Preparation of a sulfated exopolysaccharide (S-EPS) from Ophiocordyceps sinensis fungus and its antioxidant effects

Tran Minh Trang, Nguyen Thi Xuan Phuong, Nguyen Thi Lai, Le Thi Thuy Hang, Huynh Thu, Dinh Minh Hiep

***Tóm tắt*—Sulfated exopolysaccharides have been well-known to enhance biological activities. Exopolysaccharide (EPS) produced by *Ophiocordyceps sinensis* fungus is a source of natural compounds. The aim of our study is to improve the EPS biological activities by its sulfated modification using the chlorosulfonic acid (CSA)-pyridine (Pyr) method. The appropriate conditions of the sulfation reaction were explored, including CSA/Pyr ratio (v/v) of 1:3 and 6h. The degree of substitution (DS) of S-EPS_{11} was the highest (DS = 1.59). The total contents of polysaccharides and SO_4^{2-} of S-EPS_{11} were 52.25% and 47.15%, respectively. Besides, the FT-IR spectra analysis indicated the presence of C-O-S (peak of 815 cm^{-1}) and S=O (peak of 1129 cm^{-1}) stretching vibrations, while the natural EPS did not appear. Importantly, $OH^{\bullet}$ and $ABTS^{\bullet}$ radical scavenging potential of S-EPS_{11} significantly increased compared with those of the natural EPS. Together, we successfully generated sulfated EPS extracted from *O. sinensis* fungus which enhanced antioxidant activities of natural EPS.**

***Từ khóa*—Antioxidant activity, *Ophiocordyceps sinensis*, exopolysaccharide, sulfated modification, FT-IR spectra analysis**

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1 INTRODUCTION

Ophiocordyceps sinensis (syn. *Cordyceps sinensis*), an insect-parasitic fungus, is known as a natural source in Chinese traditional medicine [1–3]. Major bioactive compounds from *O. sinensis* including adenosine, cordycepin, polysaccharides, proteins and amino acids, are reported to have antioxidant activity, anti-inflammatory, anti-tumor and immunomodulatory [3, 4]. Among them, polysaccharide/exopolysaccharides represented as one of the most prominent compounds of antioxidants, especially the crude expolysaccharides (EPS) isolated from the *O. sinensis* culture broth [5, 6]. To improve such of activities, sulfation has widely applied to modify the natural polysaccharides [7, 8].

Sulfated modification is an effective method to increase the biological activities of polysaccharides. It enhances the water solubility of polysaccharides due to the presence of polyanionic charges that which leads to increasing of absorption and application as functional foods [2]. In addition, recent studies have demonstrated that sulfated polysaccharides increased remarkable bioactivities such as antitumor, antiviral, anticoagulant and antithrombotic [2, 3]. For instance, Yan *et al.* obtained four sulfated EPS-1 derivatives of *C. sinensis* fungus by using chlorosulfonic acid (CSA)-pyridine (Pyr) method [2]. The results showed that the antioxidant properties of these derivatives for $OH^{\bullet}$ and $ABTS^{\bullet}$ radical scavenging potentials considerably rose compared to non-sulfated EPS-1. Similarly, the

immunomodulatory activity of macrophages of a sulfated polysaccharide (S-CP_{1-8}) from *Cyclocarya paliurus* was also significantly improved [9]. However, the bioactivities were closely related to their physicochemical features, such as molecular weight (M.W.) and sulfate contents, in which antioxidant activity of sulfated derivatives were correlated with increasing sulfate contents, but decreasing M.W. [2, 10].

