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Sulfated modification can enhance antiglycation abilities of polysaccharides from *Dendrobium huoshanense*

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ABSTRACT

Dendrobium huoshanense is an important edible-medicinal plant with high nutritional values and health functions. A homogenous polysaccharide (DHPD1) with molecular weight of 3.2×10^3 Da was extracted from *D. huoshanense*, which was mainly composed of glucose, arabinose, galactose, mannose and xylose. Chlorosulfonic acid-pyridine (CSA-Pyr) method was performed to modify the structure of DHPD1. In order to get a high degree of substitution (DS), sulfated modification conditions were optimized by response surface methodology. The maximum DS of 1.473 was obtained when the reaction condition was fixed at reaction temperature 60 °C, reaction time 160 min and volume ratio of Pyr to CSA 2:1. NMR spectra revealed that this sulfation occurred to C-2 and C-6 of glycosyl residues in DHPD1. After 28 days of incubation, the sulfated DHPD1 at 1.0 mg/mL showed the inhibitory ability of 58.5%, which increased by 16.2% and 52.5% than that of aminoguanidine and DHPD1 at the same dosage.

1. Introduction

Glycation is a complicated reaction process between the aldehydic group of sugars and the amino group of proteins, which is a major factor responsible for the development of chronic complications of diabetes, such as diabetic cataract and diabetic atherosclerosis (Lapolla, Traldi, & Fedele, 2005; Wu, Hsieh, Wang, & Chen, 2009). It has been proposed that development of glycation cascade inhibitors should supply a promising therapeutic approach for the prevention of diabetic complications (Wu et al., 2009). Aminoguanidine, a hydrazine compound, has been considered as an effective therapeutic agent to alleviate the development of diabetic vascular complications through inhibiting the process of protein glycation. However, some toxicity problems have often been happened in clinical trials with this compound (Singh, Barden, Mori, & Beilin, 2001). Therefore, there is an increasing interest in the application of natural compounds as anti-diabetic agents (Wu et al., 2009).

Dendrobium huoshanense, an important edible-medicinal plant belonging to orchid family, has high nutritional values and health functions (Bao, Shun, & Chen, 2001). Its stems were traditionally employed to make eye bright, treat chronic superficial gastritis, promote the production of body fluids and relieve throat inflammation (Bao et al., 2001). In China, *D. huoshanense* is customarily used as the material to make functional teas and soups to entertain the distinguished guests (Bao et al., 2001; Luo, Deng, & Zha, 2008). Recent studies demonstrated that *Dendrobium* plants showed some activities of anti-tumor, anti-oxidant, anti-liver fibrosis and anti-diabetes due to their abundance of polysaccharides (Hsieh et al., 2008; Li, Xie, Su, Ye, & Jia, 2012; Lin, Shaw, Sze, Tong, & Zhang, 2011; Luo, He, Zhou, Fan, Luo, & Chun, 2010; Wang, Luo, Zha, & Feng, 2010). In our previous work, we found that polysaccharides of *D. huoshanense* (DHP) have the response of inhibiting the development of diabetic cataract of mice, attributing to their inhibitory effects on protein glycation and down-regulation of NO synthetase in crystalline lens (Luo et al., 2008). It has been reported that the biological activities of polysaccharides depended on their structural features such as types of monosaccharide compositions, molecular weights, substitution degrees, linkage types of glycosidic bonds, and types and positions of branches (Zhang, Lu, Zhang, Qin, & Zhang, 2010). Therefore, molecular modification of polysaccharides to improve their bioactivities are drawn much attention by chemists and pharmacologists. Among different polymer derivatives, sulfated polysaccharides have been reported to exhibit strong biological properties, such as anti-tumor, anticoagulant, anti-oxidant and anti-virus, in comparison with the native polysaccharides (Sun, Liang, Cai, et al., 2009). Until now, many sulfated polysaccharide drugs have been developed and some of them have been used in clinical trials (Wei, Wei, Cheng, & Zhang, 2012). However, it is still unclear the effects of sulfation occurred to polysaccharides on their antiglycation activity.

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Table 1
Experimental data and predicted of D.S. of polysaccharides from *Dendrobium huoshanense* with different combinations of reaction temperature, reaction time and volume ratio of Pyr to CSA designed by response surface methodology.

Run	Coded variables[a]			Uncoded variables			D.S.		
	X_1	X_2	X_3	X_1	X_2	X_3	Experimental (Y_0)	Predicted (Y_1)	$Y_0 - Y_1$
1	1	−1	0	80	2	3:1	1.298	1.263	−0.035
2	−1	0	−1	40	3	1:1	1.343	1.292	−0.051
3	0	−1	1	60	2	5:1	1.003	1.001	−0.002
4	0	1	−1	60	4	1:1	1.238	1.249	0.011
5	0	0	0	60	3	3:1	1.435	1.45	0.015
6	−1	1	0	40	4	3:1	1.184	1.217	0.033
7	0	0	0	60	3	3:1	1.480	1.450	−0.03
8	0	0	0	60	3	3:1	1.453	1.450	−0.003
9	1	0	−1	80	3	1:1	1.276	1.228	−0.048
10	0	0	0	60	3	3:1	1.487	1.450	−0.037
11	1	1	0	80	4	3:1	0.812	0.851	0.039
12	0	0	0	60	3	3:1	1.389	1.45	0.061
13	−1	0	1	40	3	5:1	0.89	0.934	0.044
14	0	−1	−1	60	2	1:1	1.373	1.451	0.078
15	1	0	1	60	3	5:1	0.699	0.746	0.047
16	0	1	1	80	4	5:1	0.927	0.859	−0.068
17	−1	−1	0	40	2	3:1	1.174	1.149	−0.025

[a] X_1, reaction temperature (°C); X_2, reacion time (h); X_3, volume ratio of Pyr to CSA.

In this paper, we performed the sulfated modification of *D. huoshanense* polysaccharide (DHPD1) using chlorosulfonic acid-pyridine (CSA-Pyr) method. The sulfated reaction conditions were optimized using response surface methodology (RSM). Moreover, the structure and anti-glycation activity of sulfated DHPD1 were compared with those of DHPD1.

