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Sulfated modification of epimedium polysaccharide and effects of the modifiers on cellular infectivity of IBDV

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Abstract

Nine modification conditions were designed to sulfate epimedium polysaccharide (EPS) by chlorosulfonic acid–pyridine method according to orthogonal test, and nine sulfated EPS (sEPS), $sEPS_1$, $sEPS_2$, $sEPS_3$, $sEPS_4$, $sEPS_5$, $sEPS_6$, $sEPS_7$, $sEPS_8$ and $sEPS_9$, were obtained and their effects on IBDV infecting chick embryo fibroblast (CEF) was compared by MTT assay. The results showed that modified sEPS had significantly enhanced CEF resisting IBDV infection in comparison of non-sulfated EPS, especially, $sEPS_2$ and $sEPS_5$ presented better effects, which there were certain relationships in the degree of sulfate group (DS) and carbohydrate content. The optimum modification conditions were the reaction temperature of 80 °C, the ratio of chlorosulfonic acid to pyridine of 1:8 and the reaction time of 2 h.

Keywords: EPS; Sulfated modification; IBDV; Cellular infectivity

1. Introduction

Polysaccharide is a class of macromolecular substance existing widely in organism. It has many biological activities, such as anti-infection, anti-cancer, anti-oxidation, immunologic enhancement and so on (Chen et al., 2005). Biological activities of a polysaccharide depend mainly on its molecular structure including sugar unit and glycosidic bond of the main chain, the types and polymerization degree of the branch and flexibility and apetial configuration of the chains (Alban, Schauerte, & Franz, 2002; Wang, Li, Guo, & Cai, 2002). Therefore, molecular modification and structure improvement of polysaccharide arouse wide concern. Most studies have demonstrated that biological activities of polysaccharide are greatly increased by molecular modification (Liu & Sun, 2005; Xing et al., 2005).

Sulfated polysaccharide, including the natural or chemically synthesized, is a kind of one with sulfated group in its hydroxyls. Many studies confirmed that they had stronger biological activities, especial anti-viral action (Baba, Snoeck, Pauwels, & Clercq, 1988; Talarico et al., 2004), which caused researchers' interest. Therefore, it may use sulfated modification to improve the biological activities of some polysaccharides and obtain more sulfated polysaccharides.

There are many methods to sulfate polysaccharides, such as sulfuric acid, sulfur trioxide–pyridine, chlorosulfonic acid (CSA)–pyridine (Pyr), sulfur trioxide–dimethylacetamide and so on. CSA–Pyr method is the most popular one. It possesses advantages of high yield and convenient manipulation. In application of this method, the ratio of CSA to Pyr and the reaction temperature and time are important effective factors (Vogl, Paper, & Franz, 2000; Yang, Jia, Shang, Mei, & Zhao, 2001).

Abbreviations: CSA, chlorosulfonic acid; Pyr, pyridine; IBD, infectious bursal disease; IBDV, infectious bursal disease virus; EPS, epimedium polysaccharide; sEPS, sulfated EPS; NDV, Newcastle disease virus; CEF, chick embryo fibroblast; TCA, trichloroacetic acid; SPF, specified-pathogens free; DMSO, dimethyl sulfoxide; MM, maintenance medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CMF, calcium and magnesium-free; PBS, phosphate-buffered saline; DS, the degree of sulfate group; CPE, cytopathic effect.

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Epimedium is a famous traditional herbal medicine using in China, Korea and Japan for treatment of hepatitis, leucopenia, infertility, chronic nephritis, osteoporosis, rheuma, asthma, menstrual irregularity and cardiovascular problems. The pharmacological researches have confirmed that these therapeutic actions are based on its effective ingredients of polysaccharide, flavonoid glycosides, ignans, terpenoids and alkaloid magnoflorine (Kovačević, Čolić, Backović, & Došlov-Kokoruš, 2006). Our previous researches demonstrated that epimedium polysaccharide (EPS) possessed resisting infectious bursal disease virus (IBDV) and Newcastle disease virus (NDV) (Hu et al., 2004, 2003) and immune enhancement activities (Chu, Yan, Li, & Hu, 2006; Hu et al., 2004; Sun, Hu, Wang, Zhang, & Liu, 2006; Wang et al., 2005, 2006). In this research, EPS was extracted and sulfatized by CSA–Pyr method which nine modification conditions were designed according to orthogonal test focusing on the above-mentioned three affecting factors. Nine sulfated EPS (sEPS), $sEPS_1$, $sEPS_2$, $sEPS_3$, $sEPS_4$, $sEPS_5$, $sEPS_6$, $sEPS_7$, $sEPS_8$ and $sEPS_9$, were obtained and their effects on IBDV to infect chick embryo fibroblast (CEF) were compared, taking non-modified EPS as control. The purpose of this study was to validate the probability of improving antiviral action of EPS through the sulfated modification and screen out the optimum reaction conditions.