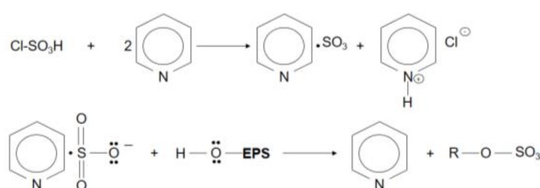

Fig. 1. The reaction mechanism for sulfation of EPS by CSA/Pyr

Currently, sulfated modification of polysaccharides was performed by a variety of various methods, including oleum dimethylformamide, sulfur trioxide pyridine (SO_3-Pyr), chlorosulfonic acid (CSA)-pyridine (Pyr) and aminosulfonic acid (ASA)- pyridine (Pyr) method [2, 8, 11, 12]. Of those, the CSA-Pyr method has widely used due to capacity of the high yield and degree of substitution (DS) [13]. The sulfated reaction underwent two steps (Fig. 1), consisting of SO_3-Pyr complex and sulfated derivative formation [2]. The yield and DS depended on CSA/Pyr ratio, reaction time and temperature [14]. Specifically, the antioxidant properties of these derivatives and DS interrelate closely [2]. In this study, we obtained sulfated EPS iosolated from *O. sinensis* fungus by CSA-Pyr method. In addition, the enhancement of the sulfated EPS in free radical scavenging potentials was experimented as well.

2 MATERIAL AND METHODS

Fungal strain and cultivation

O. sinensis strain was obtained from Dr. Truong Binh Nguyen as a kind gift (Dalat University, Vietnam). The strain was maintained on potato dextrose agar (PDA) (200 g/L potatoes, 50 g/L glucose and 15 g/L agar, initial pH 7.0) at 4 ^oC. To harvest liquid broth and biomass, the fungus was inoculated (4% of seed) in a medium containing 200 g/L potatoes, 50 g/L saccharose, 4 g/L yeast extract, 6 g/L peptone, 0.5 g/L KH_2PO_4 and 0.1 g/L $MgCl_2$, initial pH 7.0, at 22 ^oC for 40 days.

Preparation of exopolysaccharide

Liquid broth was separated after harvesting the mycelial biomass and filtered through Whatman filter paper No.1. Filtrate was concentrated in a rotary vacuum evaporator at 65 ^oC. recipitated with 4 volumes of 96% ethanol (v/v) overnight at 4 ^oC and centrifuged at 8000 rpm for 10 min. The precipitation was washed 2-3 times with 96 % ethanol and acetone prior to lyophilizing.

EPS was optimally deproteinized by using trichloroacetic acid (TCA), hydrochloric acid (HCl) or Sevag reagent (1-butanol : chloroform = 1:4, v/v). The EPS was then lyophilized and further purified by Sephadex-G100 (Φ2.4 x 100 cm) equilibrated with 0.2 M NaCl. EPS fractions were dialyzed against distilled water by using a dialysis membrane (molecular weight cut-off of 2 kDa) for 48 h, and then lyophilized. Total polysaccharide and protein content were measured by the phenol – sulfuric acid (at 490 nm) and Bradfrod (at 595 nm) method. [5]

Sulfated modification of expolysaccharide

Experimental design

13 conditions were designed according to the table 1 with three factors, including CSA/Pyr ratio (v/v), temperature (^oC) and time (h) [15].

Table 1. The modification conditions, yield, DS and carbohydrate content of S-EPS

S-EPS	CSA/Pyr ratio (v/v)	Temperature (^oC)	Time (h)
S-EPS_1	3:1	45	4
S-EPS_2	3:1	65	2
S-EPS_3	3:1	65	6
S-EPS_4	3:1	85	4
S-EPS_5	1:1	45	2
S-EPS_6	1:1	45	6

S-EPS	CSA/Pyr ratio (v/v)	Temperature (ºC)	Time (h)
$S\text{-}EPS_7$	1:1	65	4
-EPS8	1:1	85	2
S-EPS9	1:1	85	6
S-EPS10	1:3	45	4
S-EPS11	1:3	65	6
S-EPS12	1:3	65	2
S-EPS13	1:3	85	4

Preparation of sulfation reagent

CSA was added dropwise into Pyr filled in three-necked flask, with stirring and cooling in an ice water bath [2, 14]. The ratio of CSA to Pyr referred to table 1. The process was completed within 40 min and sulfation reagents were obtained.

Sulfation reaction

EPS powder (300 mg) was suspended in anhydrous dimethyl formamide (DMF) and stirred for 30 min at room temperature. The sulfated EPS reaction was performed by adding sulfation reagent during stirred according to various conditions as table 1. After indicated time of reaction, the mixture was slowly cooled to room temperature, neutralized with 2.5 M NaOH and precipitated with 4 volumes of 96% ethanol (v/v). The pellet was harvested, washed, redissolved and dialyzed against distilled water for 72 h to remove pyridine, salt and degraded compounds. The products were finally collected by lyophilizing. [2, 12, 14].

