Appl Biochem Biotechnol (2011) 163:669–678
DOI 10.1007/s12010-010-9072-3

An Exopolysaccharide from Cultivated *Cordyceps sinensis* and its Effects on Cytokine Expressions of Immunocytes

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Received: 13 May 2010 / Accepted: 16 August 2010 /
Published online: 2 September 2010

Abstract The exopolysaccharide (EPS) is a polysaccharide from cultivated *Cordyceps sinensis*, which possesses immunomodulatory and antitumor effects, was purified by DEAE-32 cellulose and Sephadex G-200 gel. The preliminary characters of EPS were analyzed by IR and GC, and the molecular weight was estimated by gel filtration. The effect of EPS on proliferation ability of lymphocytes from ICR mice was assayed by MTT method. The mRNA and protein expression levels of several cytokines in spleen and thymus cells were detected by RT-PCR and ELISA. The results showed that EPS consists of mannose, glucose, and galactose in a ratio of 23:1:2.6. Its molecular weight is about 1.04×10^5. EPS elevated proliferation ability of spleen lymphocytes only at 100 μg/ml after 48 h treatment. Tumor necrosis factor alpha (TNF-α), interferon-α (IFN-γ), and interleukin-2 (IL-2) mRNA levels in splenocytes and thymocytes were increased after EPS treatment for 2, 4, 8, or 20 h. EPS also significantly elevated splenic TNF-α and IFN-γ protein expressions at 100 μg/ml and increased thymic TNF-α and IFN-γ protein levels at 50 and 100 μg/ml. These data indicated that EPS may stimulate cytokine expressions of immunocytes.

Keywords Cultivated *Cordyceps sinensis* · Cytokine · Exopolysaccharide · Immunocyte

Introduction

Fungi, especially macrofungi, are regarded as rich sources of natural bioactive compounds and the therapeutic effects of these compounds such as anticancer, antiinfective activity and

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suppression of autoimmune diseases have been reported to be associated with their immunomodulating effects [1–3].

It is generally accepted that polysaccharide is one of main natural bioactive compounds of medicinal fungi and plays an important role in enhancing various immune responses [4–7]. We have found that crude polysaccharide extract exopolysaccharide fraction (EPSF) from cultivated *Cordyceps sinensis* has immunomodulatory function and antitumor activity. It could not only significantly inhibit tumor's growth and metastasis in mice, but also significantly enhanced the spleen lymphocyte proliferation and cytokine expressions in tumor-bearing mice [8, 9]. Thus, we assumed that EPSF yield antitumor effects mainly by activating the host's immune system. In the present study, we purified the polysaccharide EPS from EPSF by chromatography and evaluate the effect of EPS on several cytokine expressions in mouse spleen and thymus cells *in vitro*.

Materials and methods

Preparation of EPS

Exopolysaccharide fraction was extracted from the cultured supernatant of *C. sinensis* fungus G1 as previous report [8]. The exopolysaccharide fraction was further purified and analyzed. It was first chromatographed over a diethylaminoethyl (DEAE)-32 column (2.6× 20 cm) eluting successively with water and a NaCl gradient (0–2 M) and then was further filtrated over a Sephadex G-200 column (2.6×60 cm) eluting with 0.05 M NaCl to afford EPS-containing solution according to phenol-sulfuric acid monitoring and obtain EPS powder upon dialysis and lyophilization. EPS was dissolved in RPMI-1640 medium, filtered through a 0.45 μm filter for cytological assay.

Preliminary chemical analysis

The chemical characteristics of EPS were analyzed using infrared spectra (IR), gas chromatography (GC) and gel filtration. IR spectra were recorded between 4,000–400 cm in KBr pellet with 1 mg of polysaccharide EPS. For GC analysis, 10 mg of EPS was hydrolyzed at 100 °C by 3 ml of 2.0 mol/l trifluoroacetic acid for 8 h and the hydrolysate was reacted with 10 mg of hydroxylamine hydrochloride and 0.5 ml of pyridine for 30 min. Then 0.5 ml of acetic anhydride was added and the mixture was kept at 90 °C for another 30 min. Finally the sample was analyzed by GC.

The average molecular weight of EPS was estimated by gel filtration over a Sephadex G-200 column (1.1×70 cm), which was completely equilibrated for 3 days with 0.05 mol/l NaCl at the flow rate of 4.8 ml/h followed by calibration with blue dextran and dextrans (MW: 11500, 41000, 71000 and 267000). Calibrated the void volume (V_0) and elution volume (Ve) of each sample and set up the standard curve by logarithm of molecular weight (Lg M) versus Ve/V_0.

