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Effects of *Lycium barbarum* extract on production and immunomodulatory activity of the extracellular polysaccharopeptides from submerged fermentation culture of *Coriolus versicolor*

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Abstract

Polysaccharopeptides (PSPs) from *Coriolus versicolor* have been used as immunomodulatory and anticancer agents. However, most studies have concentrated on the mycelial PSPs and not those in the fermented broth. On the other hand, *Lycium barbarum* fruit has been used as a traditional Chinese herbal medicine for two millennia. Its extract contains various nutrients, minerals, and also polysaccharide–protein complexes, which are proven to be bioactive. Herein we report the effects of *L. barbarum* fruit extract on the mycelial growth and extracellular PSP (ePSP) production of *C. versicolor* LH1 by using a submerged fermentation process in 20 l fermenters. Fermentation production of *C. versicolor* biomass and its ePSP were augmented in the presence of *L. barbarum* extract. The ePSP such obtained differs from those obtained with normal culture medium in terms of simple sugar composition and protein content but shows similar overall chemical structures as analyzed by Fourier transformed infrared spectroscopy. Moreover, the ePSP from *C. versicolor* cultured with supplementary *L. barbarum* extract exhibits significant immunomodulatory activity as judged by its effects on the production of nitric oxide and several cytokines by murine RAW264.7 macrophages.

Keywords: *Coriolus versicolor*; Extracellular polysaccharopeptides; *Lycium barbarum* extract; Immunomdulatory activity; Macrophages

1. Introduction

Coriolus versicolor (syn. *Trametes versicolor*, Yunzhi), a white rot fungus found worldwide, is a medicinal mushroom with a wide range of applications (Wasser & Weis, 1999) and the fungus can be grown in submerged fermentation as mycelial biomass (Cui & Chisti, 2003). The most commercially successful products from *C. versicolor* are polysaccharopeptides (Cv-PSPs), protein-bound polysaccharide preparations obtained from cultured mycelia of the CM-101 (ATCC 20547) and Cov-1 strains (Cui & Chisti, 2003; Kobayashi, Matsunaga, & Fujii, 1993; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007). These PSP preparations are heteroglycans with $\alpha(1 \rightarrow 4)$ and $\beta(1 \rightarrow 3)$ glycosidic linkages with protein components and have documented anticancer activity *in vitro*, *in vivo* and in human clinical trials (Hsieh, Wu, Park, & Wu, 2006). The drugs are also regarded as immunomodulators or biological response modifiers (BRMs). For instance, extracts of a PSP preparation from Cov-1 strain markedly increased the

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secretion of IL-1β and IL-6 and triggered the apoptotic pathways in human leukemia HL-60 cells (Hsieh, Kunicki, Darzynkiewicz, & Wu, 2002). In an *in vivo* study, peritoneal macrophages isolated from mice that were fed with PSP showed increased production of reactive nitrogen intermediates, superoxide anions, and TNFs (Liu, Ng, Sze, & Tsui, 1993). Taken together, *C. versicolor* PSPs were shown to activate effector cells like macrophages, T lymphocytes and NK cells to secrete cytokines like TNF-α, IFN-γ, IL-1β, etc., which are antiproliferative and inductive to cell apoptosis and differentiation. It is conceivable that the drugs are able to modulate the non-specific immune system and to exert antitumor activity through the stimulation of the host's defense mechanism (Cui & Chisti, 2003; Ng, 1998).

The fruit of *L. barbarum* in the family Solanaceae is a well-known herb in the East, and is widely used as a popular functional food (Li, Ma, & Liu, 2007). Several lines of evidence suggested that the polysaccharide–protein complex (LBP) is the important bioactive component in this herb. LBP are heteroglycans with β(1 → 3) glycosidic linkages. It contains several monosaccharides and 17 amino acids, and constituents with biological effects and immunomodulatory activity. It was also reported that the crude LBP could exhibit antitumor activity *in vivo* (Gan, Zhang, Yang, & Xu, 2004).

