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In vitro skin protection effects of sulfated exopolysaccharide fragment derived from the cultivation of *Ophiocordyceps sinensis*

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Abstract Exopolysaccharides (EPS) are extracted from a cultivated medium of *Ophiocordyceps sinensis*. EPS extraction was deproteinized and filtrated to remove EPS fragments weighing higher than 100kDa. The sulfur trioxide-pyridine (SO3.Py) method was conducted to enhance the biological activity of exopolysaccharides. The degree of sulfate substitution (DS) of SEPS11 is the highest 1.79 corresponding with 34.90% of sulfate amount at 80 ^{o}C. Increasing the EPS fragment ratio decreased the DS to 1.20 and 24.67% of the sulfate amount. Sulfated exopolysaccharides exhibited higher levels of antioxidant and tyrosinase inhibitor activities, which showed a correlation with sun protection factors. The tyrosinase inhibitor, antioxidant and activities of SEPS11 are the highest, and SPF of 15.66 mg/mL and 1.05 respectively. Sulfated *O. sinensis* EPS fragments weighted less than 100 kDa have the potential in the application of cosmetics or functional food as a skin protection additive.

Keywords: *Ophiocordyceps sinensis*, Sulfated exopolysaccharide, Antioxidant activity, Anti-tyrosinase activity, Photoprotective effect

Introduction

Ophiocordyceps sinensis, previously referred to as *Cordyceps sinensis*, and commonly known as caterpillar fungus, is used in traditional Chinese medicine. Numerous clinical studies have provided evidence of the biological effects of *Ophiocordyceps sinensis*. Whether in its natural habitat or cultivated forms, this fungus offers health benefits to humans and displays various pharmacological activities. Beside secondary bioactive compound, exopolysaccharides (EPSs) are

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extracted from the cultivated medium of *Ophiocordyceps sinensis* and are recognized as external metabolites contributing various functions within living organisms such as adaptation and survival. Enhancing the biological activities and solubility of EPSs is crucial for their application in functional food, cosmetics, and related fields. Sulfated modification emerges as a practical approach to create novel pharmacological compounds with potential therapeutic uses and to innovate in the development of new products for health, cosmetics, and various applied domains. The engineered sulfated polysaccharides structures are different from natural sulfated polysaccharides, undetected in nature, and frequently have novel or improved characteristics (Arlov *et al.,* 2021). Sulfated polysaccharides, characterized by negatively charged polysaccharides with monovalent groups derived from vitriol, are formed through the sulfonation of a polysaccharide chain (Xu *et al.,* 2019). Sulfation reaction enhances the water solubility of the polysaccharide chain, leading to alterations in biological activities like immune response, antiviral effects, and antioxidant properties (Rahman *et al.*, 2019). Among various sulfation methods, employing SO3.Py for sulfating exopolysaccharides is a straightforward process, and the reaction conditions are less rigorous compared to the CSA method. The drawback of this method is using high quality chemical reagents and is only suitable for laboratory applications. Nevertheless, its significant degree of substitution, ranging from 1.65 to 2.46, linked to heightened bioactivities, has been recognized and garnered significant attention. (Pillaiyar *et al.*, 2018).

The study was to improve the skin protection activities of EPS fragment weighted less than 100 kDa derived from a cultivated medium of Ophiocordyceps sinensis by sulfation SO3.Py method.

Materials and methods

Microorganism

O. sinensis strain, which received from Dr. Truong Binh Nguyen (Dalat University, Vietnam), was inoculated in Potato dextrose broth at 28^{o}C. After 30 days, EPS was collected from cultivated medium.

Exopolysaccharide preparation

The concentrate was obtained from the culture broth using a rotary evaporator. Then, 96% ethanol was used to precipitate EPS at a volume ratio of 1:4 (v/v) at 4^{o}C for 24 hours. Next, the solution was poured out, and the precipitate was collected, dehydrated, and preserved at 4^{o}C.