Detection of proliferation of spleen and thymus lymphocytes

Spleens and thymus were aseptically obtained from ICR mice and placed in RPMI-1640 medium, respectively. Single-cell suspension was prepared. The cells were washed twice and then resuspended in medium and adjusted to 1.0×10^6 cells/ml with RPMI 1640 medium. Lymphocytes were then incubated in the presence of the mitogen concanavalin A

(10 μg/ml; Sigma) and different doses of EPS (the ultimate concentrations were 12.5, 25, 50, 100 μg/ml, respectively) at 37 °C in a humidified 5% CO_2-air mixture. Proliferation ability of lymphocytes was tested by MTT [10] method after they were incubated for 24 and 48 h.

RNA extract of lymphocytes

Single-cell suspensions of mouse splenocytes and thymocytes were adjusted to 1.0×10^6 cells/ml with RPMI 1640 medium as above described. Aliquots (800 μl) of lymphocytes were seeded into each well of a 24-well microplate and EPS was added to the ultimate concentrations were 25, 50, 100 μg/ml, respectively. The plate was incubated at 37 °C in a humidified 5% CO_2-air mixture.

After 2, 4, 8, and 20-h incubation, the cells were collected and washed with PBS, and then were lysed in 1 ml of Trizol Reagent (Invitrogen); 0.2 ml of chloroform was added to 1 ml of the cell lysate, shaked vigorously for 15 s, and incubated for 3 min at room temperature. The aqueous phase was transferred into a tube after centrifugation at 12,000 rpm for 15 min and then added with 0.5 ml of isopropyl alcohol. The tube was mixed and kept at room temperature for 10 min to precipitate RNA. The RNA pellet was washed with 1 ml of 75% ethanol and then dried and dissolved again in RNase-free water. RNA concentration was determined by measuring the optical density at 260 nm.

Detection of cytokine mRNA levels by RT-PCR

RNA sample was heated at 70 °C for 5 min and reverse-transcribed at 37 °C for 1 h in a 25-μl reaction mix containing 2 μg of RNA sample, 0.5 μg of oligo dT, 0.5 mM of each dNTP, 200 units of Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega) and 5 μl of 5× reaction buffer. Samples were stored at −70 °C until subjected to polymerase chain reaction (PCR) amplification. PCR was carried out in a 25 μl reaction mixture containing 2.0 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 μM of each primer (tumor necrosis factor alpha (TNF-α): 5′-CCACATCTCCCTCCAGAAAA-3′, 5′-CGGACTCC GCAAAGTCTAAG-3′; interferon-a (IFN-γ): 5′-AGGAACTGGCAAAAGGATGGTG-3′, 5′-GTGCTGGCAGAATTATTCTTATTG-3′; interleukin-2 (IL-2): 5′-CATTGA CACTTGTGCTCCTT-3′, 5′-GAAAGTCCACCACAGTTGCT-3′), 1 U of *Taq* DNA polymerase (Promega) and 2 μg cDNA. The reaction mixture was denatured at 94 °C for 5 min and subjected to 35 circles of 30 s at 94 °C, 30 s at 54 °C, 40 s at 72 °C, and a final extension step of 10 min at 72 °C. β-actin mRNA was used as an internal control (Primers: 5′-AGGCATCCTGACCCTGAAGTAC-3′, 5′-TCTTCATGAGGTAGTCTGTCAG-3′).

After PCR, agarose gel electrophoresis was performed in the presence of 0.5 μg/ml ethidium bromide. For each sample, the bands were quantified by densitometry using Quantity One software.

Assay of TNF-α, IFN-γ, and IL-2 by ELISA

Mouse plenocytes and thymocytes were prepared and adjusted to 1.0×10^6 cells/ml. After they were treated with EPS (25, 50, 100 μg/ml) for 20 h, the culture supernatant was obtained and TNF-α, IFN-γ, and IL-2 levels were detected by enzyme-linked immunosorbent assay (ELISA) kits (R&D, USA) according to the instructions of the manufacturer.

Statistical analysis

Data are expressed as mean ± SD. They were evaluated by the one-way analysis of variance, followed by SPSS software. $p<0.05$ was considered statistically significant.