Mycelial growth from submerged fermentation of fungal mycelia is known to be affected by the fermentation conditions, especially the medial constituents (Wang & Lu, 2005). Process parameters such as temperature, rotatory speed, and initial pH were shown to affect the mycelial growth and extracellular PSP (ePSP) production by *Boletus* spp. ACCC 50328 (Wang & Lu, 2005). On the other hand, it was shown that accumulation of mycelial growth and ePSP in *C. versicolor* Wr-74 and ATCC-20545 could be augmented by milk permeate (Cui, Goh, Archer, & Singh, 2007). The chemical compositions and thus the properties of the targeted products might also be affected by the medial constituents. For instance, Chen, Hsu, Lin, Lai, and Wu (2006) reported that the sugar compositions of ePSPs of *Tremella mesenterica* were altered by culture media with different carbon sources and that the preparations were able to stimulate the productions of nitric oxide and a number of cytokines in murine macrophage RAW264.7 cells.

In our attempts to study the production and bioactivity ePSP of *C. versicolor* LH1 (ePSP-Cv) produced from fermentation cultures with herbal supplements, we herein report our findings on the characterization of ePSP-Cv prepared from cultures supplemented with *L. barbarum* extract in terms of chemical properties and immunomodulatory activities.

2. Materials and methods

2.1. Strain and culture conditions

The *Coriolus versicolor* strain LH1 was originally collected from the mountains of Nantou, Taiwan and stored at the Da-Yeh University, Changhua County, Taiwan. The culture was maintained on potato dextrose agar (PDA) plates at 25 °C. For seeding, the cultures were cultured in normal culture medium (4.0% glucose, 0.3% peptone, 0.15% KH_2PO_4 and 0.15% $MgSO_4 \cdot 7H_2O$) in Erlenmeyer flasks at 25 °C on a rotary shaker at 150 rpm; 5-day-old cultures were used.

2.2. Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, Utah, USA). Sodium nitrite, Griess reagent and lipopolysaccharide (LPS, *Escherichia coli*, Serotype 055:B5) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The TNF-α, IL-1β and IL-6 ELISA kits were purchased from e-Bioscience (CA, USA). All other reagents were purchased from Sigma unless otherwise specified.

2.3. Preparations of Lycium barbarum extracts (LBE) and polysaccharopeptides (PSP-Lb)

Fruits of *L. barbarum* were purchased from a local market. One hundred grams of dried fruit was boiled in 1 l of water for 2 h. The soup was concentrated in a rotary vacuum evaporator until the liquid volume reduced to 100 ml. The solution was filtered and labeled as *L. barbarum* extract (LBE), which was added to the culture medium for fermentor production of *C. versicolor* when needed. Alternatively, the LBE was added four volumes of 95% ethanol and allowed to settle for 24 h. The precipitates thus obtained were collected by centrifugation and then lyophilized, giving the desired crude PSP (designated as PSP-Lb hereafter).

2.4. Preparations of extracellular PSPs from fermentation cultures of C. versicolor (ePSP-Cv)

Batch fermentation of *C. versicolor* LH1 was carried out in a 20 l fermenter (Bio-top, Taiwan) in normal culture medium with or without 0.5% (w/v) of LBE. Fermentations were carried out at 25 °C, pH 4.5–5.0, 100 rpm for 7 days. After fermentation, cell mass were collected by centrifugation at 6000 rpm for 30 min, lyophilized, and weighed. The supernatants, respectively, from culture media with or without added LBE, were processed for PSP preparations as described above, and labeled as ePSP-Cv-LBE and ePSP-Cv. PSP contents were determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), using glucose as the standard.

2.5. Preparations of PSP solutions for cell treatments

The PSPs, including the PSP-Lb, ePSP-Cv, and ePSP-Cv-LBE obtained above were dissolved in DMEM at the

concentration of 10 mg/ml. After 24 h continuous shaking at room temperature, undissolved materials were removed by centrifugation and the supernatants were sterilized by passing through 0.22 μm filters. The concentrations of the PSP solution were diluted with cell culture medium to 250 μg/ml before use.

2.6. Characterization of the PSP preparations

The protein contents of the PSP preparations were determined by the AOAC method (1995). For determinations of monosaccharide composition, the PSP samples were hydrolyzed with 5 ml of 2 M trifluoroacetic acid (TFA) at 100 °C for 16 h. The contents of neutral sugars was analyzed by a HPLC system (Jasco PU-2080 Plus) equipped with a refractive detector (RI-2031 PLUS) and a SUGAR SP0810 (Shodex) column (8 mm × 300 mm) was used. The mobile phase was ddH_2O at 0.8 ml/min and the temperature of column was maintained at 80 °C. Additionally, the samples were analyzed by an absorbance IR spectroscopy by the KBr pellet method using a Perkin Elmer LEE-59 spectrometer from 500 cm^{-1} to 4000 cm^{-1}.