Sevag solution was added to the EPS solution in a 1:1 (v/v) ratio for deproteinization. The deproteinized solution obtained was filtered using a tangential flow filtration system(TFF) equipped with membranes having a molecular weight cut-off of 100 kDa. The final solution contains EPS fragments weighing less than 100 kDa and is stored at 4°C. Total amount of polysaccharides and proteins of EPS was measured by phenol-sulfuric acid method at 490 nm and Bradford method at 595 nm, respectively.

Sulfated modification of EPS fragment (SEPS)

Experimental design and sulfation reaction

1 mL of dimethyl sulfoxide was use to dissolve 200 mg of EPS powder by using a magnetic mixing over a half hour. Then, the temperature and the volume ratio of SO_3.Py (v/v) were adjusted according to the experimental design table. The reaction occurred in a closed system with inert gas for 2 hours.

Table 1. Experimental design table

SEPS	Ratio of EPS and SO_3.Py (w/w)	Temperature (^oC)
SEPS1	1:10	50
SEPS2	1:15	50
SEPS3	1:20	50
SEPS4	1:10	60
SEPS5	1:15	60
SEPS6	1:20	60
SEPS7	1:10	70
SEPS8	1:15	70
SEPS9	1:20	70
SEPS10	1:10	80
SEPS11	1:15	80
SEPS12	1:20	80

After sulfation, the pH SEPS solution was adjusted to 7.0 by adding NaOH 2M. Then, SEPSs were placed into a dialysis membrane with a molecular weight cut-off of 1 kDa and dialyzed against distilled water for 72 hours.

Analysis of sulfated exopolysaccharides

The amount of sulfate in the sample was determined using the method described by Hartiala and Terho (1971) using the Na_2SO_4 solution (50 µg/mL) to construct standard curve (Terho and Hartiala, 1971). Calculation of degree of sulfated substitution (DS) was performed by formula below (Equation 1).

$$\mathbf{DS} = \frac{\mathbf{1.62} * \%\boldsymbol{S}}{\mathbf{32 - 1.02} * \%\boldsymbol{S}} \qquad \textit{(Equation 1)}$$

Then, 200 mg of Kali bromide and 2 mg of the sample were dried and ground to investigate the structure of SEPSs. The mixture was then compressed into transparent pellets suitable for direct measurement. Finally, the IR spectra of EPS/SEPS were recorded using an FTIR spectrometer within the mid-infrared region of 4000–400 cm-1.

ABTS•+ radical scavenging assay

Potassium phosphate buffer solution was used to dilute ABTS•+ stock in an amber flask until the solution's absorbance reached 0.7 at the wavelength of 734 nm. Then, 100 µL of the sample solution was added to test tubes, followed by adding 3 mL of the ABTS• substrate solution to each tube. The tubes were incubated in the dark for half hour at 25ºC and then measured at a wavelength of 734 nm. PBS was use as the negative control and vitamin C stock as the positive control. The sample was prepared and investigated at concentrations ranging from 0 to 4 mg/mL. IC_{50} of sample was calculated by equation 2.

$$IC_{50} = \frac{50-k}{h}(\mu g/mL) \qquad \textit{(Equation 2)}$$

k and h are value of regression equation (y=hx+k)

In vitro tyrosinase inhibition assay

The experiment was conducted according to the protocol of Alam *et al. (*2010) with several modifications. Into a 96-well plate, 100 µL of the kojic acid/sample solution, 100 µL of potassium phosphate buffer, and 40 µL of tyrosinase enzyme were added. Then, 40 µL of L-tyrosine was added to the plates and allowed to incubate at 37°C for 20 minutes. Following incubation, the absorbance of the solution was assessed at a wavelength of 490 nm, with potassium phosphate buffer serving as the negative control and a kojic acid solution were used as the positive control. The sample was prepared and measured at concentrations ranging from 0 to 4 mg/mL. IC_{50} value of samples were calculated by the equation 2.