2. Materials and methods

2.1. Plant materials and source of sample

D. huoshanense C. Z. Tang et S. J. Cheng was collected in Huoshan county, Anhui province of China in July of 2004. The protocorm-like bodies (PLBs) were induced and propagated on Murashige and Skoog (MS) medium under a 16:8 h light–dark cycle (approx. 30 μmol m^{-2} S^{-1}) at 25 ± 2 °C (Zha & Luo, 2008). Nitro blue tetrozolium and sodium barbital were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (MO, USA). Girards Reagent T and Aminoguanidine Hydrochloride were purchased from Adamas Reagent Co., Ltd. (Shanghai, China). All other reagents were of analytical grade levels.

2.2. Extraction and purification of polysaccharides

The fresh PLBs of *D. huoshanense* were dried in an oven at the temperature ranged from 40 °Cto 50 °C, and grinded into powders. The dried powders (500 g) were pre-extracted with methanol for 72 h in a Soxhlet system and subsequently with acetone for another 72 h. The extracts were discarded. The residues were dried at room temperature and extracted with ten volume of deionized water in a water bath (50–60 °C) for three times. The combined water extracts were concentrated to a certain volume with a rotary evaporator under 60 °C, subsequently mixed with four volume of 99.99% ethanol to precipitate polysaccharides. After centrifugation at a speed of 12,000 rpm, the precipitates were dissolved in deionized water again and Sevag method was performed to remove proteins from this solution (Luo et al., 2008). The crude polysaccharide was fractionated on a DEAE-Cellulose column using distilled water, 0.1, 0.2, 0.3, and 0.6 M aqueous NaCl solution as eluent in a stepwise gradient manner. The fraction obtained by distilled water elution were collected and dialyzed for 72 h against tap water using a bag filter with molecular weight cutoff 3500 Da. The nondialysate was freeze-dried to give a purified polysaccharide, named DHPD1.

2.3. Optimization of parameters for DHPD1 sulfation

2.3.1. Single-factor test

Sulfation of DHPD1 was performed using CSA-Pyr method (Chen et al., 2010). For preparation of esterification reagent, chlorosulfonic acid (CSA) was added to pyridine (Pyr) drop by drop in a three-necked flask, under agitating and cooling in ice water bath. Various volume ratio of Pyr to CSA (1:1, 3:1, 5:1, 7:1 and 9:1) were used. The DHPD1 powder (100 mg) were dissolved in the solvent of formamide (FA), dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) with different dosage (2.5, 5.0, 7.5, 10.0 and 12.5 mL), respectively. Subsequently, the polysaccharide solution was added to a three-necked flask filled with esterification reagent, and the mixture was stirred at a preset reaction temperature. Various reaction temperature (20, 40, 60, 80 and 100 °C) and reaction time (1.0, 2.0, 3.0, 4.0 and 5.0 h) were performed.

2.3.2. Optimization of reaction conditions for DHPD1 sulfation

The reaction parameters including reaction temperature (X_1), reaction time (X_2) and volume ratio of Pyr to CSA (X_3) were optimized by RSM. According to the results of single-factor experiments, the range and center point values of three independent variables were selected and shown in Table 1. Each variable was coded at three levels (−1, 0, +1). The Box–Behnken design was carried out to determine the best combination of reaction variables for polysaccharide sulfation, which contained 12 factorial points and 5 replicates of the central point (Table 1). Accordingly, a second-order regression analysis of the experimental data was performed to get an empirical model for visualizing the relationship between the response and the levels of independent variables and deducing the optimum conditions.

For all experiments, the mixture was cooled in ice water bath, neutralized with sodium hydroxide and precipitated with ethanol at a final concentration of 80% after sulfated reaction. The precipitate was dissolved in distilled water and dialyzed in a bag filter (molecular weight cutoff 3500 Da) for 72 h against double-distilled water. The nondialysate was lyophilized to give the sulfated polysaccharide of DHP (SDHPD1).

2.4. Determination of sugar content in polysaccharide

The polysaccharide content was measured by phenol-sulfuric acid method, using D-glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.5. Degree of sulfate substitution

According to the reference (Dodgson & Price, 1962), the degree of sulfate substitution (DS) was determined by barium chloride-gelatin method. Potassium sulfate was used as standard. The DS was calculated according to the equation: $DS = (1.62 \times S\%)/(32 - 1.02 \times S\%)$.

2.6. Analysis of homogeneity, molecular weight, monosaccharide composition and glycosyl linkages

The homogeneity and molecular weight of DHPD1 and SDHPD1 were determined by high-performance liquid chromatography (HPLC) according to our previous reports (Zha et al., 2012). The monosaccharide composition of DHPD1 was measured by gas chromatography (GC) with detection condition described in the reference (Zha et al., 2012). The glycosyl linkages were analyzed by methylation method (Sun, Liang, Zhang, Tong, & Liu, 2009).

2.7. FT-IR and UV analysis

For IR spectroscopy, polysaccharide powders were mixed with KBr, grounded, and pressed into a 1 mm pellet. FT-IR spectra were recorded on a Nicolet Nexus 470 spectrometer. For UV spectra, the aqueous solution of polysaccharide was scanned with the wavelength from 190 to 400 nm on a UV–vis spectrophotometer.

2.8. NMR spectroscopy

The polysaccharide was dried using P_2O_5 for several days in vacuum dryer. Exchangeable protons were replaced with deuterium by suspending polysaccharide in D_2O, and lyophilizing. The ^{13}C NMR spectrum was recorded in D_2O on a Bruker Avance DPX-400 instrument (Fällanden, Switzerland) at 27 °C. Data processing was performed using standard Bruker NMR software.

