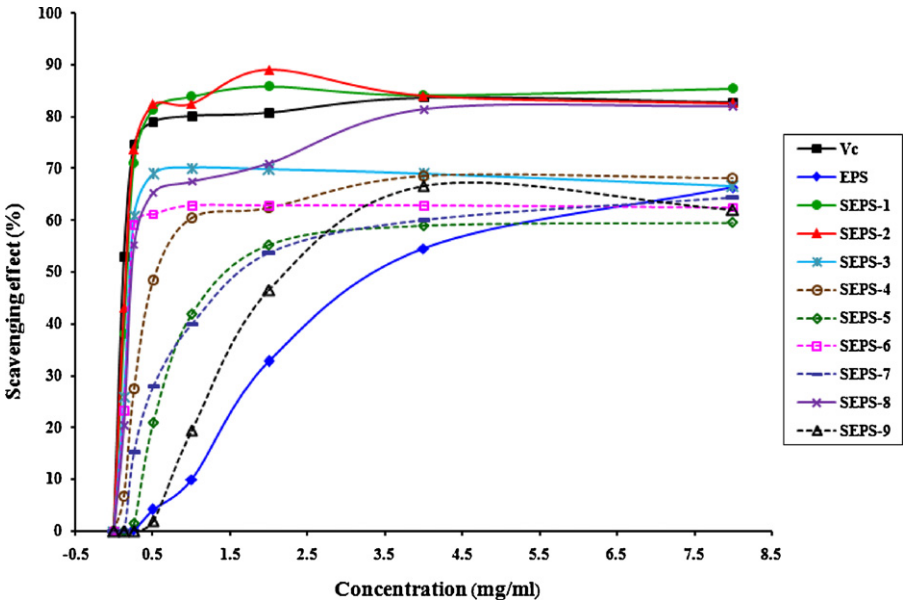

Fig. 2. Scavenging activity of EPS and its sulfated derivatives on superoxide radical. Data are presented as mean values ($n = 3$).

in cell or food systems [29]. In this study, the hydroxyl radical was generated through the Fenton reaction, which is the most important mechanism *in vivo* [31], and a transition metal is involved as pro-oxidant in the catalysed decomposition of superoxide and hydrogen peroxide [32].

The hydroxyl radical scavenging activity of EPS and its sulfated derivatives is shown in Fig. 3. The original EPS showed a very low scavenging ability on hydroxyl radical, while the sulfated derivates were found to have higher scavenging ability than EPS. The EC_{50} values of SEPS-2 and SEPS-3 were 4.45 and 7.13 mg/ml, respectively; however, the EC_{50} values of other samples could not read in Fig. 3. Moreover, sulfated derivatives with high DS, such as SEPS-2, SEPS-3, SEPS-1, SEPS-8 and SEPS-6, possessed higher hydroxyl radical scavenging activity than other derivatives with low DS. The results indicated that sulfate group played an important role in the scavenging of hydroxyl radical. This was in accordance with the previous results that substitution of –OH with $–OSO_3H$ group would enhance the scavenging activity of hydroxyl radical [5,33].

In addition, the relationship between antioxidant activities and DS of sulfated derivatives of EPS was not exact direct correlation. As the superoxide radical and hydroxyl radical scavenging activities of SEPS-1 with highest DS of 0.68 was not the best, while SEPS-2 with DS of 0.60 showed the best activities. This suggested that DS of sulfated polysaccharides must be within optimum scope, and a moderate DS of the sulfated derivatives was necessary for high biological activities. This was in accordance with the previous results that the highest DS had not direct inevitable relation with highest biological activities, and the higher DS was likely to be concerned with the cytotoxicity [3,5]. Besides DS, some other structural characteristics, such as molecular weight and monosaccharide composition, are also considered as main factors influencing radical scavenging capacity of polysaccharide [34]. The antioxidant activity of polysaccharides was not a function of single factors but a combination of several factors [28,35]. The detailed relationship between antioxidant activity and structure characteristics of sulfated derivatives of EPS still requires further investigation.