In vitro photoprotective effect of sulfated EPS by ultraviolet spectrophotometry

The experiment was conducted according to the protocol of Mesías *et al.* (2017) with several modifications (Mesías *et al.,* 2017). The stock solution was prepared by dissolving SEPSs powder in 96% ethanol to get a 200 µg/mL SEPSs solution. The solution was analyzed at different wavelengths spanning from 290 to 320 nm, with intervals of 5 nm, using 96% ethanol as the negative control.

Homosalate 8% solution mixed with ethanol 96% was used as the positive control solution with concentration of 200 µg/mL. SPF calculated based on equation 3 (Mansur *et al.*, 1986) for each sample.

$$SPF = CF \times \sum_{290}^{320} I(\lambda) \times A(\lambda) \times EE(\lambda) \qquad \textit{(Equation 3)}$$

Statistical analysis

The data underwent processing using Microsoft Excel, one-way ANOVA, and Tukey's HSD test, where the parameters were set with an n-value of 3, an α-value of 0.05, and a confidence level of 95%. Additionally, the averages of the results, representing three replicates, were presented as mean ± standard deviation (M±SD).

Results

Preparation of exopolysaccharide

After rotary evaporation and ethanol precipitation, the EPS concentration reached 4.743 g/L. Before deproteinization, the initial quantities of proteins and polysaccharides in EPS, were 4.54% and 77.68% respectively. The Sevag method was applied with an EPS collection efficiency of 25.63% after deproteinization. After deproteinization and TFF, fragments of EPS weighted less than 100 kDa were obtained, with a polysaccharide concentration is 81.26% and protein is 2.39%. The polysaccharide content of SEPS10, SEPS10, and SEPS12 exhibited a significant decrease, with values of 72.98%, 70.30%, and 68.06%, respectively (Table 2).

Sulfated modification of EPS fragment (SEPS)

The FTIR spectra of EPS weighing less than 100 kDa and the sulfation results are presented in the table below (Table 3). The absorption bands of EPS linked to ranges of 3570-3200, 3000-2800, 1700-1400, and 1260-700 cm^{-1}.

Table 2. Proportion of polysaccharide and protein of EPS before and after deproteinization and SEPS fragments

Sample	Polysaccharide (%)	Protein (%)
Initial EPS	77.68 ± 1.00^{c}	4.54 ± 0.09^{c}
Deproteinized EPS	81.26 ± 0.34^{a}	2.39 ± 0.17^{a}
SEPS10	72.98 ± 1.15^{b}	0.81 ± 0.06^{b}
SEPS11	70.30 ± 1.49^{b}	0.93 ± 0.02^{b}
SEPS12	70.44 ± 1.02^{b}	0.95 ± 0.04^{b}

Values in a column have the different letter(s) above them exhibit significant differences on average ($p<0.05$).

Table 3. FTIR assignments of sulfated EPSs

Assign-ment	EPS	SEPS at 50ºC			SEPS at 60ºC			SEPS at 70ºC			SEPS at 80ºC		
		1:10	1:15	1:20	1:10	1:15	1:20	1:10	1:15	1:20	1:10	1:15	1:20
C-H stretch	3000-2800	2938	-	-	-	-	-	-	-	-	3000-2800	3111	3100-3000
O-H stretch	3648 1451	3421	3336	3385	3321	3219	3473	3381	3430	3435	3443 1489	3434 1488	3430 1488
COO^- stretch	1688	1640	1647	1640	1654	1656	1644	1643	1647	1635	1635	1633	1633
C-O-C/ C-O-S stretch	1032	1129	1125	1127	1136	1124	1126	1127	1123	1118	1111	1110	1111
C-O-S stretch	-	789	789	789	816	-	822	814	901	835	899	899	900
S=O stretch	-	-	-	-	-	-	-	-	-	-	1185	1190	1186

The IR analysis revealed that temperature of the environments range from 50 to 70 °C were not conducive to sulfating EPS at ratios of 1:20, 1:15, and 1:10, bccause undetected absorption bands within the detection range of sulfate. However, SEPS at 80°C, with ratios of 1:20, 1:15, and 1:10, exhibited both the structure of a polysaccharide and signals of sulfate groups, with absorption bands in the estimated range of 1190-1185 and 835-751 $cm^{-1.}$ (Table 3).