2. Materials and methods

2.1. Epimedium and reagents

Epimedium was bought from Dahua Chinese traditional medicine company in Nanjing, Jiangsu province.

Chlorosulfonic acid (CSA) was produced in Jinshang ting Institute of Chemical Engineering in Shanghai, Pyridine (Pyr), Hongsheng Chemical Engineering in Wu-xi, Dimethyl sulfoxide (DMSO), Zheng-xing Institute of Chemical Engineering in Suzhou. All other chemicals used were of analytical grade.

MEM (GIBCO) supplemented with benzylpenicillin 100 IU/ml, streptomycin 100 IU/ml and 5% fetal bovine serum, was used as nutritive medium. For maintenance medium (MM), the serum concentration was reduced to 2%. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco) was dissolved with calcium and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.4) to 5 mg/ml. These three reagents were filtered through a 0.22 μm filter.

2.2. Cell and virus

The CEF were obtained from 10 days old chick embryo (specified-pathogens free, SPF; purchased from Nanjing Pharmaceuticals and Instruments Factory) as follows: First, the gas chamber of chorion was disinfected with ethanol and opened. The chick embryo was taken out and put into a Petri dish to eliminate the head, extremities and viscera, and washed with Hanks' solution to eliminate blood. Second, the chick embryo was cut into pieces in a beaker of about 1–2 mm^3 and washed for three times with Hanks' solution, and trypsinized at 0.25% concentration at 37 °C for 30 min. Third, gently removed the trypsin solution and washed the tissues three times by Hanks' solution, then suspended in MEM beating upon fully by pipette of big orifice and filtered through a 3-tier gauze. Finally cells were cultured in 96-well plates (100 μl/well, 1×10^6/ml) in nutritive medium at 37 °C in the CO_2 incubator for 24 h into single layer, and then replaced with MM for maintaining.

IBDV (NF8, provided by NanJing Pharmaceuticals and Instruments Factory) was obtained from allantois by the means of innoculation and passage of allantoic cavity of chicken embryo. $TCID_{50}$ of the virus was 1×10^{-5} by Reed–Mueeh assay (Hu et al., 2003) and diluted with MM to 1×10^{-3} (100 $TCID_{50}$) in the experiment.

2.3. Extraction and purification of EPS

EPS was extracted by water decoction and ethanol precipitation (Kong, Hu, Rui, Wang, & Li, 2004). And then EPS was purified seriatim by trichloroacetic acid (TCA) method to eliminate protein (Chen et al., 2005), permeating through macroporous adsorption resin (ADS-7) to eliminate protein and pigment, and DEAE Sephadex™ A-25 to separate from other carbohydrate according to Li (2005). All dialysis in the process of the isolation were substituted for ultrafiltration using 1 kDa ultrafiltration membrane (Green Bird Science & Technology Development Co.Ltd., China). Finally the liquor was freeze drying. The carbohydrate concentration (%) of total EPS was 20.59 comparing with D-glucose.

2.4. Sulfated modification of EPS

2.4.1. Design of modification condition

To investigate the effect of three factors on sulfation, such the ratio of CSA to Pry, reaction temperature and time were tested at different concentrations. Three levels per factor were used with the ratio of CSA to Pry of 1:6, 1:8 and 1:10, the reaction temperature of 60 °C, 80 °C and 95 °C, and the reaction time of 1, 2 and 3 h, respectively (Chen et al., 2005). Nine reacting conditions were designed according to orthogonal test as L_9 (3^4) (Table 1).

2.4.2. Preparation of sulfating reagent

CSA was dropped one by one in pyridine (25 ml) filled in three-necked flask, under agitating and cooling in ice water bath (Liu & Sun, 2005), and the ratio of CSA to pyridine referred to Table 1. All determinations were completed in 40 min and nine kinds of sulfating reagents were obtained.