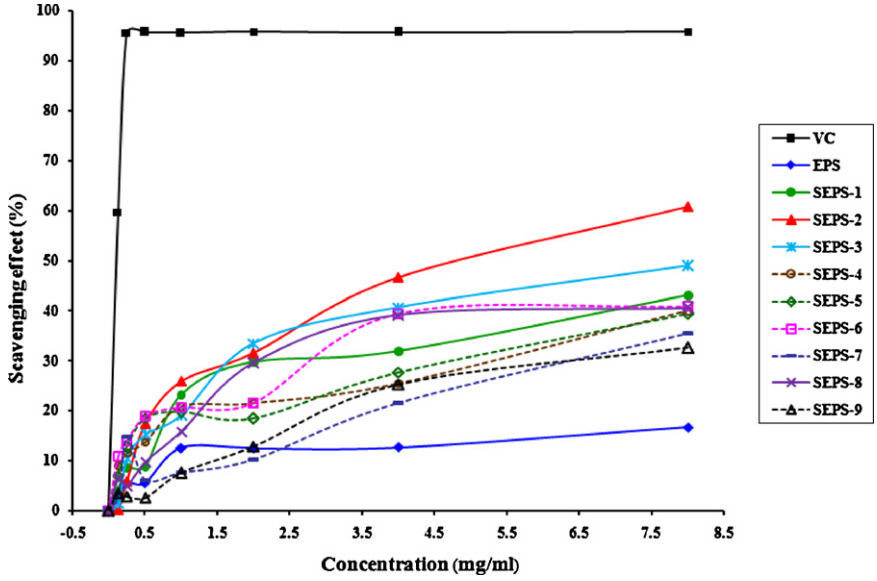

Fig. 3. Scavenging activity of EPS and its sulfated derivatives on hydroxyl radical. Data are presented as mean values ($n = 3$).

4. Conclusion

In the present study, nine sulfated derivatives of EPS produced by *E. cloacae* Z0206 with different DS were synthesized by CSA–Pyr method. The primary structure and corresponding antioxidant activities, such as scavenging abilities on superoxide radical and hydroxyl radical, were evaluated *in vitro*. Sulfated derivatives of EPS showed noticeable effects on scavenging superoxide radical and hydroxyl radical compared with native one. It suggested that sulfate modification could be considered as the effective approach to enhance the antioxidant activities of EPS. Moreover, SEPS-2 with moderate DS of 0.60 showed highest antioxidant activities. It indicated that a moderate DS of the sulfated derivatives was necessary for high biological activities, and the optimum sulfated conditions of EPS should be 1:2 of CSA–Pyr ratio, 2 h of reaction time and 70 °C of reaction temperature according to antioxidant activities and DS.