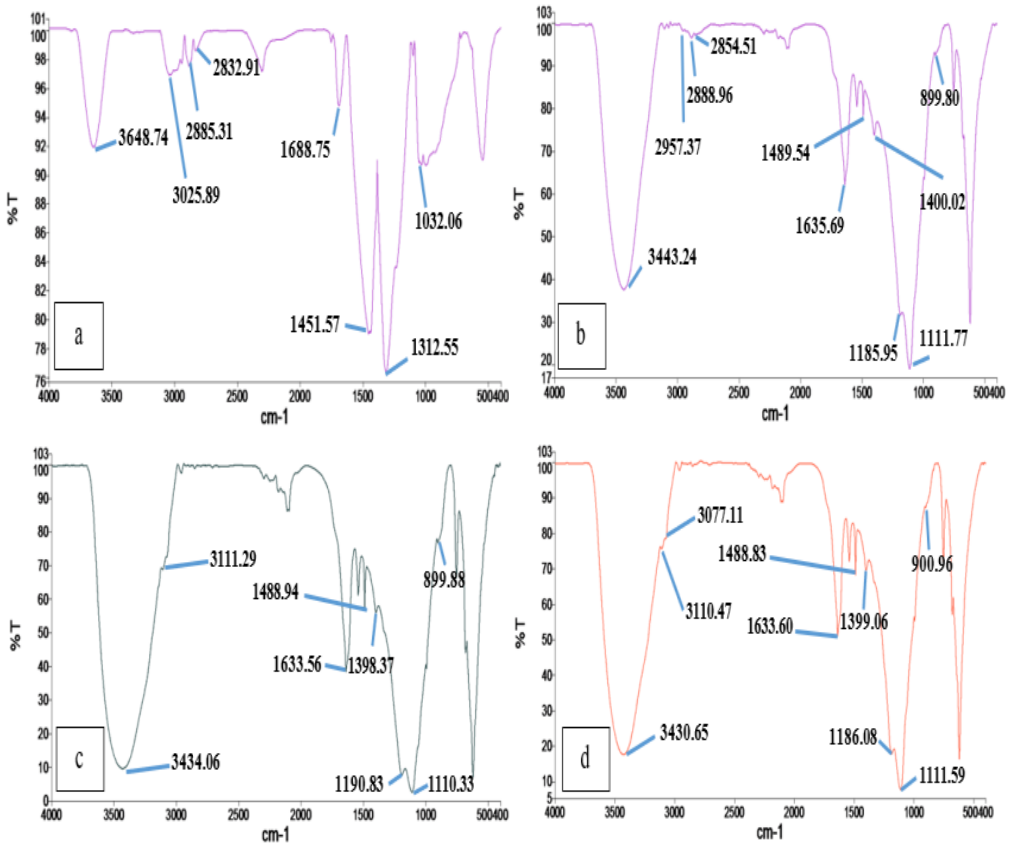

Figure 1. IR spectra (400-4000 cm-1) of EPS below 100kDa before sulfation (a) and SEPS10 (b), SEPS11 (c), and SEPS12(d)

Among the sulfated fragments, SEPS10 and SEPS11 exhibited the highest degree of sulfation (DS) at 1.70 and 1.79, respectively, equivalent to 33.31% and 34.90% of the sulfate content (Table 4). SEPS12 had a degree of sulfation about 1.26, representing 24.67% of the sulfate amount.

Table 4. Sulfate content and degree of sulfation of EPS after sulfation at 80°C for 2 hours

Sample	Sulfate content (%)	DS
SEPS10	33.31 ± 5.84[a]	1.70 ± 0.30[a]
SEPS11	34.90 ± 4.65[a]	1.79 ± 0.24[a]
SEPS12	24.67 ± 1.06[b]	1.26 ± 0.05[b]

Values in a column have the different letter(s) above them exhibit significant differences on average ($p<0.05$).