2.4.3. Sulfation reaction

EPS (500 mg) was added in the three-necked flask filled with sulfating reagent, and the mixture was stirred for various durations and/or temperatures (Table 1). The

Table 1
The yield, DS and the carbohydrate content of sulfated EPS

sEPSs	Sulfated condition			Yield (mg)	DS (%)	Carbohydrate content (%)
	(*A*) Temperature (°C)	(*B*) CSA: Pyr	(*C*) Time (h)			
$sEPS_1$	60	1:6	1	153	0.097	31.843
$sEPS_2$	60	1:8	2	159	0.484	62.474
$sEPS_3$	60	1:10	3	167	1.120	50.949
$sEPS_4$	80	1:6	3	142	0.772	49.938
$sEPS_5$	80	1:8	1	133	0.696	72.380
$sEPS_6$	80	1:10	2	124	0.834	28.049
$sEPS_7$	95	1:6	2	241	0.351	21.027
$sEPS_8$	95	1:8	3	255	0.074	27.699
$sEPS_9$	95	1:10	1	148	0.089	36.190

determinations were performed nine times for nine kinds of sulfating reagents. After the reaction, the mixture was cooled to room temperature and the pH was adjusted to pH 7–8 with 15% NaOH solution. The nine obtained polymers were precipitated with EtOH (95%), washed, and redissolved in water, and then dialyzed using 1 kDa ultrafiltration membrane in lotic distilled water for 72 h to remove pyridine, salt and potential degradation products (Talarico et al., 2004). At last the sulfated polysaccharides were collected after lyophilizing and kept in dryness box and named $sEPS_1$, $sEPS_2$, $sEPS_3$, $sEPS_4$, $sEPS_5$, $sEPS_6$, $sEPS_7$, $sEPS_8$ and $sEPS_9$. EPS and 9 sEPSs were dissolved in MM to the concentration of 2 mg/ml and filtered through a 0.22 μm filter respectively in the experiment.

2.5. Content determination of sEPS

Total carbohydrate was estimated by the phenol–sulfuric acid method using D-galactose as standard. The substitution degree with sulfate was established on the basis of the sulphate content, determined by barium chloride–gelatin assay. The DS (w/w) was calculated according to the equation: $DS = 162 \times (SO_4^{2-}\%)/100 - (96/98 \times SO_4^{2-}\%)$ (Zhang, 1999).

2.6. Cytotoxicity analysis

CEF viability was measured by the MTT assay (Kaneko, Fujiwara, Mori, & Shigeta, 2001). Confluent cultures, grown in 96-well plates for 24 h to monolayer, were exposed to different concentrations of the EPS and 9 sEPSs diluted with MM, with six wells for each concentration, using MM alone as control. Treated cultures were incubated at 37 °C in the CO_2 incubator. The cultures were daily observed for evidence of cytopathic effect (CPE, partial or complete loss of the monolayer, rounding or shrinkage of the cells). After 72 h incubation, 20 μl MTT (5 mg/ml) was added into each well and the plates were reincubated for 4 h. The supernatant was removed carefully and 100 μl of DMSO were added into each well to dissolve the formazan crystals. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance of cell in each well was measured by microliter enzyme-linked immunosorbent assay reader (Model DG-3022, East China Vacuum Tube Manufacturer) at a wave length of 570 nm.

2.7. Antiviral assay

When CEF cultured into monolayer, the serial twofold dilutions of polysaccharides (diluted with MM) and IBDV were added respectively as follows:

Pre-adding polysaccharide: polysaccharides solution were added on CEF firstly (100 μl/well, four wells per concentration), removed after incubated for 2 h at 37 °C, then the virus solution was added after the cell were washed twice with Hanks' solution.

Post-adding polysaccharide: virus solution was added on CEF firstly, then removed after incubated for 2 h at 37 °C, then polysaccharides solution were added after the cell were washed twice with Hanks' solution, four wells for each concentration.

Simultaneous adding polysaccharides and IBDV: polysaccharides solution and virus solution were preincubated for 2 h at 4 °C, and then added on CEF, four wells for each perform.