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References

[1] J.M. Wang, Y.L. Hu, D.Y. Wang, J. Liu, J. Zhang, S. Abula, B. Zhao, S.L. Ruan, Cell. Immunol. 263 (2010) 219–223.
[2] M.L. Jin, Y.M. Wang, C.L. Xu, Z.Q. Lu, M. Huang, Y.Z. Wang, Carbohydr. Polym. 81 (2010) 607–611.
[3] Y. Lu, D.Y. Wang, Y.L. Hu, X.Y. Huang, J.M. Wang, Carbohydr. Polym. 71 (2008) 180–186.
[4] Y.H. Liu, C.H. Liu, H.N. Tan, T. Zhao, J.C. Cao, F.S. Wang, Carbohydr. Polym. 77 (2009) 370–375.
[5] J.L. Wang, H.Y. Guo, J. Zhang, X.F. Wang, B.T. Zhao, J. Yao, Y.P. Wang, Carbohydr. Polym. 81 (2010) 897–905.
[6] X.Y. Huang, D.Y. Wang, Y.L. Hu, Y. Lu, Z.H. Guo, X.F. Kong, J.L. Sun Junling, Int. J. Biol. Macromol. 42 (2008) 166–171.
[7] X.Y. Huang, Y.L. Hu, X.N. Zhao, Y. Lu, J.M. Wang, F. Zhang, J.L. Sun, Carbohydr. Polym. 73 (2008) 303–308.
[8] S.G. Chen, J.F. Wang, C.H. Xue, H. Li, B.B. Sun, Y. Xue, W.G. Chai, Carbohydr. Polym. 81 (2010) 560–566.
[9] Y.L. Zhang, X.Y. Lu, Y.N. Zhang, L.G. Qin, J.B. Zhang, Int. J. Biol. Macromol. 46 (2010) 67–71.
[10] Y.L. Zhang, J.B. Zhang, X.Y. Mo, X.Y. Lu, Y.N. Zhang, L.G. Qin, Carbohydr. Polym. 82 (2010) 515–520.
[11] A. Chaidedgumjorn, H. Toyoda, E.R. Woo, K.B. Lee, Y.S. Kim, T. Toida, T. Imanari, Carbohydr. Res. 337 (2002) 925–933.
[12] X.H. Nie, B.J. Shi, Y.T. Ding, W.Y. Tao, Int. J. Biol. Macromol. 39 (2006) 228–233.
[13] A.M. Staub, Methods Carbohydr. Chem. 5 (1965) 5–6.
[14] L. Wang, X.X. Li, Z.X. Chen, Int. J. Biol. Macromol. 44 (2009) 211–214.
[15] M. Dubois, K.A. Gilles, J.K. Harmilton, P.A. Rebers, F. Smith, Anal. Chem. 28 (1956) 350–356.
[16] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[17] S.C. Chang, B.Y. Hsu, B.H. Chen, Int. J. Biol. Macromol. 47 (2010) 445–453.
[18] L. Diez, M.H. Livertoux, A.A. Stark, M. Wellman-Rousseau, P. Leroy, J. Chromatogr. B 763 (2001) 185–193.
[19] N. Smirnoff, Q.J. Cumbes, Phytochemistry 28 (1989) 1057–1060.
[20] M. Baba, R. Snoeck, R. Pauwels, E. Clercq, Antimicrob. Agents Chemother. 32 (1998) 1742–1745.
[21] Y.X. Sun, H.T. Liang, G.Z. Cai, S.W. Guan, H.B. Tong, X.D. Yang, J.C. Liu, Int. J. Biol. Macromol. 44 (2009) 14–17.
[22] H. Vogl, D.H. Paper, G. Franz, Carbohydr. Polym. 41 (2000) 185–190.
[23] J.H. Yang, Y.M. Du, R.H. Huang, Y.Y. Wan, T.Y. Li, Int. J. Biol. Macromol. 31 (2002) 55–62.
[24] O. Bloknina, E. Virolainen, K.V. Fagerstedt, Ann. Bot. 91 (2003) 179–194.
[25] W. Liu, H.Y. Wang, X.B. Pang, W.B. Yao, X.D. Gao, Int. J. Biol. Macromol. 46 (2010) 451–457.
[26] B. Halliwell, FEBS Lett. 92 (1978) 321–326.
[27] Z.S. Zhang, F. Wang, X.M. Wang, X.L. Liu, Y. Hou, Q.B. Zhang, Carbohydr. Polym. 82 (2010) 118–121.
[28] J. Wang, Q.B. Zhang, Z.H. Zhang, Z. Li, Int. J. Biol. Macromol. 42 (2008) 127–132.
[29] T.T. Hu, D. Liu, Y. Chen, J. Wu, S.S. Wang, Int. J. Biol. Macromol. 46 (2010) 193–198.
[30] X.Y. Zhu, J.M. Wu, Z.S. Jia, Chin. Chem. Lett. 15 (2004) 808–810.
[31] F.R. Lai, Q.B. Wen, L. Li, H. Wu, X.F. Li, Carbohydr. Polym. 81 (2010) 323–329.
[32] S.J. Stohs, D. Baguchi, Free Radic. Biol. Med. 18 (1995) 321–336.
[33] H.M. Qi, Q.B. Zhang, T.T. Zhao, R. Chen, H. Zhang, X.Z. Niu, Z. Li, Int. J. Biol. Macromol. 37 (2005) 195–199.
[34] E. Tsiapali, S. Whaley, J. Kalbfleisch, H.E. Ensley, I.W. Browder, D.L. Williams, Free Radic. Biol. Med. 30 (2001) 393–402.
[35] B.W. Zhu, D.Y. Zhou, T. Li, S. Yan, J.F. Yang, D.M. Li, X.P. Dong, Y. Murata, Food Chem. 121 (2010) 712–718.