2.9. Anti-glycation analysis

2.9.1. In vitro glycation of BSA

The glycation system of BSA was prepared according to the reference method (Wu et al., 2009) with some modifications. The model group contained 10 mL barbitone sodium-hydrochloric acid buffer solution (200 mM, pH 7.4), 20 mmol/mL BSA and 500 mM glucose. Each tested group contained 10 mL barbitone sodium-hydrochloric acid buffer solution (200 mM, pH 7.4), 20 mmol/mL BSA, 500 mM glucose and 0.1 mg/mL or 1.0 mg/mL polysaccharide. The positive control group contained 10 mL barbitone sodium-hydrochloric acid buffer solution (200 mM, pH 7.4), 20 mmol/mL BSA, 500 mM glucose and 0.1 mg/mL or 1.0 mg/mL aminoguanidine. The negative control (NC) was free of aminoguanidine and polysaccharide. All reagents were dissolved in 200 mM barbitone sodium-hydrochloric acid buffer solution with pH 7.4, and filtrated through 0.22 μm membrane to remove microorganisms. The reaction mixtures were cultured at 37 ± 0.5 °C. The medium of 1.5 mL was taken out from the culture system every 7 days and stored at −20 °C until analysis.

2.9.2. Analysis of amadori products

Amadori products were measured according to the reference Baker, Zyzak, Thorpe, and Baynes, 1994) with minor modification.

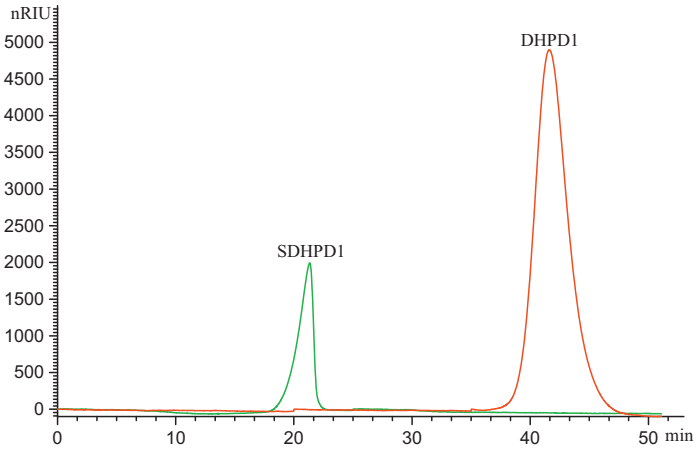

Fig. 1. UV spectrum and HPLC profiles of DHPD1 and SDHPD1. (A) UV and (B) HPLC.

The glycated sample (0.5 mL) was added to 2.0 mL sodium carbonate buffer (100 mM, pH 10.35) containing 0.3 mM nitro blue tetrozolium. The mixture was incubated at room temperature for 15 min, and then the absorbance was measured at 530 nm.

2.9.3. Analysis of dicarbonyl compounds

The contents of α-dicarbonyl compounds were determined by the method of Wells-Knecht, Zyzak, Litchfield, Thorpe and Baynes (1995). The mixture which contained 0.4 mL glycosylated substance, 0.2 mL Girard-T stock solution (500 mM) and 3.4 mL sodium formate (500 mM, pH 2.9), was incubated at room temperature for 1 h. The absorbance was recorded at 294 nm.

2.9.4. Analysis of AGEs

The glycated sample (0.1 mL) was diluted with sodium barbital buffer (200 mM, pH 7.4) for 30 times, and the fluorescence of AGEs was recorded at 370 nm of excitation wavelength and 440 nm of emission wavelength using a Sanko 930 A spectrofluorometer.

The inhibitory ratio was calculated as $[(A_{NC} - A_{sample})/A_{NC}] \times 100$, where A_{NC} and A_{sample} represent the absorbance of negative control group and polysaccharide or aminoguanidine group.

2.10. Data analysis

All data were expressed as means ± standard deviations (SD) from three independent replicates. All data were evaluated by one-way analysis of variance (ANOVA). $p < 0.05$ were considered to be significant on statistics.

3. Results and discussion

3.1. Analysis of homogeneity and molecular weight

No absorptions were observed at 260 nm and 280 nm, indicating that there was no proteins and nucleic acids linked to DHPD1. As shown in Fig. 1, the symmetrical and narrow peak on HPLC revealed that DHPD1 was a homogeneous fraction. The molecular weight (MW) was calculated as 3.2×10^3 Da based on the calibration curve of the elution time of standard Dextrans on HPLC. GC analysis showed that DHPD1 was mainly composed of glucose, arabinose, galactose in a molar ratio of 1.023:0.023:0.021, along with trace of mannose and xylose.