All groups were keeping on cultivation at 37 °C in the CO_2 incubator. When the IBDV control group appeared obviously CPE (72 h), CEF viability was measured by the MTT assay. All determinations were performed triplicate and with virus and cell as control. OD_{570} value is the indicator of antiviral activity. If the OD mean value of one polysaccharide is significantly higher than that of virus control group, the polysaccharide has obviously antiviral activity.

2.8. Statistical analysis

Data are expressed as the mean ± SEM. Duncan's multiple range test was used to determine the difference polysaccharides and control groups. Differences between means were considered significant at $P < 0.05$.

3. Results

3.1. The DS and carbohydrate content of sEPSs

The modification conditions of orthogonal design and the yield, DS and carbohydrate content of the obtained

polymers are listed in Table 1. The yield of $sEPS_8$ and $sEPS_7$ were the highest, respectively, 255 and 241 mg. The highest DS was $sEPS_3$ (1.120%), and next were $sEPS_6$, $sEPS_4$, $sEPS_5$ and $sEPS_2$. The highest carbohydrate content (comparable with those of glucose) was $sEPS_3$ (72.380%), and next were $sEPS_2$, $sEPS_3$ and $sEPS_4$.

3.2. Cytotoxicity

The maximum safe concentration of EPS, $sEPS_1$, $sEPS_8$ and $sEPS_9$ was 500 μg/ml, while that of $sEPS_2$, $sEPS_3$, $sEPS_4$, $sEPS_5$, $sEPS_6$ and $sEPS_7$ was 250 μg/ml. The maximum concentration tested of all the polysaccharides was 250 μg/ml in order to be easy to compare.

3.3. Antiviral activity in pre-adding polysaccharides

The results are listed in Table 2. The OD value of EPS group had no significant difference compared with IBDV control group at all concentrations ($P > 0.05$). At 250–125 μg/ml, the OD value of all sEPS groups except $sEPS_8$ group were significantly larger than that of IBDV control group ($P < 0.05$). The OD value of $sEPS_1$, $sEPS_2$, $sEPS_4$, $sEPS_5$ and $sEPS_6$ groups at 62.5 μg/ml and $sEPS_2$, $sEPS_5$ and $sEPS_6$ groups at 31.25 μg/ml were significantly larger than that of IBDV control group ($P < 0.05$). At 7.813–1.953 μg/ml, the OD value of all treatment groups were not significantly different with IBDV control group ($P > 0.05$, data omitted).

3.4. Antiviral activity in post-adding polysaccharides

The results are listed in Tables 3 and 4. The OD value of the EPS, $sEPS_1$, $sEPS_2$, $sEPS_6$, $sEPS_8$ and $sEPS_9$ groups at 250–15.625 μg/ml, the $sEPS_1$ and $sEPS_2$ groups at 7.813 μg/ml and the $sEPS_2$ group at 3.906 μg/ml were significantly larger than that of IBDV control group ($P < 0.05$).

3.5. Antiviral activity in simultaneous adding polysaccharides with IBDV

The results are listed in Table 5.The OD value of all treatment groups at 250–15.625 μg/ml were significantly larger than that of IBDV control group ($P < 0.05$) (the data of 250–31.25 μg/ml omitted). At 7.813 μg/ml the OD value of all treatment groups except EPS and $sEPS_4$ groups and at 3.906 μg/ml the $sEPS_5$, $sEPS_7$ and $sEPS_9$ groups were significantly larger than that of IBDV control group ($P < 0.05$).

4. Discussion

The OD value is an index to reflect living cells. The larger OD value becomes, the more living cells is and the better antiviral activity polysaccharide has. The experimental results showed that the antiviral activity of sulfated sEPSs had obviously enhanced. During pre-adding polysaccharides, the sulfated sEPSs presented significant antiviral activity while non-sulfated EFS had not significant action (Table 3); During post-adding polysaccharides (Tables 3 and 4) and simultaneous adding polysaccharides with IBDV (Table 5), the effective concentration of sulfated sEPS were lower than that of non-sulfated EPS. The antiviral activity of $sEPS_2$ and $sEPS_5$ were the best among three administrations modes since their OD values were the highest at high concentration, and the effective concentration was the lowest.