Analysis of S-EPS

The content of polysaccharide was determined by the phenol-sulphuric acid method, using saccharose as a standard. The sulfur content of S-EPS was determined by the method of Terho and Hartiala [16], with sodium sulfate as a standard. The DS was calculated according to the equation [15, 17]:

$$DS = \frac{1.62 \times S\%}{32 - 1.02 \times S\%} \qquad (\textit{Equa. 1})$$

Where, S% is the sulfur content of sulfated derivatives. For Fourier Transform Infrared Spectroscopy (FT-IR) analysis, samples were recorded on a Perkin-Elmer Spectrum One Spectrometer in the region between 400 and 4000 cm^{-1}.

In vitro antioxidant activities

$ABTS^{\bullet -}$ radical scavenging assay

The $ABTS^{\bullet -}$ radical scavenging assay was measured according to the previous method with minor modifications [18]. Briefly, $ABTS^{\bullet -}$ was generated by mixing 7 mM ABTS stock solution with 2.45 mM $K_2S_2O_8$ in a 1:1 (v/v) ratio and stored in the dark at room temperature for 10–16 h. The solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. The reaction was initiated by adding 3 mL of $ABTS^{\bullet -}$ solution to 0.1 mL of samples. After 30 min incubation, the absorbance was measured at the wavelength of 734 nm. The control experiments were conducted by replacing of 0.1 mL sample by 0.1 mL distilled water. Ascorbic acid was used as a standard. The scavenging percentage of the samples was calculated by using the following equation:

$$I\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \qquad (\textit{Equa. 2})$$

$OH^{\bullet}$ radical scavenging assay

The hydroxyl radical scavenging assay was measured according to the previous method with minor modifications [12]. The mixture consists of 1 mL of $FeSO_4$ (3 mM), 0.35 mL of H_2O_2 (3 mM), 0.3 mL of $Na_2S_2O_8$ (40 mM) and 1 mL of sample with different concentrations which was incubated at room temperature for 1 h. The absorbance was then measured at the wavelength of 562 nm. Ascorbic acid was used as a standard. The scavenging percentage was determined by using the following equation:

$$I\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \qquad (\textit{Equa. 3})$$

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS software (Ver. 20).

All experiments were performed in triplicate. A p-value < 0.05 was considered to be statistically significant.

3 RESULTS AND DISCUSSION

Preparation of exopolysaccharide

The EPS crude has reported containing polysaccharides (64.96 %), proteins (12.72 %) and others (22.32 %) [5]. The protein molecules exist either in free forms [19] or polysaccharide-protein complex [20]. These properly shield and block functional groups, especially the hydroxyl groups of the polysaccharide chain, that prevents the sulfation process. Hence, the deproteinization of EPS was necessary to remove protein from EPS and enhance the yield of the sulfated EPS modification.

Table 2. Comparison of three different deproteinization methods

Parameters, unit	Methods		
	TCA	HCl	Sevag
Isolation yield (%)	25.62 ± 1.45^a	28.77 ± 4.23^a	42.98 ± 3.19^b
Deproteinization (%)	91.64 ± 1.22^a	90.60 ± 1.88^a	87.70 ± 0.45^a
Polysaccharide loss (%)	76.85 ± 3.70^a	76.92 ± 0.87^a	62.25 ± 3.45^b

Average values in a row with the same letter(s) above them do not differ significantly (Duncan test, p<0.05)

Our results revealed that the Sevag method was the best technique for deproteinization of the EPS (table 3). Although the rate of deproteinization of the Sevag method was not different in comparison to HCl and TCA method, the isolation yield of EPS was the highest (about 42.98 %) and the polysaccharide loss was the lowest (about 62.25 %). Therefore, Sevag method was used for deproteinization of EPS in further.

The physicochemical and structural features of a polysaccharide include monosaccharide composition, carbohydrate and protein content, molecular weight, type of glycosidic linkage, and sequence of monosaccharide, *etc* [4]. These would affect the bioactivities and chemical modification of EPS extracted from the cultured *O. sinensis*. Thus, the comprehension of these properties of EPS would help qualitative management of material resource for the sulfation process.