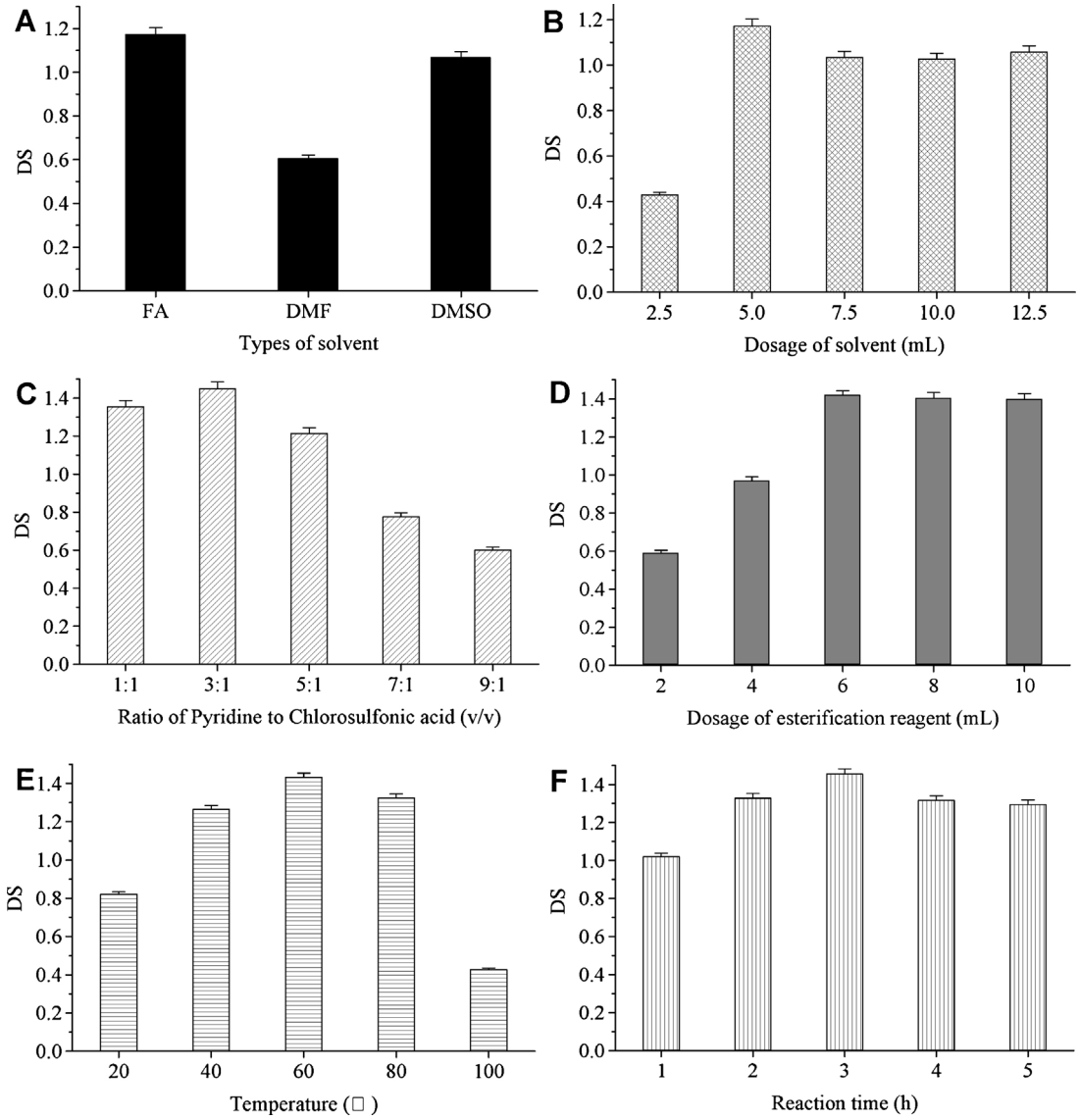

Fig. 2. Effects of different (A) solvent type, (B) FA dosage, (C) volume ratio of Pyr to CSA, (D) dosage of esterification reagent, (E) reaction temperature and (F) reaction time on the degree of sulfate substitution (DS).

3.2. Sulfation of DHPD1

3.2.1. Solvent selection for DHPD1 sulfation

The solvent, as the pivotal reaction medium, plays an important role in all chemical reactions. In this study, formamide (FA), dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were used as the solvent to dissolve DHPD1. To investigate the effects of these solvents on DHPD1 sulfation, the solvent dosage was set at 5.0 mL. The other conditions were ratio of Pyridine (Pyr) to Chlorosulfonic acid (CSA) 5:1 (v/v), reaction temperature 60reaction temperature and reaction time 3.0 h. As shown in Fig. 2A the DS of FA, DMF and DMSO was 1.174, 0.606 and 1.068, respectively. The results indicated the capability for DHPD1 sulfation could be in the order of FAYDMSOYDMF. Therefore, FA was considered to be the optimal solvent for DHPD1 sulfation. As far as the effect of solvent dosage on DHPD1 sulfation was concerned, the DS reached a critical value of 1.174 when the dosage of FA was 5.0 mL. Beyond 5.0 mL, the DS decreased in a mild slope with the dosage increasing (Fig. 2B). Thus, 5 mL FA was selected as the solvent to dissolve DHPD1 in the following experiments.

3.2.2. Ratio selection of Pyr to CSA for DHPD1 sulfation

The volume ratio of Pyr to CSA is an important factor affecting the sulfation of polysaccharides. For each ratio, the solvent was 5.0 mL FA and the reaction was conducted at 60 °C for 3.0 h. Results showed that high percent of CSA was beneficial for DHPD1 sulfation. The DS reached the peak when the volume ratio of Pyr to CSA was set at 3:1 (Fig. 2C). After this point, the DS decreased rapidly. Thus, the optimal volume ratio of Pyr to CSA for DHPD1 sulfation was considered to be 3:1 in the present experiment. Many other studies also indicated that polysaccharide sulfation of could be enhanced by enlarging the percentage of CSA (Zhang et al., 2010a).

3.2.3. Dosage selection of esterification reagent for DHPD1 sulfation

The dosage of esterification reagent is another factor that would influence polysaccharide sulfation. According to our above results, the esterification reagent was composed of Pyr and CSA with volume ratio of 3:1. In order to study the effect of different dosage of esterification reagent on DHPD1 sulfation, sulfation reaction was carried out using 2, 4, 6, 8 or 10 mL esterification reagent, and the

other reaction conditions were fixed at reaction temperature 60 °C and reaction time 3.0 h. The DS value of DHPD1 increased gradually when the dosage of esterification reagent was from 2 to 6 mL. As shown in Fig. 2D, the maximum DS was 1.421 when the dosage of esterification reagent was 6 mL. When the dosage varied from 6 to 10 mL, the DS of DHPD1 was decreased in a mild slope with the dosage enhancement, but there is no significant difference in statistics ($p > 0.05$). Thus, 6 mL dosage of esterification reagent was beneficial for DHPD1 sulfation in the present work.

3.2.4. Reaction temperature selection for DHPD1 sulfation

To investigate the influence of reaction temperature on sulfated efficiency of DHPD1, the reaction temperature was set at 20, 40, 60, 80 and 100 °C, respectively. The other conditions were fixed at 6 mL of Pyr and CSA with volume ratio of 3:1, and 3 h of reaction time. Fig. 2E displayed that the DS of DHPD1 continued to enhance with the increase of reaction temperature from 20 to 60 °C and reached the peak value at 60 °C. However, the DS was reduced when the reaction temperature exceeded 60 °C. Similar phenomenon was also found in the sulfation of Chinese lacquer polysaccharides (Yang, Du, Wen, Li, & Hu, 2003). These results suggested that the reaction temperature ranged from 40 to 80 °C was favorable to DHPD1 sulfation using CAS-Pyr method.