SO_4^{2-} content of sulfated polysaccharide was one of important effective factors for its antiviral action. It was reported that polyglucosan did not have any antiviral activities, while sulfated polyglucosan had strong anti-HIV effect. In addition some sulfated polysaccharides lost their antiviral activities when de-sulfated (Alban et al., 2002; Wang et al., 2002). The mechanism is that acid polyanion, e.g. SO_4^{2-}, can combine with the positive ions on the virus or cell surface, thus stereo-inhibit viral adsorption

Table 2
OD_{570} values of every group at 250–15.625 μg/ml during pre-adding polysaccharides

Groups	Concentration (μg/ml)				
	250	125	62.5	31.25	15.625
EPS	0.133 ± 0.022^{c}	0.140 ± 0.022^{bc}	0.135 ± 0.021^{c}	0.103 ± 0.005^{c}	0.102 ± 0.025^{c}
$sEPS_1$	0.178 ± 0.005^{b}	0.170 ± 0.032^{b}	0.173 ± 0.029^{b}	0.145 ± 0.017^{c}	0.135 ± 0.014^{c}
$sEPS_2$	0.200 ± 0.015^{b}	0.187 ± 0.012^{b}	0.204 ± 0.064^{b}	0.210 ± 0.016^{b}	0.160 ± 0.008^{bc}
$sEPS_3$	0.212 ± 0.043^{b}	0.178 ± 0.035^{b}	0.158 ± 0.025^{bc}	0.160 ± 0.017^{bc}	0.155 ± 0.031^{bc}
$sEPS_4$	0.235 ± 0.013^{b}	0.220 ± 0.010^{b}	0.228 ± 0.015^{b}	0.203 ± 0.051^{b}	0.183 ± 0.041^{c}
$sEPS_5$	0.230 ± 0.010^{b}	0.223 ± 0.006^{b}	0.183 ± 0.021^{b}	0.170 ± 0.010^{b}	0.166 ± 0.017^{c}
$sEPS_6$	0.223 ± 0.013^{b}	0.203 ± 0.021^{b}	0.180 ± 0.000^{b}	0.165 ± 0.024^{b}	0.156 ± 0.032^{bc}
$sEPS_7$	0.173 ± 0.040^{b}	0.168 ± 0.009^{b}	0.160 ± 0.010^{bc}	0.130 ± 0.008^{cd}	0.130 ± 0.018^{c}
$sEPS_8$	0.158 ± 0.029^{bc}	0.158 ± 0.015^{bc}	0.163 ± 0.015^{bc}	0.135 ± 0.013^{bc}	0.122 ± 0.021^{c}
$sEPS_9$	0.177 ± 0.015^{b}	0.168 ± 0.005^{b}	0.153 ± 0.006^{bc}	0.160 ± 0.010^{bc}	0.160 ± 0.011^{bc}
Virus control	0.113 ± 0.005^{c}	0.113 ± 0.005^{c}	0.113 ± 0.005^{c}	0.113 ± 0.005^{c}	0.113 ± 0.005^{c}
Cell control	0.305 ± 0.026^{a}	0.305 ± 0.026^{a}	0.305 ± 0.026^{a}	0.305 ± 0.026^{a}	0.305 ± 0.026^{a}

$^{a-d}$: Column data marked without the same superscripts differ significantly ($p < .05$). The same is as follows.

Table 3
OD_{570} values of every group at 250–31.25 μg/ml during post-adding polysaccharides

Groups	Concentration (μg/ml)			
	250	125	62.5	31.25
EPS	0.210 ± 0.040^{b}	0.283 ± 0.084^{b}	0.207 ± 0.031^{b}	0.203 ± 0.015^{b}
$sEPS_1$	0.193 ± 0.012^{b}	0.220 ± 0.014^{b}	0.203 ± 0.015^{b}	0.228 ± 0.028^{b}
$sEPS_2$	0.203 ± 0.021^{b}	0.213 ± 0.015^{b}	0.223 ± 0.017^{b}	0.235 ± 0.013^{b}
$sEPS_3$	0.161 ± 0.011^{bc}	0.160 ± 0.007^{bc}	0.158 ± 0.019^{bc}	0.185 ± 0.006^{b}
$sEPS_4$	0.133 ± 0.019^{c}	0.123 ± 0.025^{c}	0.128 ± 0.009^{c}	0.158 ± 0.010^{bc}
$sEPS_5$	0.152 ± 0.015^{bc}	0.153 ± 0.037^{bc}	0.155 ± 0.003^{bc}	0.153 ± 0.028^{bc}
$sEPS_6$	0.195 ± 0.013^{b}	0.198 ± 0.050^{b}	0.185 ± 0.064^{b}	0.193 ± 0.067^{b}
$sEPS_7$	0.200 ± 0.030^{b}	0.206 ± 0.032^{b}	0.203 ± 0.047^{b}	0.168 ± 0.071^{bc}
$sEPS_8$	0.193 ± 0.032^{b}	0.233 ± 0.071^{b}	0.233 ± 0.015^{b}	0.203 ± 0.031^{b}
$sEPS_9$	0.198 ± 0.049^{b}	0.183 ± 0.021^{b}	0.203 ± 0.047^{b}	0.200 ± 0.026^{b}
Virus control	0.118 ± 0.010^{c}	0.118 ± 0.010^{c}	0.118 ± 0.010^{c}	0.118 ± 0.010^{c}
Cell control	0.311 ± 0.048^{a}	0.311 ± 0.048^{a}	0.311 ± 0.048^{a}	0.311 ± 0.048^{a}