2.7. Cells and cell culture

Mouse macrophage cell line RAW264.7 (ATCC TIB-71) was purchased from the Bioresources Collection and Research Center, Hsinchu, Taiwan. The cells were cultured in DMEM medium supplemented with 10% heated-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37 °C in a humidified incubator under 5% CO_2. For drug treatments, cell were trypsinized, suspended, adjusted to a concentration of 1–2 × 10^5 cells/ml, and dispensed to appropriate culture vessels. The cells were allowed to adhere for 24 h before treatment.

2.8. Effects of the PSP preparations on RAW264.7 cells

2.8.1. Cell growth and treatment protocol

Murine RAW264.7 macrophages were used to assess the immunomodulatrory effects of the PSP preparations, i.e., ePSP-Cv, ePSP-Cv-LBE and PSP-Lb, cell growth and proliferation was determined by the MTT assay, which was performed as previously described with modifications. In order to establish a treatment protocol, cells were treated with various amounts of the PSPs and the non-cytotoxic dose was determined to be 62.5–125 μg/ml. Thus the treatment protocol was used in all subsequent experiments. Cells in 96-well plates (10^4 cells/100 μl/well) were incubated with ePSP-Cv, ePSP-Cv-LBE, and PSP-Lb, at a final concentration of 62.5 μg/ml for 48 h. After treatments, 30 μl of MTT at 5 mg/ml in phosphate buffered saline (PBS, Invitrogen, Gibco) was added to each well and the samples were incubated at 37 °C for 2 h. The media were then removed and 100 μl of dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan precipitates. Absorbance at 570 nm of the dissolved solution was determined by a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The reading from the untreated cells was regarded as 100%.

2.8.2. Nitrite production

Measurements of nitrite production in the treated cells were carried out as described previously (Chen et al., 2006). Briefly, 50 μl of the PSP preparations were added to cells in 96-well plates (10^4 cells/100 μl/well) and then 50 μl LPS (at 4 μg/ml in DMEM and filter-sterilized) or equal volumes of medium were added. After 48 h of incubation, nitrite contents in the culture media were determined by the Griess reaction. For each sample, 100 μl of the conditioned media was mixed with equal volume of Griess reagent (1% Sulfanilamide, 0.1% NED, and 2.5% H_3PO_4) in a fresh 96-well plate and incubated at room temperature for 15 min. Absorbance at 540 nm was determined by a microtiter plate reader. The production of nitrite was quantified by comparing with a sodium nitrite ($NaNO_2$) standard curve.

2.8.3. Cytokine production

For cytokine production assays, cells in 24-well plates (10^5 cells/500 μl/well) were used. To the cells was added 250 μl of the PSP preparations and 250 μl LPS or equal volumes of medium. After 48 h of treatment, the conditioned media were collected from each well and assayed for the cytokines or stored at −80 °C before used. The cytokines, IL-1β, IL-6 and TNF-α, produced by the treated cells were analyzed immunochemically with commercial ELISA kits according to the manufacturer's instructions.

2.9. Statistical analysis

The data of this study were analyzed using the one-way ANOVA by using the Statistical Analysis System 8.0, followed by Duncan's multiple range test. The p values <0.05 were considered to be significantly different.

3. Results and discussion

3.1. Effect of L. barbarum extract (LBE) on production of mycelial biomass and extracellular polysaccharopeptides (ePSP) of C. versicolor LH1 in submerged fermentation

Measurements of mycelial biomass and extracellular PSP (ePSP) were carried in duplicate on a daily basis (Fig. 1). The results showed that both mycelial growth and ePSP production were increased in the presence of 0.5% LBE. In the presence LBE, ePSP contents in the fermented broth steadily increased during the course of fermentation and reached 1.66 g/l after a 7-day period. By contrast, without added LBE, the amount of PSP in the fermented broth remained relatively constant and reached only 0.61 g/l (Fig. 1, Table 1). In a recent study on submerged culture of *C. versicolor*, Tavares, et al. (2005)