3.2.5. Reaction time selection for DHPD1 sulfation

To study the effects of reaction time on DHPD1 sulfation, sulfated process was carried out for 1, 2, 3, 4 or 5 h. The other sulfated conditions were 6 mL esterification reagent consisted of Pyr and CSA with volume ratio of 3:1 and 60 °C reaction temperature. As shown in Fig. 2F, The DS of DHPD1 had been increasing when the reaction time increased from 1 to 3 h. The shorter and longer time had a negative effect on DHPD1 sulfation. The highest DS was obtained when reaction time was 3 h. Therefore, the reaction time ranged from 2 to 4 h was considered to be optimal in the present experiment.

3.2.6. Optimization of sulfated conditions using response surface methodology

In general, the bioactivities of sulfated polysaccharides were improved with increasing DS (Yang et al., 2003). In order to get the sulfated DHPD1 with the highest DS, it is very necessary to optimize the sulfated conditions. Response surface methodology (RSM) is an effective statistical technique for optimizing complex processes, which has been successfully applied to scientific researches and industrial processes of chemistry, biology and engineering (Yoshida, Tsubaki, Teramoto, & Azuma, 2010). Therefore, based on the single-factor-test, RSM was employed to optimize the combinations of reaction temperature, reaction time and volume ratio of Pyr to CSA. The design matrix and the corresponding results of RSM experiments were displayed in Table 1. Results showed that the DS ranged from 0.699 to 1.487 were observed in all combinations of different reaction condition using RSM. The peak DS were obtained at $X_1 = 60$ °C, $X_2 = 3$ h and $X_3 = 3{:}1$. Applying multiple regression analysis to the experimental data (shown in Table 1), a mathematical model describing the predicted response Y was expressed as the following second-order polynomial equation:

$$Y = 1.45 - 0.063X_1 - 0.086X_2 - 0.21X_3 - 0.12X_1X_2 - 0.031X_1X_3 - 0.015X_2X_3 - 0.21X_1^2 - 0.12X_2^2 - 0.19X_3^2$$

where Y is the predicted DS of DHPD1, X_1, X_2 and X_3 represent the reaction temperature, reaction time and ratio of Pyr to CSA, respectively. The analysis of variance for this model was given in Table 2. The F-value (24.81) and p-value (0.0002) implied that this model was very significant. The independent variables (X_1, X_2, X_3), interaction coefficient (X_1X_2) and quadratic term coefficients (X_1^2, X_2^2, X_3^2) were observed to affect DHPD1 sulfation significantly ($p < 0.05$). In contrast, the interaction between reaction temperature and the ratio of Pyr to CSA (X_1X_3) and the interaction between reaction time and the ratio of Pyr to CSA (X_2X_3) were not significant. As shown in Fig. 3, the predicted DS was calculated using this model and compared with the corresponding experimental results. The determination coefficient (R^2) was calculated as 0.9696 with one-way analysis of variance of the quadratic regression model, indicating that the model was efficient for prediction of the process of DHPD1 sulfation within the range of experimental variables. The adjusted coefficient of determination (Adj-$R^2 = 0.9305$) also confirmed that the model was highly significant. Moreover, the coefficient of variation (CV) was observed to be 5.46%, implied a high degree of veracity and good reliability of the experiment data.

Table 2
Estimated regression model of relationship between response variables (Y) and independent variables (X_1, X_2, and X_3).

Source	SS	DF	MS	F-value	p-value[a]
Model	0.96	9	0.11	24.81	0.0002
X_1	0.032	1	0.032	7.41	0.0297
X_2	0.059	1	0.059	13.66	0.0077
X_3	0.37	1	0.37	84.73	<0.0001
X_1X_2	0.062	1	0.062	14.24	0.0069
X_1X_3	0.0038	1	0.0038	0.89	0.3769
X_2X_3	0.0087	1	0.0087	0.20	0.6671
X_1^2	0.18	1	0.18	41.99	0.0003
X_2^2	0.065	1	0.065	15.06	0.0061
X_3^2	0.15	1	0.15	34.93	0.0006
Residual	0.03	7	0.0043		
Lack of fit	0.024	3	0.008	5.15	
Pure error	0.0062	4	0.0016		
Cor total	0.99	16			

SS, sum of squares; DF, degree of freedom; MS, mean square.
[a] p-value < 0.05 represents significance.

3.2.7. Interactions between independent variables

The three-dimensional representation of response surface and the two-dimensional contours were often used to elucidate the effects of the independent variables and interactive effects of independent variables on the response (Zhu, Wang, Wang, Chen, Zhu, & Zhu, 2011). The effects of two variables on DHPD1 sulfation were

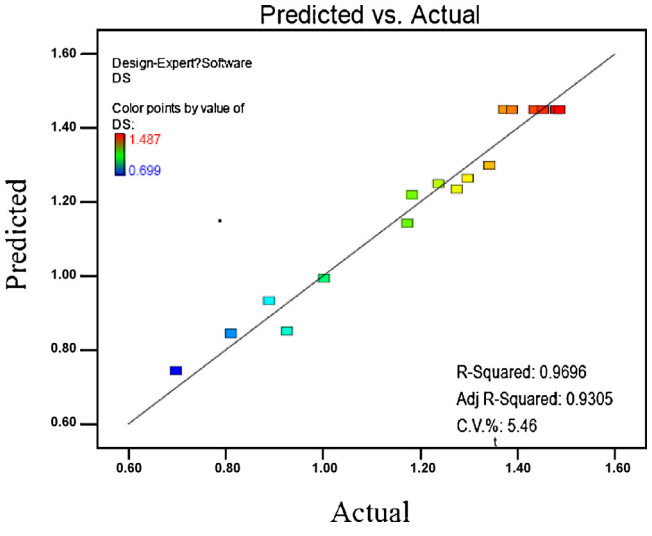

Fig. 3. Comparison between predicted and actual degree of sulfate substitution.