Table 4
OD_{570} values of every group at 15.625–1.953 μg/ml during post-adding polysaccharides

Groups	Concentration (μg/ml)			
	15.625	7.813	3.906	1.953
EPS	0.197 ± 0.025^{b}	0.151 ± 0.033^{bc}	0.149 ± 0.022^{bc}	0.124 ± 0.046^{b}
$sEPS_1$	0.220 ± 0.017^{b}	0.190 ± 0.018^{b}	0.154 ± 0.009^{bc}	0.112 ± 0.077^{b}
$sEPS_2$	0.198 ± 0.004^{b}	0.183 ± 0.006^{b}	0.165 ± 0.028^{b}	0.143 ± 0.064^{b}
$sEPS_3$	0.150 ± 0.000^{bc}	0.144 ± 0.014^{bc}	0.135 ± 0.024^{bc}	0.118 ± 0.018^{b}
$sEPS_4$	0.123 ± 0.015^{bc}	0.103 ± 0.029^{c}	0.090 ± 0.012^{c}	0.091 ± 0.015^{b}
$sEPS_5$	0.151 ± 0.017^{bc}	0.103 ± 0.005^{c}	0.105 ± 0.013^{c}	0.101 ± 0.015^{b}
$sEPS_6$	0.168 ± 0.050^{b}	0.158 ± 0.050^{bc}	0.105 ± 0.013^{c}	0.095 ± 0.006^{b}
$sEPS_7$	0.157 ± 0.041^{bc}	0.148 ± 0.055^{c}	0.110 ± 0.034^{c}	0.118 ± 0.052^{b}
$sEPS_8$	0.173 ± 0.025^{b}	0.153 ± 0.055^{bc}	0.133 ± 0.042^{c}	0.129 ± 0.025^{b}
$sEPS_9$	0.170 ± 0.010^{b}	0.131 ± 0.030^{c}	0.121 ± 0.035^{c}	0.114 ± 0.015^{b}
Virus control	0.118 ± 0.010^{c}	0.118 ± 0.010^{c}	0.118 ± 0.010^{c}	0.118 ± 0.010^{b}
Cell control	0.311 ± 0.048^{a}	0.311 ± 0.048^{a}	0.311 ± 0.048^{a}	0.311 ± 0.048^{a}

Table 5
OD_{570} values of every concentration group 15.625–1.953 μg/ml during simultaneous adding polysaccharides with IBDV