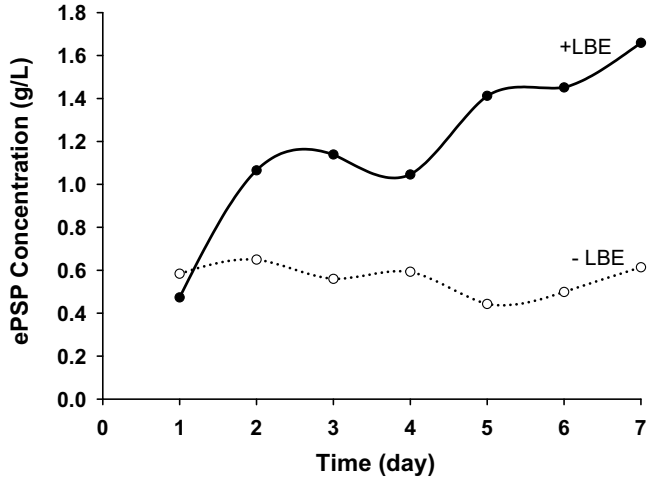

Fig. 1. Effect *L. barbarum* extract (LBE) on production of extracellular polysaccharopeptides (ePSP) of *C. versicolor* grown in 20 l fermenters.

Table 1
Productions of mycelial biomass and extracellular polysaccharopeptides (ePSP) in *C. versicolor* cultures with or without supplements

Cultures	Supplement	ePSP (g/l)	Biomass (g/l)	ePSP/ biomass ratio	References
Cv (LH1)	None	0.61	2.38	0.26	This study
Cv (LH1)-LBE	0.5% LBE	1.66	3.71	0.45	This study
Cv (INETI)-YM	Yeast malt extract	0.70	3.8	0.18	Tavares et al. (2005)
Cv (Wr-74)	Milk permeate	1.15	8.9	0.13	Cui et al. (2007)
Cv (ATCC 20545)	Milk permeate	1.32	10.6	0.12	Cui et al. (2007)

showed *C. versicolor* grown in yeast malt yielded 3.8 g/l biomass and 0.70 g/l ePSP. More recently, Cui et al. (2007) showed ePSP in *C. versicolor* Wr-74 and ATCC-20545 fermented with milk permeate respectively produced 8.9 and 10.6 g/l biomass; and, 1.15 and 1.32 g/l ePSP.

It is known that composition of culture medium plays pivotal roles in mycelial growth and PSP production in *C. versicolor* and other fungal species. Using the methods specified by the AOAC (1995), the moisture, carbohydrate, ash, crude protein, crude fat, and crude fiber contents of the LBE (100 g/l) were determined to be 90.83, 5.6, 0.52, 1.23, 0.60, and 1.22 g/100 g, respectively. Additionally, it was shown that *L. barbarium* fruits contained various amounts of protein, carbohydrates, β-carotene, succinic acid, vitamin C, riboflavin (Li et al., 2007), as well as numerous minerals such as Cu, Fe, Zn, Mn, Mg, Se, Ca, etc. (Wen, Chung, Chou, Lin, & Hsieh, 2006). Although the exact component stimulant(s) in LBE was not determined in this study, it is clear that supplementary LBE, which contains a variety of bioactive compounds as mentioned, would significantly stimulate mycelial growth and ePSP production of *C. versicolor* LH1. Our data also showed that *C. versicolor* LH1 cultured in the presence of LBE yielded the highest ePSP/biomass ratio (Table 1).

3.2. Characterizations of the PSPs from C. versicolor fermented broth and L. barbarum fruits

3.2.1. Protein contents

Protein contents of the ePSPs of *C. versicolor* and *L. barbarum* fruits are shown in Table 2. Addition of the LBE in the culture medium led to a 63.8% increase in protein content of the ePSP produced by *C. versicolor* (22.6 vs. 13.8%). In general, PSPs from *C. versicolor* was determined to be around 15% (Yang & Zhou, 1993) and it was reported that the protein content of Krestin (PSK), a commercialized *C. versicolor* PSP from Japan was 28–35% (Ueno et al., 1980). Our results nevertheless showed that the protein content of the ePSP produced by *C. versicolor* can be significantly increased by adding LBE in the culture medium; a 1.7-fold increase in protein content in ePSP production was observed. The increased biological activity and protein content of PSP are likely to be closely correlated, so that increased production of polysaccharides would also increase the protein content since the two components are covalently linked (Lee, Yang, & Wan, 2006). Most recently, it was suggested that polysaccharide polymers can reach maximum complexity when they are covalently attached to other molecules such as polypeptides and proteins as polysaccharide–peptide complexes (Moradali et al., 2007).