Results

Preliminary chemical characteristics of EPS

The DEAE elution profile contains two sharp peaks (Fig. 1a). The left peak is neutral polysaccharide by elution with distilled water, while the right one is eluted by gradient NaCl solution. Then we got the EPS after Sephadex G-200 column chromatography with the right peak fraction composition (Fig. 1b). IR spectra of EPS showed some polysaccharide specific absorption bands (3,600~3,200 cm^{-1}, 3,000~2,800 cm^{-1}, 1,200~1,000 cm^{-1}) (Fig. 2). GC of EPS indicated that mannose, glucose and galactose were in an approximate molar ratio of 23:1:2.6. The average molecular weight of EPS was estimated by gel filtration over a Sephadex G-200 column. The V_0 and V_e of EPS were 23.1 and 33.7 ml, respectively. So the average molecular weight of EPS was determined to be 1.04×10^5 according to the calibration curve (Fig. 3).

Effects of EPS on the proliferation of mouse lymphocytes

The effects of EPS on the proliferation of lymphocytes are shown in Table 1. The result showed that only the average absorbency of spleen lymphocytes treated with 100 μg/ml of EPS for 48 h was statistically higher than that of normal control ($p<0.05$). EPS could not significant influence proliferation ability of thymus lymphocytes at all the four concentrations after 24 or 48 h treatment.

Effects of EPS on TNF-α mRNA expression of mouse immunocytes

After cDNA and internal standard β-actin were simultaneously amplified in the same condition, the two bands of amplified products were observed by agarose gel electrophoresis. Bands were quantified by densitometry and the densitometric ratio of the corresponding bands was calculated with Quantity One software.

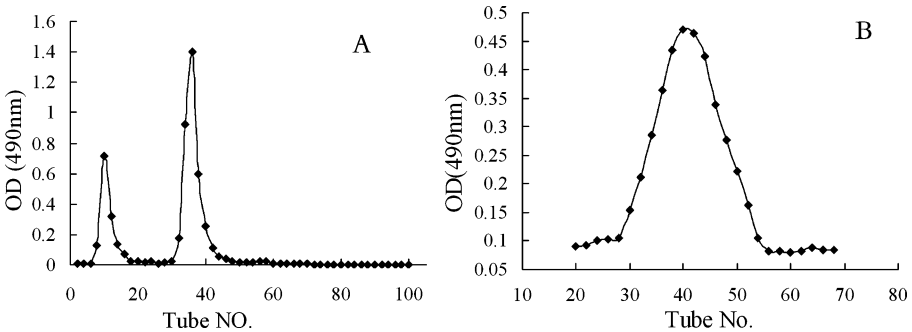

Fig. 1 EPS elution profile. **a** DEAE-32 chromatography. **b** Sephadex G-200 chromatography

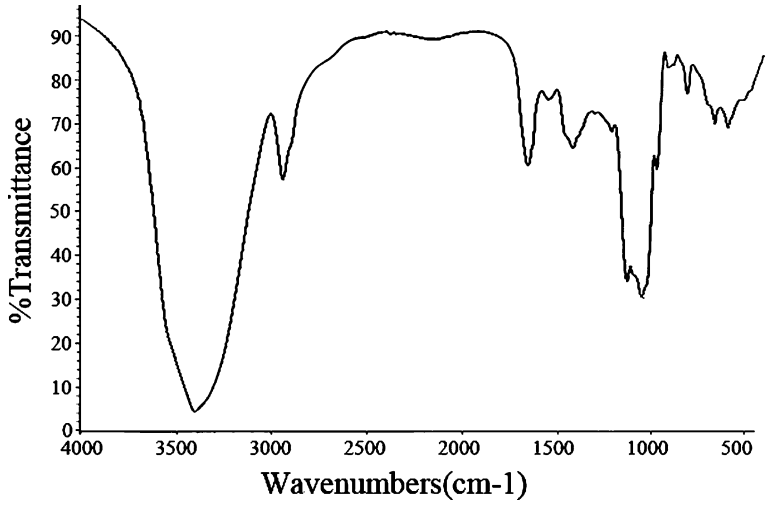

Fig. 2 Infrared spectra of the exopolysaccharide from cultivated *Cordyceps sinensis*

As shown in Fig. 4a, TNF-α expression was significantly elevated in EPS treated splenocytes for 2 h (25, 50, 100 μg/ml), 4 h (50, 100 μg/ml), 8 h (50, 100 μg/ml), 20 h (25, 50, 100 μg/ml), and reached peak at 2 h ($p<0.01$). TNF-α mRNA expression in thymocytes was shown in Fig. 4b, compared with the control, it was significant higher when treated with EPS at 50 μg/ml (2 h, $p<0.01$; 4 h, $p<0.05$), 100 μg/ml (8 h and 20 h, $p<0.01$).