Groups	Concentration (μg/ml)			
	15.625	7.813	3.906	1.953
EPS	0.174 ± 0.035^{b}	0.133 ± 0.026^{e}	0.101 ± 0.016^{c}	0.109 ± 0.061^{b}
$sEPS_1$	0.205 ± 0.045^{b}	0.193 ± 0.023^{b}	0.148 ± 0.064^{bc}	0.130 ± 0.021^{b}
$sEPS_2$	0.188 ± 0.016^{b}	0.173 ± 0.015^{b}	0.158 ± 0.025^{bc}	0.128 ± 0.014^{b}
$sEPS_3$	0.209 ± 0.025^{b}	0.163 ± 0.078^{b}	0.155 ± 0.017^{bc}	0.131 ± 0.064^{b}
$sEPS_4$	0.175 ± 0.056^{b}	0.160 ± 0.008^{bc}	0.158 ± 0.015^{bc}	0.131 ± 0.024^{b}
$sEPS_5$	0.220 ± 0.024^{b}	0.170 ± 0.010^{b}	0.169 ± 0.021^{b}	0.141 ± 0.036^{b}
$sEPS_6$	0.219 ± 0.054^{b}	0.168 ± 0.036^{b}	0.140 ± 0.014^{bc}	0.133 ± 0.006^{b}
$sEPS_7$	0.200 ± 0.032^{b}	0.183 ± 0.025^{b}	0.163 ± 0.015^{b}	0.155 ± 0.021^{b}
$sEPS_8$	0.220 ± 0.015^{b}	0.188 ± 0.021^{b}	0.156 ± 0.065^{bc}	0.119 ± 0.021^{b}
$sEPS_9$	0.233 ± 0.006^{b}	0.200 ± 0.049^{b}	0.164 ± 0.031^{b}	0.151 ± 0.021^{b}
Virus control	0.128 ± 0.009^{c}	0.128 ± 0.009^{c}	0.128 ± 0.009^{c}	0.128 ± 0.009^{b}
Cell control	0.330 ± 0.018^{a}	0.330 ± 0.018^{a}	0.330 ± 0.018^{a}	0.330 ± 0.018^{a}

and stop the virus to enter the cell or to replicate in the cell. This inhibiting effect is changed along with the sulfated degree of the polysaccharides (Wang & Guan, 2000; Wu & Mao, 2002). In this research, we found that the relationship between the antiviral activity and DS of sulfated EPS was not exact direct correlation, because the antiviral effect of $sEPS_3$ with highest DS (1.120) was not the best, while $sEPS_2$ and $sEPS_5$ with middle DS (0.484% and 0.696%) presented the best activity. This indicated that DS of sulfated polysaccharides must be within optimum scope. The higher DS was likely to be concerned with the cytotoxicity (Liu & Yang et al., 2004; Liu, He, Yang, Pu, & Zhang, 2004).

This experiment showed that the antiviral activity of sulfated polysaccharides was direct correlation with carbohydrate content. $sEPS_2$ and $sEPS_5$ with higher carbohydrate content (62.474% and 72.380%) displayed better efficacy.

This was likely related to the finding that sulfated dextran could inhibit the syncytium formation (Yang & Meng, 2002).

Lots of researches confirmed that the activity of sulfated polysaccharides is concerned with administration mode and dose (Wang & Guan, 2000). To compare three administration modes, simultaneous adding of polysaccharides and IBDV after mixed for 2 h presented better effect which all sEPSs displayed a certain extent of antiviral action. This was likely that polysaccharides directly killed virus or their acid polyanion combined with the positive ions of virus surface in advance during mixing (Liu & Yang et al., 2004; Liu & He et al., 2004). During pre-adding polysaccharides, the effect which most sEPSs displayed a certain extent of antiviral action was next to simultaneous adding. It was likely that sEPSs combined with cell firstly to competitively inhibit IBDV to adhere to cell. Also it was probable that sEPSs inhibited the antigen expression, plasmodial formation and enzyme activity of IBDV (Katsuraya, 1999).

The CSA–Pyr method has many advantages, such as high DS, high yield and convenient production reclamation and so on (Baba et al., 1988). Therefore, it was selected in this experiment. Many studies indicated that esterification could be enhanced by means of increasing reaction temperature, enlarging the percentage of CSA and extending reaction time (Chen et al., 2005). To analyze the orthogonal results of this experiment, the range (R) values of temperature (A), proportion of CSA–Pyr (B) and time (C) were 0.596, 0.274 and 0.387, respectively, that is $R_A > R_C > R_B$, which showed that the affecting DS of three factors were temperature (A) > time (C) > proportion of CSA–Pyr (B).

It was reported that the DS could be enhanced when reaction temperature was gone up to 80 °C from 60 °C, but the DS and carbohydrate content were obviously decreased when up to 95 °C. If the proportion of CSA was over high, it could cause hardening, difficult stirring and uneven reaction in practical manipulation (Shi, Xu, Ao, Sun, & Tao, 2003).

According to antiviral effect, DS and carbohydrate content of sEPS, the optimum sulfated conditions of EPS should be 80 °C of the reaction temperature, 1:8 of CAS–Pyr ratio and 2 h of reaction time.

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