3.2.2. Monosaccharide compositions

Simple sugars including glucose, galactose, mannose, xylose, and arabinose in the PSP samples were determined and presented as mg/g ratio as shown in Table 3. Compared to the PSP from *C. versicolor*, those from *L. barbarum* contained relatively high levels of arabinose and galactose, a finding similar to that reported by Wu, Ng, and Lin (2004). Additionally, mannose was not detectable in our preparation (Table 3). After 7 days of fermentation, however, arabinose was not detectable but significant amounts of mannose were found, suggesting that the *L. barbarum* PSP (PSP-Lb) in the supplementary LBE was completely utilized and converted.

In this study, glucose and galactose contents in ePSP-Cv and ePSP-Cv-LBE were basically the same, around 80% and 9%, respectively. By contrast, the relative amount of mannose decreased by 80% (1.92% vs. 8.18%), while that

Table 2
Protein contents of the PSPs from *C. versicolor* fermented broths and *L. barbarum* fruits

Cultures	Protein contents (%)	Changes (%)
ePSP-Cv (LH1)	13.8 ± 0.3	–
ePSP-Cv (LH1)-LBE	22.6 ± 1.5	63.8
PSP-Lb	18.6 ± 1.5	–

Table 3
Monosaccharide compositions of extracellular polysaccharopeptides (ePSPs) from *C. versicolor* fermented broths

ePSPs	Monosaccharide contents (mg/g ratio)					
	Glucose	Galactose	Mannose	Xylose	Arabinose	References
PSP-Lb	49.21	17.06	n.d.	1.33	32.40	This study
ePSP-Cv (LH1)	82.27	8.67	8.18	0.87	n.d.	This study
ePSP-Cv (LH1)-LBE	80.06	9.29	1.92	8.73	n.d.	This study
ePSP-Cv (INETI)-Tak	98	–	1.2	0.8		Tavares et al. (2005)
ePSP-Cv (INETI)-MCM	88	–	7.4	1.6		Tavares et al. (2005)
ePSP-Cv (INETI)-YM	95	–	3.4	1.6		Tavares et al. (2005)

n.d., not detectable.Tak, Tien and Kirk medium.

of xylose increased 10-fold (8.73 vs. 0.87%) in the ePSP preparations obtained from fermented broth supplemented with LBE (Table 3). The monosaccharide compositions of ePSPs from *C. versicolor* LH1 cultures with different supplements were reported. It was shown that the glucose content remained above 95% and that mannose and xylose contents increased slightly in culture media with specified mushroom complete medium and yeast malt extract (Tavares et al., 2005; Table 3). The variations may also arise due to different *C. versicolor* strains and growing conditions. Nevertheless, our data demonstrated that ePSP produced by *C. versicolor* LH1 in the presence of LBE contains a different composition of monosaccharides. The data showed that ePSP-Cv (LH1)-LBE, in terms of monosaccharide composition, was distinctly different from PSP-Lb, suggesting that there was little residue PSP-Lb in the fermented broth.

3.2.3. Fourier transformed infrared (FTIR) analysis

The FTIR spectra of ePSP-Cv and ePSP-Cv-LBE were highly similar with clear absorption bands at 3419, 1641, 1078 cm^{-1} (Fig. 2). The absorption band at 1078 cm^{-1} was also reported for myclial PSP from *C. versicolor* by

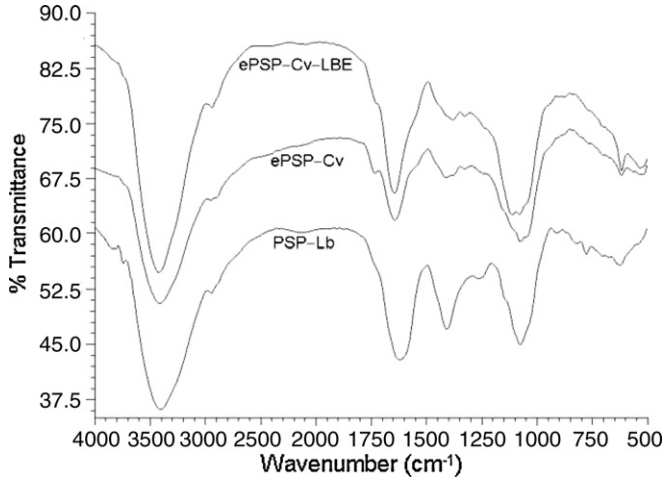

Fig. 2. FTIR spectra of the ePSPs from *C. versicolor* cultures. ePSP-Cv-LBE and ePSP-Cv were extracellular PSPs obtained from *C. versicolor* fermentation with or without LBE (0.5%, w/v), after 7 days of culture. PSP-Lb was from the LBE and run in parallel as an additional control. The spectra were obtained from solid samples by KBr disc method using a Perkin Elmer LEE-59 spectrometer from 500 cm^{-1} to 4000 cm^{-1}.