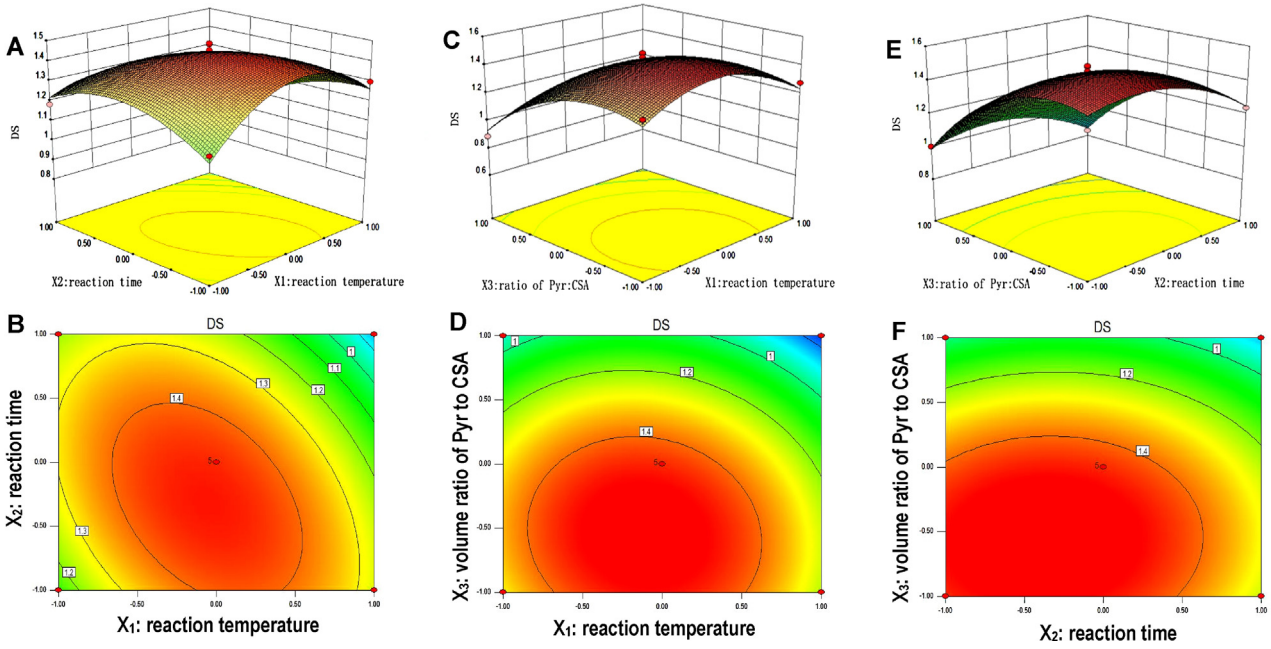

Fig. 4. Three-dimensional mesh plot and two-dimensional contour plot showing the effects of two variables on the degree of sulfate substitution (DS). (A) and (B) The effects of reaction time and reaction temperature on DS of DHPD1; (C) and (D) the effects of reaction temperature and volume ratio of Pyr to CSA on DS of DHPD1; (E) and (F) the effect of reaction time and volume ratio of Pyr to CSA on DS of DHPD1.

displayed in Fig. 4 when the third variable was kept at the zero level (Table 1). Fig. 4A and B showed the effects of reaction temperature (X_1) and reaction time (X_2) on the DS of DHPD1 when the volume ratio of Pyr to CSA (X_3) was fixed at 3:1. Fig. 4A indicated that the DS value increased first and then decreased gradually with the increasing of reaction temperature (X_1) and reaction time (X_2). As shown in Fig. 4B, when the reaction temperature (X_1) ranged from 50 to 70 °C and reaction time (X_2) from 2 to 3.5 h, the DS reached more than 1.4. Fig. 4C and D displayed the influence of reaction temperature (X_1) and volume ratio of Pyr to CSA (X_3) on the DS value of DHPD1 when the reaction time was fixed at 3.0 h. Fig. 4C suggested that when reaction temperature (X_1) and volume ratio of Pyr to CSA (X_3) increased, the DS increased slowly, and then decreased obviously. When reaction temperature (X_1) and volume ratio of Pyr to CSA (X_3) ranged from 50 to 70 °C and 1:1 to 3:1, respectively, the DS achieved a high value than 1.4 (Fig. 4D). The effects of reaction time (X_2) and volume ratio of Pyr to CSA (X_3) on the DS of DHPD1 were shown in Fig. 4E and F when the reaction temperature (X_1) was fixed at 60 °C. The DS value increased gradually first and decreased drastically with the increasing of reaction time (X_2) and volume ratio of Pyr to CSA (X_3). As presented in Fig. 4F, the DS reached the value over 1.4 when reaction time (X_2) and volume ratio of Pyr to CSA (X_3) ranged from 2 to 3.5 h and 1:1 to 3:1, respectively.

3.2.8. Confirmative test

According to the quadratic polynomial equation regressed with the data of RSM experiment, the predicted highest value of 1.527 could be obtained under the following reaction conditions: 60.08 °C of reaction temperature, 2.62 h of reaction time and 1.84:1 of Pyr to CSA volume ratio. To confirm this data and consider the operability in actual experiment, the optimal conditions were modified as following: 60 °C of reaction temperature, 160 min of reaction time and 2:1 of Pyr to CSA volume ratio. Under the modified conditions, the DS was 1.473 ± 0.017 (N = 3), which is very close to the predicted value. Compared with the original DHPD1, the MW of sulfated sample SDHPD1 was much higher than that of DHPD1 (Fig. 1), suggesting that polymerization might be occurred among DHPD1 molecules during the course of sulfation.