Effects of EPS on IFN-γ mRNA expression of mouse immunocytes

IFN-γ expression in splenic lymphocytes was significantly elevated when EPS treated at dose of 100 μg/ml for 4 and 8 h ($p<0.05$) (Fig. 5a). IFN-γ mRNA expression in thymus lymphocytes was significantly elevated when EPS treated at 25, 50, and 100 μg/ml for 8 h and at 50 and 100 μg/ml for 20 h ($p<0.05$) (Fig. 5b).

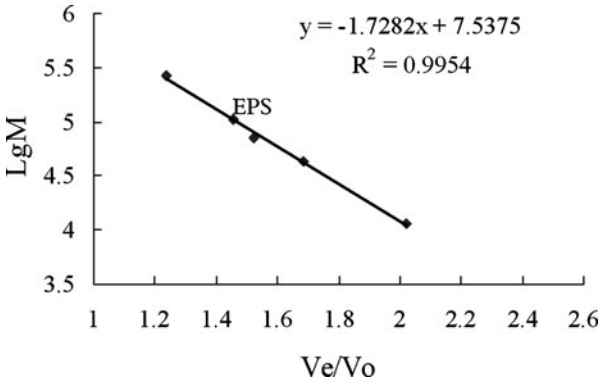

Fig. 3 The standard curve of molecular weight by filtration. *Ve/Vo* ratio of void volume and elution volume, *LgM* logarithm of molecular weight

Table 1 Effects of EPS on the proliferation of splenocytes and thymocytes (x±s, n=6)

Concentration (μg/ml)	Splenocytes		Thymocytes	
	A 570 nm (24 h)	A 570 nm (48 h)	A 570 nm (24 h)	A 570 nm (48 h)
0	0.5169±0.0415	0.6363±0.0772	0.7294±0.1163	0.7876±0.1297
12.5	0.5116±0.0651	0.6687±0.0152	0.6740±0.0321	0.8292±0.0097
25	0.5106±0.0916	0.6745±0.0126	0.7614±0.1524	0.8028±0.0054
50	0.4603±0.1050	0.6593±0.0252	0.7471±0.0533	0.8003±0.01718
100	0.4740±0.1096	0.7193±0.0424*	0.7768±0.0765	0.7964±0.0195

*p<0.05, compared with control

Effects of EPS on IL-2 mRNA expression of mouse immunocytes

The densitometric ratio of the IL-2 and β-actin bands was demonstrated in Fig. 6. IL-2 mRNA expression in splenocytes was significantly higher when EPS treated at dose of 100 μg/ml for 4 h (p<0.05). Its mRNA level in thymocytes was significantly

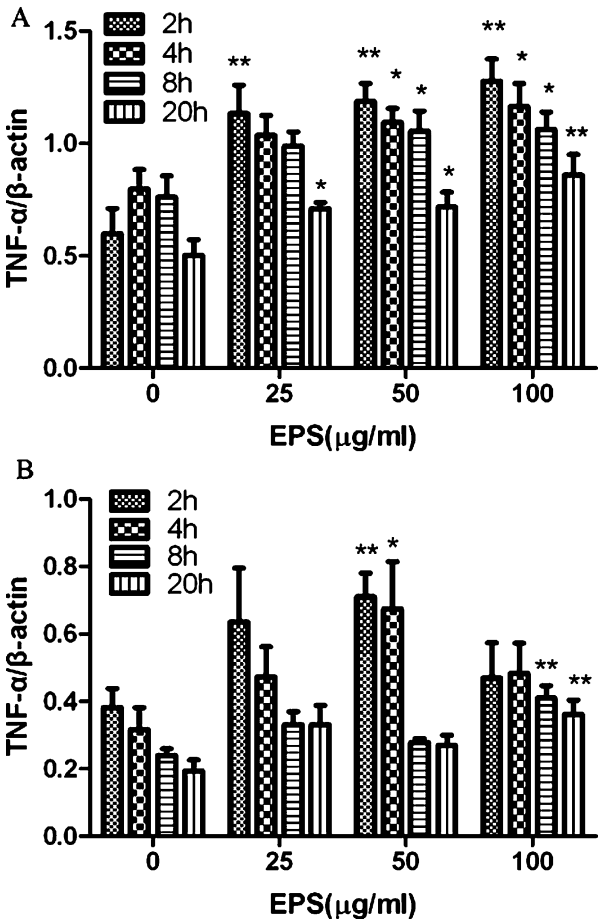

Fig. 4 Effects of EPS on TNF-α mRNA level of splenocytes (**a**) and thymocytes (**b**) of ICR mice. Data are expressed as mean±SD (n=6). *p<0.05; **p<0.01; compared with control