Ng and Chan (1997). In an earlier study (Yang & Zhou, 1993), the absorption bands at 3400, 1650, 1050, and 893 cm^{-1} are suggested to be, respectively, indicative of –OH, $–NH_2$, C–O–C, and β-glycosidic linkages. However, it was also suggested that not only 893, but also 930, 1038, 1078 and 1161 cm^{-1} are indicative of β(1 → 3) glycosidic bonds (Sandula, Kogan, Kacurakova, & Machova, 1999). In this study, the 893 cm^{-1} band was not found, but instead the 1078 cm^{-1} band was found in all PSPs preparation. The data led us to conclude that the ePSP-Cv and ePSP-Cv-LBE are highly similar in their gross chemical structures and that both samples contained β(1 → 3) glycosidic bonds, indicating that they both may be bioactive. On the other hand, the FTIR spectrum of PSP-Lb was significantly different to those of *C. versicolor*, especially from 1,650 to 1,050 cm^{-1}, again suggesting that the ePSP recovered from the fermented broth (ePSP-Cv-LBE) was not PSP-Lb.

Taken together, the addition of LBE in the culture medium not only changed the yield of ePSP of *C. versicolor* LH1, but also changed the chemical characteristics such as protein content and monosaccharide composition. Nevertheless, the FTIR spectra indicated that all PSP preparations contain β(1 → 3)glucans, which are deemed to be bioactive; therefore, the samples were subjected to the following experiments together.

3.3. Immunomodulatory effects of PSPs from C. versicolor fermented broths and L. Barbarum fruits

3.3.1. Effects of ePSP-Cv, ePSP-Cv-LBE and PSP-Lb on proliferation of RAW264.7 cells

After treatment of RAW 264.7 cells at 62.5 μg/ml PSPs for 48 h, the relative cell proliferations were determined to be 101.3 ± 14.0, 130.0 ± 10.0, and 109.8 ± 1.4% for ePSP-Cv, ePSP-Cv-LBE and PSP-Lb, respectively. The data showed that ePSP-Cv-LBE is slightly more mitgenic, compared to those of ePSP-Cv and PSP-Lb, which seems to be neutral in terms of cell proliferation.

3.3.2. Effects of ePSP-Cv, ePSP-Cv-LBE and PSP-Lb on nitric oxide production in RAW264.7 cells

Cells were treated with 62.5 μg/ml of the PSPs in the presence or absence of 1 μg/ml LPS. After 48 h of treat-

ment, the nitrite contents in the condition media were determined by the Griess reaction. In cells treated with the PSPs without LPS, there was no difference between ePSP-Cv and the untreated control; but those treated with ePSP-Cv-LBE and PSP-Lb secreted much larger amounts of NO in the conditioned media (Fig. 3). In the presence of LPS, cells produced approximately ten times of NO compared to the untreated control; however, there was no significant difference among different treatment groups (Fig. 3).

NO production by macrophages was deemed to be one of the protective functions of the cells and stimulation of NO production by LPS has frequently employed as a way of checking immunomodulatory activity in general. Wasser (2005) showed that the β-glucan-related polysaccharides of the macrofungi are able to activate macrophages to release NO, which is a well known and important chemical messenger in many biological responses. PSPs from *C. versicolor* were shown to increase NO production in *ex vivo* and *in vivo* models. Pang, Zhou, Chen, and Wan (1998) showed that when mouse peritoneal macrophages were treated with LPS in the presence of polysaccharide Krestin (PSK, a form of PSP produced by *C. versicolor* CM-101), NO production was much higher compared to those treated with LPS alone. It was also shown that C57BL/6 mice treated with LPS would also induce NO production, which could be further stimulated by feeding the mice with intra- and extra-cellular PSPs from *C. versicolor* Cov-1 (Wang, Ng, Liu, Ooi, & Chang, 1996). In this study, it is noteworthy that the NO content