3.3. Anti-glycation analysis of DHPD1 and SDHPD1

In general, the reaction process of protein non-enzymatic glycation could be divided into three stages including early, intermediate and late stages. To inhibit any process can reduce the formation of advanced glycation end products (AGEs). In the early stage, the reducing sugars (glucose, mannose, fructose, etc.) react with the free amino groups of proteins to form an unstable Schiff base and then rearrange to form the stable Amadori products (Wu et al., 2009). As shown in Fig. 5A, all of SDHPD1, DHPD1 and aminoguanidine (AG) exhibited inhibitory effects on the formation of Amadori products. All samples displayed an increase profile of inhibitory activities with the culture proceeding from 0 to 21 day and then decrease to different levels. In the three cases, the capability of inhibiting the formation of Amadori products was indicated to be in the order of SDHPD1 > DHPD1 > aminoguanidine throughout the culture period when the agents were fixed at the same concentration. The peak inhibition of 80.2% was observed in the group with 1.0 mg/mL SDHPD1 treatment at the culture time of 21 day, which was 1.49-fold and 2.21-fold that of DHPD1 and aminoguanidine, respectively.

In the intermediate stage of protein non-enzymatic glycation, the Amadori products were converted to methylglyoxal, glyoxal and deoxyglucosones through oxidation and dehydration reactions (Wu et al., 2009). These dicarbonyl compounds are more reactive than their donors and easily continue to react with the free amino groups of proteins to produce the AGEs. As shown in Fig. 5B, all samples could inhibit the formation of dicarbonyl compounds. At the same dose, the ability of the three agents to inhibit dicarbonyl compound formation was in the order of aminoguanidine > SDHPD1 > DHPD1. It has been reported that the inhibitory mechanism of polysaccharide from *Misgurnus anguillicaudatus* on protein non-enzymatic glycation *in vitro* was mainly due to its strong inhibitory effects on dicarbonyl compound formation (Zhang et al., 2010b).

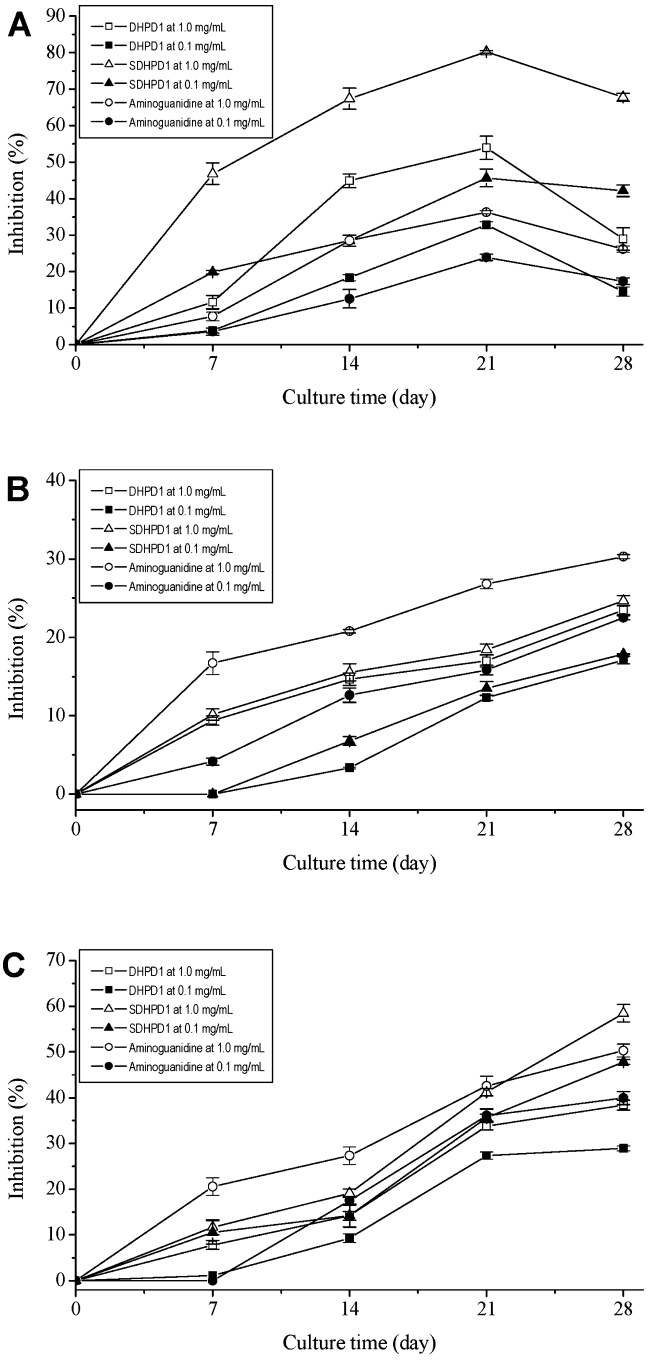

Fig. 5. Inhibitory effects of DHPD1, SDHPD1 and aminoguanidine on anti-glycated activities. (A) Inhibit Amadori product formation; (B) inhibit dicarbonyl compound formation; (C) inhibit AGEs formation.

In the late stage, the dicarbonyl compound resulted from the intermediate stage of protein non-enzymatic glycation, continues to react with the free amino groups and irreversibly form the yellow-brown compounds, named AGEs (Wu et al., 2009). As shown in Fig. 5C, SDHPD1 and aminoguanidine showed stronger inhibitory effects on AGEs formation than DHPD1 within the range of tested concentrations. As far as the inhibitory process was concerned, the three tested samples displayed similar increase patterns of inhibitory ability with the culture proceeding. After 28 days of incubation, SDHPD1 at 1.0 mg/mL showed the highest inhibitory ability of 58.5%, which increased by 16.2% and 52.5% than that of aminoguanidine and DHPD1 at the same dosage.