Expectedly, the main composition of our EPS was polysaccharide, which occupied about 68.81 % (table 3). It strongly agrees with the previous studies demonstrated that polysaccharide content of EPS accounted for an outnumbered majority ranging from 60 % and 85 %. For example, EPS extracted from another *O. sinensis* strain cultured broth contained 83.9 % of polysaccharides and 11.8 % of proteins [21]; 65 % – 70 % of polysaccharides and 25% of proteins [22]; 70 % of polysaccharides and 20 % – 25 % of proteins [23]. By contrast, only light amount of proteins (approximate 0.19 %) appears in our EPS samples indicated that the deproteinization using the Sevag method has done. Noticeably, these differences were due to various fungus train, cultured conditions and compositions as well as the extraction technique.

Table 3. Physicochemical characterization of EPS and S-EPS_{11}

Parameters, unit	EPS	S-EPS_{11}
Polysaccharide content (%)	68.81± 3.9^a	52.25 ± 8.45^b
Protein content (%)	0.185± 0.004	No detected
Sulfate content (%)	No detected	47.15 ± 1.96
Main chemical groups	-OH, C-H, C-O-C	-OH, C-H, C-O-C, S=O, C-O-S
Number of fractions	EPS-1; EPS-2	S-EPS_{11}-1

Average values in a row with the same letter(s) above them do not differ significantly (Duncan test, p<0.05)

In addition, the FT-IR spectra analysis of EPS were also performed as shown in fig. 2A. The bands in the range of 3600 – 3000 cm^{-1}, 3000 – 2800 cm^{-1}, 1700 – 1500 cm^{-1} and 1200 – 700 cm^{-1} corresponded with the absorption of polysaccharide chain [17]. The strong peak at 3386 cm^{-1} indicated stretching vibration of O-H groups and the band at 2993 cm^{-1} was C-H stretching [17, 24]. The bands at 1603 cm^{-1} and 1416 cm^{-1} suggested the presence of some residual proteins and O-H groups of phenolic compounds, respectively. At 1051 cm^{-1} peak represented C-O-C stretching [25, 26]. The stretching vibrations of S=O and C-O-S did not appeare in the natural EPS sample.

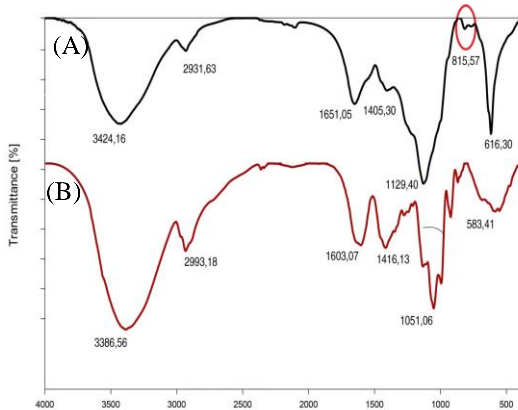

Fig 2. FT-IR analysis of S-EPS_{11} (A) and EPS (B)

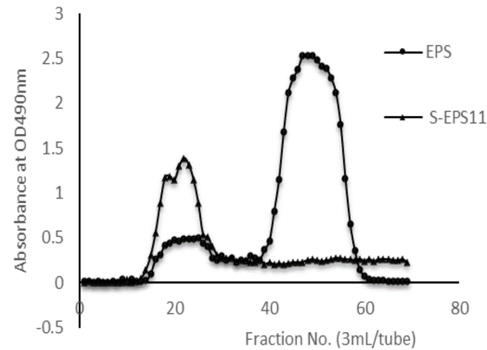

Fig 3. Elution profiles of EPS and S-EPS_{11} in Sephadex G-100 chromatography. Elution was analyzed by measuring the absorbance at 490 nm for carbohydrate

Furthermore, the gel filtration chromatography on Sephadex G-100 showed that EPS had two fractions, namely EPS-1 and EPS-2 (fig. 3, table 3).

This was also an important parameter to qualitative management of EPS extracted from *O. sinensis* cultured broth.