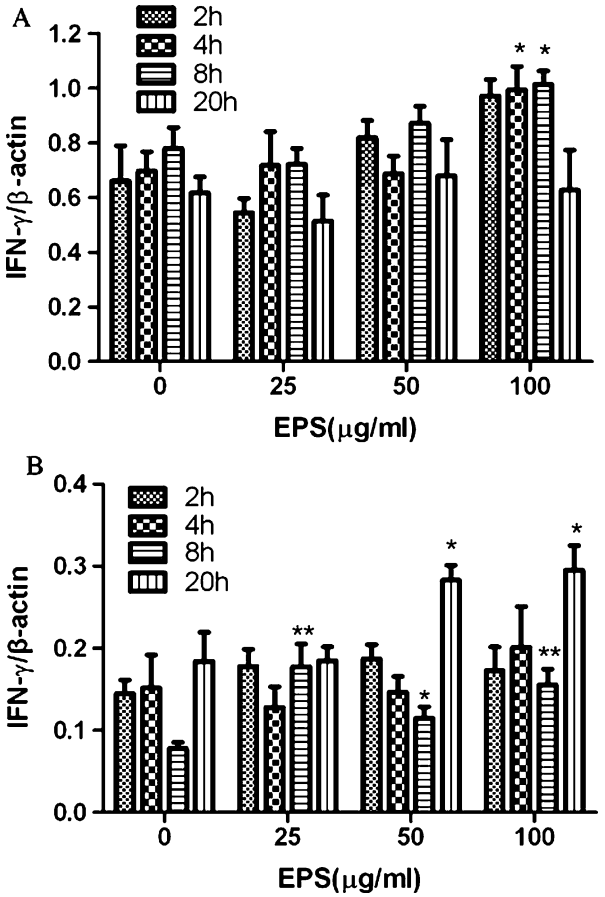

Fig. 5 Effects of EPS on IFN-γ mRNA level of splenocytes (**a**) and thymocytes (**b**) of ICR mice. Data are expressed as mean±SD (n=6). *p<0.05; **p<0.01; compared with control

elevated when EPS treated at dose of 100 μg/ml for 4 h (p<0.05), 8 h (p<0.01), and 20 h (p<0.05).

Effects of EPS on protein expression of TNF-α, IFN-γ, and IL-2 of mouse immunocytes

To evaluate the effect of EPS on protein levels of cytokines produced by mouse spleen or thymus immunocytes, the culture supernatant was used for assay by ELISA. As shown in Table 2, 100 μg/ml of EPS significantly increased TNF-α and IFN-γ levels of splenocytes (p<0.05 or p<0.01); EPS at 50 and 100 μg/ml significantly increased TNF-α and IFN-γ levels of thymocytes as well (p<0.05, p<0.01, or p<0.001). However, neither splenocytes nor thymocytes did not yielded significant changes in IL-2 protein level.

Discussion

It is well known that polysaccharides possess immunomodulatory functions. Moreover, our previous *in vivo* study has disclosed that EPS fraction not only significantly inhibited the