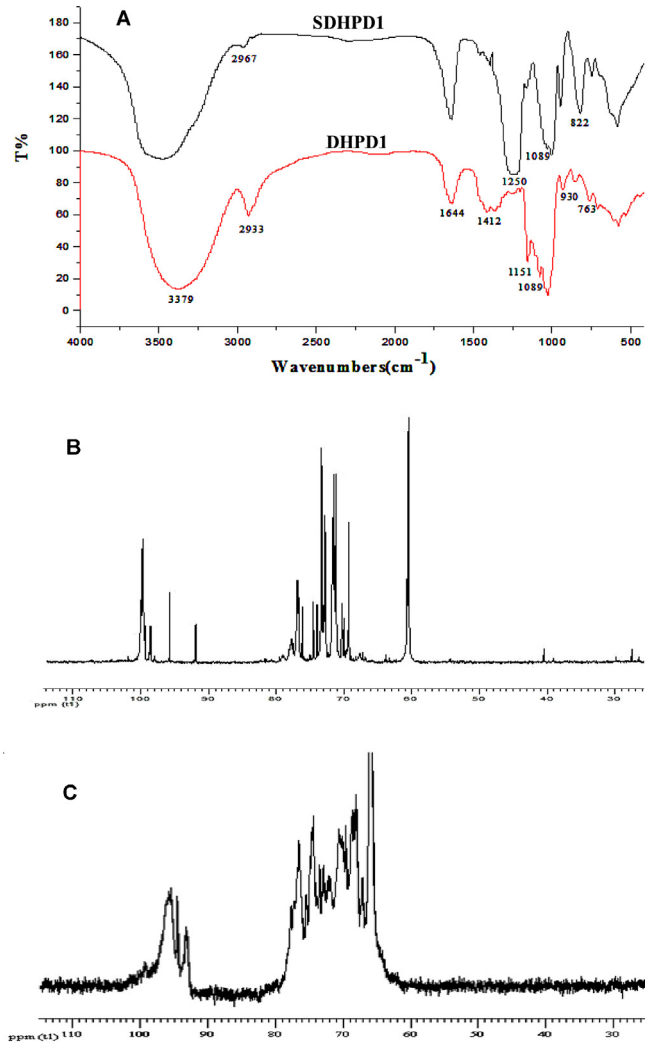

Fig. 6. FT-IR and ^{13}C NMR spectra of DHPD1 and SDHPD1. (A) FT-IR; (B) ^{13}C NMR spectrum of DHPD1; (C) ^{13}C NMR spectrum of SDHPD1.

3.4. Structure characterization of DHPD1 and SDHPD1

The FT-IR spectrum of DHPD1 showed a broad stretching intense characteristic peak at around 3379 cm^{-1} for —OH and a narrow stretching characteristic peak at around 2933 cm^{-1} for C—H (Fig. 6A). The intense peaks at around 1644 cm^{-1} and 1412 cm^{-1} were referred to C=O asymmetric stretching vibration and symmetric stretching vibration in carbonyl groups. The peaks of 1151 and 1089 cm^{-1} were referred to C—O stretching vibration of ring ether C—O—C and O—H variable angle vibration of C—O—H, respectively. In comparison with the spectrum of DHPD1 (Fig. 6A), two new intense absorption peaks were observed at 1250 cm^{-1} and 822 cm^{-1} attributed to asymmetrical S=O stretching vibration and symmetrical C—O—S vibration, respectively. Similar results were also found in other sulfated polysaccharides (Sun et al., 2009a; Sun, Liang, Zhang, et al., 2009; Wei et al., 2012; Yang Du and Huang, 2002). Moreover, the absorption peak at 2933 cm^{-1} of C—H stretching vibration became weaker and moved to 2967 cm^{-1}, suggested that sulfate substitution may occur on the position of C-6. The variable angle vibration absorption peak of 1089 cm^{-1} decreased significantly, which could be the result of hydroxyl group replaced partly by sulfate groups (Sudipta, Navid, Shruti, Paul, & Ray, 2012).

Methylation analysis revealed that DHPD1 contained the glycosyl linkages of $(1\rightarrow4)$-linked-Glc*p*, $(1\rightarrow4,6)$-linked-Glc*p*, $(1\rightarrow6)$-linked-Gal*p*, $(1\rightarrow6)$-linked-Glc*p*, $(1\rightarrow5)$-linked-Ara*f*,

1-linked-Glc*p* in a molar ratio of 7.32:1.5:0.21:0.18:0.26:1.24 and a trace of 1-linked-Xyl*p* and (1 → 3,6)-linked-Man*p*. Fig. 6B showed the ^{13}C NMR spectra of DHPD1. The chemical shifts at 99.7 ppm, 99.9 ppm, 99.6 ppm, 98.6 ppm and 99.4 ppm were assigned to the anomeric carbon signals of (1 → 4)-linked-Glc*p*, 1-linked-Glc*p*, (1 → 4,6)-linked-Glc*p*, (1 → 6)-linked-Gal*p* and (1 → 6)-linked-Glc*p*, respectively (Kardošová et al., 2004; Nie et al., 2011; Roy, Maiti, Mondal, Das, & Islam, 2008; Sudipta et al., 2012; Zheng, Liu, & Fang, 2004). However, the same signals of (1 → 3,6)-linked-Man*p*, (1 → 5)-linked-Ara*f* and 1-linked-Xyl*p* were not observed, due to their low content in DHPD1. In comparison with DHPD1, the ^{13}C NMR spectra of its sulfated derivatives became more complicated due to the existence of sulfated groups in SDHPD1. However, the chemical shift changes were easily observed around 99 ppm and 60 ppm. The signal of chemical shifts around 99 ppm of SDHPD1 was weakened while the signal around 96 ppm was strengthened (Fig. 6C). The reason of these changes were attributed to the fact that anomeric carbon splits into two peaks when C-2 replaced by the sulfuric acid (Wei et al., 2012). The chemical shifts near 60 ppm were assigned to the signal of C-6 in DHPD1. After sulfated modification, these signals were disappeared and a new strong signal was appeared at around 66 ppm, indicating that C-6 was substituted by the sulfated groups (Wei et al., 2012). Combined with the results of activity investigation, it is concluded that sulfation occurred to C-2 and C-6 were beneficial to improve DHPD1's anti-glycation activity.

4. Conclusions

An active polysaccharide DHPD1 of *D. huoshanense* was sulfated using CSA-Pyr method. Based on the single-factor-test, RSM was performed to estimate and optimize the sulfation conditions. Results showed that the maximum DS of 1.473 was obtained under the optimal sulfation condition of reaction temperature 60.08 °C, reaction time 2.62 h and Pyr to CSA volume ratio 1.84:1. Structural analysis revealed that sulfation occurred to C-2 and C-6 of glycosyl residues in DHPD1 was beneficial to enhance DHPD1's anti-glycation activity.

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