Table 4. The yield, DS and carbohydrate content of S-EPS

S-EPS	CSA/Pyr ratio (v/v)	Temperature (°C)	Time (h)	DS	Yield (mg)	Carbohydrate content (%)
S-EPS_1	3:1	45	4	0.96 ± 0.04^{bc}	44.49 ± 0.69^{bcd}	30.41 ± 0.16^{d}
S-EPS_2	3:1	65	2	0.80 ± 0.05^{b}	51.12 ± 14.92^{de}	14.84 ± 0.14^{bc}
S-EPS_3	3:1	65	6	0.31 ± 0.02^{a}	29.90 ± 1.45^{ab}	12.54 ± 2.34^{bc}
S-EPS_4	3:1	85	4	0.32 ± 0.02^{a}	18.26 ± 3.20^{a}	4.80 ± 0.03^{a}
S-EPS_5	1:1	45	2	0.86 ± 0.04^{b}	81.36 ± 8.18^{h}	8.73 ± 1.58^{abc}
S-EPS_6	1:1	45	6	1.22 ± 0.07^{c}	64.46 ± 4.50^{e}	11.25 ± 0.93^{c}
S-EPS_7	1:1	65	4	0.77 ± 0.29^{b}	77.09 ± 20.53^{f}	16.16 ± 1.46^{c}
S-EPS_8	1:1	85	2	0.43 ± 0.10^{a}	46.87 ± 11.30^{cd}	13.3 ± 1.54^{bc}
S-EPS_9	1:1	85	6	0.83 ± 0.20^{b}	43.80 ± 1.57^{bcd}	14.47 ± 0.11^{bc}
S-EPS_{10}	1:3	45	4	1.04 ± 0.26^{bc}	105.86 ± 0.79^{g}	17.02 ± 2.34^{c}
S-EPS_{11}	1:3	65	6	1.59 ± 0.09^{d}	41.36 ± 5.39^{bcd}	52.25 ± 8.45^{f}
S-EPS_{12}	1:3	65	2	0.20 ± 0.04^{a}	31.34 ± 6.03^{abc}	37.94 ± 2.78^{e}
S-EPS_{13}	1:3	85	4	1.03 ± 0.09^{bc}	25.96 ± 6.86^{a}	50.28 ± 3.42^{f}

Average values in a column with the same letter(s) above them do not differ significantly (Duncan test, $p<0.05$)

Furthermore, most of S-EPS, their carbohydrate contents decreased compared to the EPS sample (table 4). Of those, that of from S-EPS_1 to S-EPS_9 significantly dropped between 38 % and 60 % in comparison with the natural EPS. This result is similar to the previous study demonstrating that the polysaccharide chain was hydrolyzed by a high CSA/Pyr ratio [15]. In contrast, only a part of carbohydrate of S-EPS_{11} was removed approximately 16 % due to decreasing the CSA/Pyr ratio in reaction. From the above data, we suggest the optimal condition for sulfation of the EPS was CSA/Pyr ratio = 1:3, at 65 °C and 6 h. Therefore, S-EPS_{11} was selected to further study.

The physicochemical properties of S-EPS_{11} were determined to assess the efficiency of the sulfation modification (table 3). The sulfate content of S-EPS_{11} significantly increased about 47.15 % compared to the natural EPS (table 4).

Notably, the FTIR analysis indicated that S-EPS_{11} had the presence of C-O-S (at 815 cm^{-1}) and S=O (at 1129 cm^{-1}) stretching vibrations (fig. 2B) [4, 12, 28]; meanwhile, natural EPS did not. This demonstrated that we sucessfuly synthesize sulfated EPS from *O. sinensis* fungus. Besides, S-EPS_{11} had only one fraction, namely S-EPS_{11}-1 (fig. 3) because EPS-2 fraction could be hydrolyzed by redundant CSA in the sulfation process.