SEPS fragments skin protection activities

In vitro antioxidant assay result

In contrast to the original and deproteinized EPS, the SEPS fragments demonstrated notably improved ABTS• free radical scavenging activity, highlighting the role of the sulfate group in antioxidant activity (Table 5). The percentage of free radical scavenging for natural EPS was half at a concentration of 3.37 mg/mL, while the sulfated counter part exhibited this effect at a lower concentration.

Table 5. Antioxidant activity (IC_{50}) of sulfated exapolysaccharide and non sulfated exapolysaccharide derived from *O. sinensis* cultivation medium

Sample	Antioxidant activity (IC_{50}) (mg/mL)
Initial EPS	3.37 ± 0.06^{c}
Deproteinized EPS	3.04 ± 0.09^{c}
SEPS12	2.29 ± 0.12^{b}
SEPS11	2.01 ± 0.05^{b}
SEPS10	2.20 ± 0.01^{b}

Values in a column have the different letter(s) above them exhibit significant differences on average ($p<0.05$).

In vitro tyrosinase inhibition assay result

Sulfated EPS fragments exhibited greater tyrosinase inhibition activity compared to natural EPS. Within the concentration range of 0-4 mg/mL, the non sulfated EPS demonstrated no tyrosinase inhibition effect, while the deproteinized EPS had an IC50 value of 2.9 mg/mL. In contrast, the sulfated fragments, that is SEPS10, SEPS11, and SEPS12, exhibited a significant drop in the IC compared to the deproteinized EPS, with values decreasing from 2.90 to 1.67, 1.05, and 1.63, respectively (refer to Table 6). Notably, among the sulfated fragments, SEPS11, which had the highest degree of sulfation and sulfate content, exhibited the most potent tyrosinase inhibition activity with an IC50 of 1.05 mg/mL.

Table 6. Half maximal inhibitory concentration of anti-tyrosinase activity of EPS fragments after sulfation

Sample	Anti-tyrosinase activity (IC_{50}) (mg/mL)
Initial EPS	>4
Deproteinized EPS	2.90 ± 0.04^{c}
SEPS12	1.63 ± 0.05^{b}
SEPS11	1.05 ± 0.10^{d}
SEPS10	1.67 ± 0.19^{b}

Values in a column have the different letter(s) above them exhibit significant differences on average ($p<0.05$).

In vitro photoprotective assay result

EPS fragments and their sulfated counterparts exhibited a limited to moderate level of photoprotective efficacy, with SPF values ranging from 7 to 16. The EPS after deproteinization had an SPF of 12.43. Notably, sulfated fragments with SPF values exceeding 13 demonstrated enhanced photoprotective activity compared to non-sulfated fragments with SPF values below 13, as indicated in Table 7. Among these, SEPS 11 displayed the most significant photoprotective effect, with an SPF of 15.66 (Table 7).

Table 7. Sun protection factor (SPF) of sulfated exapolysaccharide and non sulfated exapolysaccharide derived from *O. sinensis* cultivation medium

Sample	SPF
Initial EPS	7.47 ± 0.15^{c}
Deproteinized EPS	12.43 ± 0.14^{d}
SEPS12	13.56 ± 0.16^{e}
SEPS11	15.66 ± 0.18^{b}
SEPS10	13.83 ± 0.19^{e}

Values in a column have the different letter(s) above them exhibit significant differences on average ($p<0.05$).

Discussion

O. sinensis exopolysaccharide extraction with a minor presence of proteins, aligning with the findings Tran *et al.* and previous studies (Tran *et al.*, 2018). The protein and polysaccharide content of deproteinized EPS showed a marked increase compared to natural EPS because the presence of proteins impeded the determination of total carbohydrates using the phenol-sulfuric method. Thus, the removal of proteins during the deproteinization process led to a more pronounced purification of the polysaccharide chain. Following the sulfation and dialysis procedures, SEPS10, SEPS11, and SEPS12 experienced a notable reduction in polysaccharide content because of the loss of polysaccharides and proteins. This implied the elimination of EPS with a molecular weight below 1 kDa along with inorganic substances and pyridine, salt, etc.