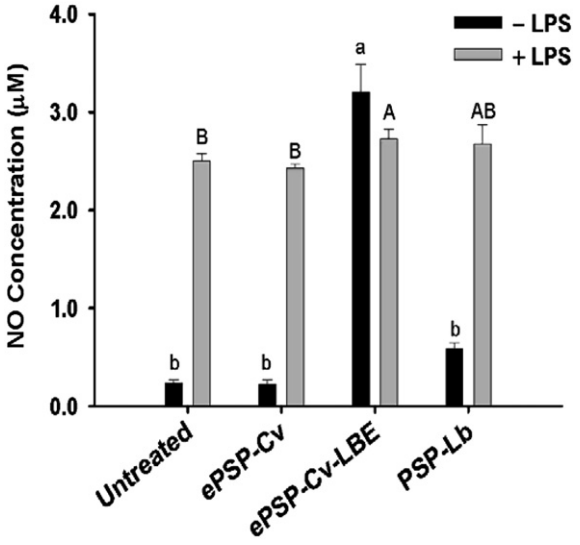

Fig. 3. Effect of PSPs on nitric oxide production of macrophage RAW264.7 cells. Cells were treated with 62.5 μg/ml of PSPs for 48 h and the nitric oxide contents in the conditioned media were determined by Griess reaction. The data were mean ± SD from three independent experiments. Statistical analyses were performed separately for samples with or without LPS and denoted by using capital and small letters respectively. Different labels (A–C or a–c) indicate that means significantly differ at $p < 0.05$ (based on the Neuwman–Keuls test).

reached 3.20 g/ml in the ePSP-Cv-LBE group, compared to 0.24 g/ml in the control and an average of 2.59 g/ml in the samples co-treated with LPS (Fig. 3). As mentioned in the previous section, there are β(1 → 3) glycosidic bonds in the ePSP preparations, but the relative amount of polysaccharides and proteins are different. The altered chemical constituents of the ePSP-Cv-LBE, compared to ePSP-Cv, may account for the enhanced production of NO in the treated cells.

3.3.3. Effects of ePSP-Cv, ePSP-Cv-LBE and PSP-Lb on cytokine production in RAW264.7 cells

The amounts of three cytokines, i.e., TNF-α, IL-1β and IL-6 were determined in the conditioned media after the RAW264.7 cells were treated with the PSP samples with or without LPS. It was found that all three PSP preparations (ePSP-Cv, ePSP-Cv-LBE and PSP-Lb) exhibit stimulatory effects on TNF-α production in RAW 264.7 cells, a 4 to 5-fold increase was observed and those treated with PSP-Lb produced slightly larger amount of TNF-α (Fig. 4A). However, when the cells were co-treated with LPS, the effects of the PSPs were completely masked; cells treated only with LPS produced 4.5-fold of TNF-α but there was no further induction of the cytokine when the cells were co-treated with the PSP preparations and there were no differences among the treatments (Fig. 4A). Cells treated with the PSPs produced significantly higher levels of IL-1β; ePSP-Cv, ePSP-Cv-LBE and PSP-Lb respectively enhanced the production of IL-1β by 4.2, 35.3, and 7.6 folds. Again, the effects were also masked by LPS; compared to the untreated control, an average of 12.1-folds of IL-1β production was observed and there was no difference among treatments (Fig. 4B). Both ePSP-Cv-LBE and PSP-Lb exhibit significant effects on IL-1β production, regardless of the presence of LPS. When RAW264.7 cells were co-treated with LPS and ePSP-Cv-LBE, production of IL-6 were enhanced by 12.62-folds, compared to the medium control (0.842 ± 0.008 vs. 0.065 ± 0.013 μg/ml) and by 20.75-folds, compared to that treated with the PSP alone (1.349 ± 0.002 vs. 0.065 ± 0.013 μg/ml) (Fig. 4C). On the other hand, ePSP-Cv did not affect the production of IL-6, regardless of the presence of LPS (Fig. 4C).

Ability to enhance production of interleukins by macrophages or other leucocytes has been widely used as an *ex vivo* experimental model for studying immunomodulatory activity of PSP preparations (Moradali et al., 2007). In our study, the ePSP-Cv-LBE preparation was shown to significantly increase the production of TNF-α, IL-1β, as well as IL-6 in RAW264.7 cells. TNF-α and IL-1β are cytokines respectively exhibiting cytotoxicity toward tumor cells and activation of T lymphocytes (Liu, Fung, Ooi, & Chang, 1996; Müller & Meineke, 2007). Müller and Meineke (2007) have also shown that while both IL-1 and TNF-α induce synthesis of IL-6, which in turn inhibits IL-1 and TNF-α synthesis. Both TNF-α and IL-1 have the capacity to induce IL-6 expression and all three cyto-