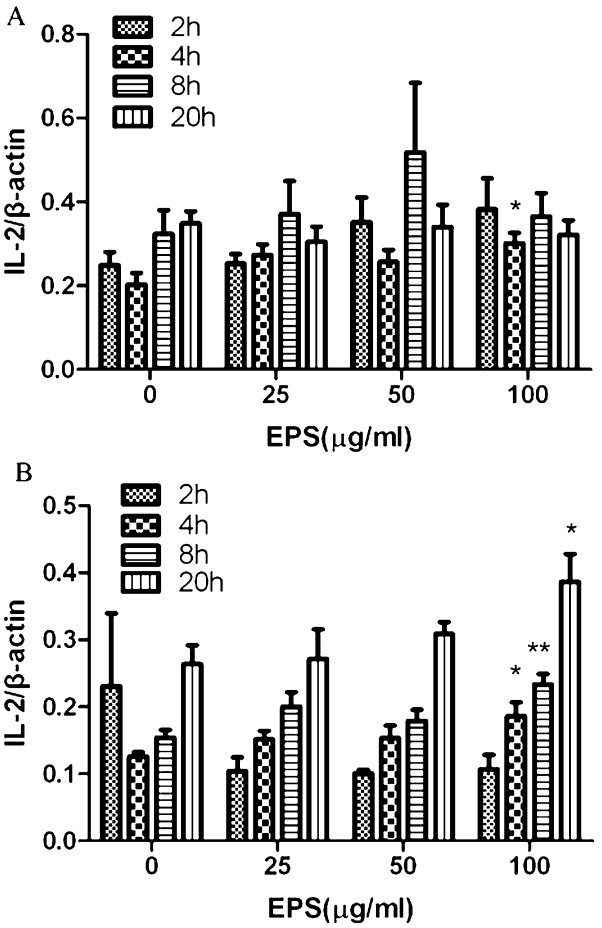

Fig. 6 Effects of EPS on IL-2 mRNA level of splenocytes (**a**) and thymocytes (**b**) of ICR mice. Data are expressed as mean±SD (n=6). *p<0.05; **p<0.01; compared with control

Table 2 TNF-α, IFN-γ and IL-2 protein levels of ICR mice splenocytes and thymocytes after 20 h EPS treatment (x±s, n=6)

Cells	Concentration (μg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)	IL-2 (pg/ml)
Splenocytes	0	21.969±2.742	26.676±6.661	27.188±3.838
	25	24.663±2.002	30.833±3.144	30.682±1.341
	50	26.450±4.287	27.468±4.164	26.277±1.134
	100	27.525±1.974**	36.308±1.569*	29.220±3.004
Thymocytes	0	24.258±2.091	25.845±3.134	28.237±2.049
	25	26.490±3.070	30.842±7.434	26.743±3.866
	50	28.281±2.629*	33.695±5.432*	27.613±1.592
	100	29.679±2.272**	39.544±4.278***	27.322±2.479

*p<0.05; **p<0.01; ***p<0.001, compared with control

tumor growth in mice, but also significantly elevated immunocytes' activity in tumor-bearing mice [8, 9]. In view of that, we supposed that EPS inhibited tumor cells mainly by activating the host's immune system. In order to further confirm that, effects of EPS on immunocytes *in vitro* were investigated in the present study. Our results showed that polysaccharide EPS mainly stimulated cytokine expressions of mouse immunocytes instead of impacting their proliferation ability.

Cytokines are intercellular mediators that regulate survival, growth, differentiation, and the effector functions of cells [11]. They are glycoproteins of low molecular weight and may also significantly affect the growth of tumors *in vivo* [12–15]. TNF-α is a multifunctional cytokine playing a key role in apoptosis and cell survival as well as in inflammation and immunity. It also has a cytotoxic effect on tumor vasculature and thus it is an important host defense cytokine that affects tumor cells [16, 17]. IFN-γ has an additional role in preventing development of primary and transplanted tumors besides playing physiological roles in promoting innate and adaptive immune responses. In cancer xenografts, the antiproliferative action of IFN-γ is probably due to enhanced cell death by up-regulation of some caspases [18–20] and an antiangiogenic activity [21]. Our results indicated that EPS treatment could significantly promote the mRNA and protein levels of TNF-α and IFN-γ and thus support the assumption, antitumor activity was related to the promoted cytokine expression of immunocytes. IL-2 is also one of the cytokines most widely used for the treatment of cancers [22]. However, significant changes did not be found in IL-2 protein level of EPS treated immunocytes although there was obvious increase in mRNA level. This means EPS could not stimulate IL-2 secretion and it also indicated that protein level of cytokines is sometimes not consistent with their mRNA level.

Conclusions

In conclusion, we purified the exopolysaccharide EPS from the cultivated *C. sinensis* and analyzed its preliminary characters. Our results also indicated that EPS may activate the immunocytes and promote cytokines' expressions.

However, polysaccharides usually have a wide range of bioactivities and stimulating cytokine expression is just one cause of tumor inhibition. Thus investigations on other functions of EPS are in progress in our laboratory to further confirm the relationship of antitumor activity and immunomodulatory function.

Acknowledgements The study was supported by the National Nature Science Foundation of China (No. 30873188 to W.Y. Zhang).

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