Exopolysaccharide derived from *O. sinensis* didn't have C-O-S and S=O groups absorption band at the range of 900-800 and 1260-1186 cm^{-1} (Hong *et al.*, 2021; Ptak *et al.*, 2021; Nandiyanto *et al.*, 2019). EPS sulfated at 80°C with three ratios of 1:20, 1:15, and 1:10 exhibited both the structure of a polysaccharide and signals of sulfate groups, with absorption bands in the estimated range of 1190-1185 and 835-751 cm^{-1} (Hong *et al.*, 2021; Ptak *et al.*, 2021). FTIR analysis result indicates that the sulfation reaction doesn't occur at 50, 60, and 70°C with all three ratios of 1:10, 1:15, and 1:20.

In terms of ABTS• free radical scavenging activity, sulfate groups possess a negative charge, allowing them to electrostatically bind to positively charged molecules. Sulfated polysaccharides exhibited higher antioxidant activity than their non-sulfated counterparts, a trend consistent with findings from various research studies (Tran *et al*., 2018; Liu *et al*., 2018 and Zou *et al.,* 2008).

Melanin plays a important role in UV-induced skin photodamage. However, excessive melanin production can lead to pigment abnormalities such as moles and freckles (Wang *et al*., 2020b). Several enzymes associated with melanocytes participate in the natural process of melanogenesis. Tyrosinase serves as the limiting factor in melanin synthesis. Consequently, a substance capable of efficiently decreasing the quantity or activity of tyrosinase may possess potential in inhibiting melanogenesis. In general, sulfated fragments exhibited higher tyrosinase inhibition activity compared to non-sulfated EPS (Wang *et al*., 2020a). The increased presence of sulfated polysaccharide was linked to elevated tyrosinase inhibition activity, as observed through the assessment of the intracellular tyrosinase inhibition of sulfated polysaccharide derived from Hizikia fusiforme on B16F10 cells. (Wang *et al*., 2020b).

UVB, a subset of ultraviolet (UV) radiation, is commonly believed to impose more stress on the skin compared to other UV subtypes. UVB induce the

generation of intracellular reactive oxygen species (ROS), causes damage to both the epidermis and dermis (Wang *et al*., 2018). The US Food and Drug Administration categorizes sunscreen products into three types based on their Sun Protection Factor (SPF): those with SPF 2 to 12, offering minimal sun protection; those with SPF 12 to 30, providing moderate sun protection (Cheng *et al*., 2018). Polysaccharides obtained from the mycelial biomass of the Cs-HK1 strain of Ophiocordyceps sinensis demonstrated SPF of 16.2, 11.3, and 10.2, respectively, through ethyl acetate, ethanol, and hot water extraction processes. (Cheng *et al*., 2018). SEPS with an SPF above 13 exhibited greater photoprotective efficacy than non-sulfated fragments with an SPF lower than 12.5. SPF is calculated as the ratio of UV energy required to induce the minimal erythema dose (leading to skin redness or sunburn) on shielded skin to the UV energy necessary for the same effect on unprotected skin (Li *et al*., 2021). Thus, the capacity to shield the skin from the detrimental effects of UV radiation was elevated. Moreover, the effectiveness of sunscreen is influenced by various factors such as solvents, concentration, esters, emulsifiers, emollients, and others. Sulfated O. sinensis EPS shows promise for the development of sunscreens with enhanced safety, SPF, and protection against both UV-A and UV-B rays.

In conclusion, the in vitro bioacitivities assay result showed that sulfated O. sinensis EPS fragments weighted less than 100 kDa have the potential in the application of cosmetics or functional food as a skin protection additive. However, the exact effect of SEPS on the human skins and bodies still require further in vivo research.

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