Antioxidant of sulfated EPS from *O. sinensis*

To examine the enhancement of biological activities of S-EPS_{11} compared with the natural EPS, we performed *in vitro* the antioxidant activity using the $OH^{\bullet}$ and $ABTS^{\bullet}$ radical scavenging assays (table 5). Expectedly, the $ABTS^{\bullet}$ radical scavenging potential of S-EPS_{11} at concentration of 4000 µg/mL rose from 62.94 % (the natural EPS) to 98.02 %. It is highly superior to four S-EPS derivatives of the previous study of Yan *et al.*; the figure for SEPS-1D was only about 60 % [2]. Evidently, the DS of S-EPS_{11} was higher than that of SEPS-1D, 1.59 and 1.38, respectively. Noticeably, there was a double increase in the $OH^{\bullet}$ scavenging ability from 46.03 % to 95.24 % between EPS and S-EPS_{11}. The results strongly agree with Ma *et al.* reported that antioxidant activity of sulfated polysaccharides were correlated with increasing sulfate contents and DS of products [10]. Collectively, our results revealed that the sulfated EPS reliably increase the bio-activities of EPS extracted from *O. sinensis* culture broth.

Table 5. Comparison of antioxidant activities of EPS and S-EPS_8

Samples	The rate of free radical scavenging capacity at the concentration of 4000 µg/mL (%)	
	$ABTS^{\bullet}$	$OH^{\bullet}$
EPS	62.94 ± 0.05^a	46.03 ± 0.08^a
S-EPS_{11}	98.02 ± 0.00^b	95.24 ± 0.06^b

Average values in a column with the same letter(s) above them do not differ significantly (Ducan test, $p<0.05$)

4 CONCLUSION

We successfuly synthesized sulfated EPS extracted from *O. sinensis* fungus by using CSA/Pyr method with an optimal condition including CSA/Pyr ratio = 1:3, at 65 °C and 6 h. The physicochemical properties of EPS and S-EPS_{11} including sulfate and polysaccharide contents, main chemical groups and number of fractions, have been determined to control the quality of sulfation products. Expectedly, our S-EPS derivative reliably improved the $OH^{\bullet}$ and $ABTS^{\bullet}$ radical scavenging potential compared with the natural EPS. However, to evaluate accurately the efficiency of sulfated modification by CSA/Pyr method for boosting the bioactivities of EPS, other bioactive properties such as immunomodulatory and antitumoral activity were performed as well. In conclusion, the sulfated modification of EPS by CSA/Pyr was an effective strategy to enhance its bioactivities so that we can thoroughly exploit the bioactive ingredients of this medicinal mushroom.

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Điều chế exopolysaccharide sulfate từ nấm *Ophiocordyceps sinensis* và tác dụng kháng oxy hóa của chúng

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Abstract—**Exopolysaccharide sulfate đã được biết đến làm cải thiện các hoạt tính sinh học. Expolysaccharide (EPS) sản xuất bởi nấm *Ophiocordyceps sinensis* là một nguồn dược chất tự nhiên. Do vậy, mục đích chính của nghiên cứu này là nâng cao hoạt tính sinh học của EPS thông qua sự thay đổi thành phần nhóm sulfate bằng phương pháp acid chlorosulfonic (CSA) và pyridin (Pyr). Điều kiện phù hợp cho phản ứng sulfate hóa được xác định, bao gồm tỷ lệ CSA/Pyr là 1:3 (v/v), và 6 giờ. S-EPS_{11} có độ thay thế cao nhất là 1,59, với thành phần đường tổng và sulfate lần lượt là 52,25% và 47,15%. Bên cạnh đó, phân tích FT-IR chứng minh S-EPS_{11} có sự hiện diện của dao động dãn C-O-S (ở 815 cm^{-1}) và S=O (ở 1129 cm^{-1}), trong khi EPS không hiện diện những dao động này. Đặc biệt, khả năng bắt gốc tự do $OH^{\bullet}$ và $ABTS^{\bullet}$ của S-EPS_{11} tăng lên đáng kể so với EPS không sulfate. Kết luận, chúng tôi đã tổng hợp thành công dẫn xuất exopolysaccharide sulfate từ nấm *O. sinensis* với hoạt tính kháng oxy hóa được cải thiện rõ rệt.**

***Index Terms*—*Ophiocordyceps sinensis,* exopolysaccharide, sulfate hóa, kháng oxy hóa, phân tích FT-IR**