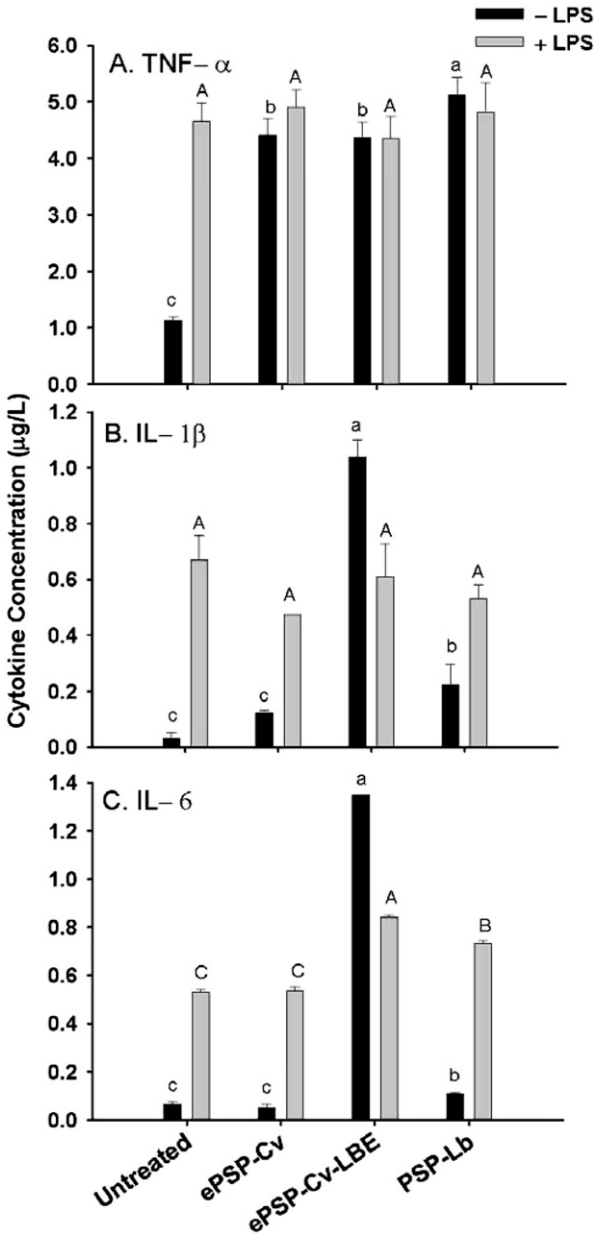

Fig. 4. Effects of PSPs on cytokine (IL-1β, IL-6 and TNF-α) secretion of macrophage RAW264.7 cells. Cells were treated with 62.5 μg/ml of PSPs for 48 h and the cytokine contents in the conditioned media were determined by ELISA. The data were mean ± SD from three independent experiments. Statistical analyses were performed separately for samples with or without LPS and denoted by using capital and small letters, respectively. Different labels (A–C or a–c) indicate significant difference at $p < 0.05$ (based on the Neuwman–Keuls test).

kines are important mediators of inflammation. In addition to the aforementioned findings, Cui et al. (2007) reported that milk permeates may be used as a culture supplement for fermentation of *C. versicolor* and the intra- and extracellular polysaccharopeptides (designated at IPS and EPS in their report) such obtained exhibit similar immunomodulatory activity. In their experiments, murine splenocytes were used as the target cells and productions of IL-12 and IFN-γ were determined. It was found that the optimal doses for immunostimulatory effect are 0.1–1.0 μg/ml, and the responses were inversely correlated with higher doses (Cui et al., 2007). Therefore, the immunomodulatory effects of PSPs may vary diversely and may attribute to their differences in chemical properties. Accordingly, the altered chemical constituents of the ePSP-Cv-LBE, compared to ePSP-Cv, reported here may account for the altered production of the cytokines in RAW264.7 cells.

4. Conclusion

The composition of culture medium plays pivotal roles in mycelial growth and PSP production in fungal species. In this study, we added *L. barbarum* extract (LBE) to *C. versicolor* LH1 culture, which resulted in significantly higher production of ePSPs. After fermentation in the presence of LBE, the composition of the simple sugar was altered, the protein content was increased, but both ePSP-Cv and ePSP-Cv-LBE still contained β(1 → 3)-glucan. We have prepared PSP-Lb from LBE by alcohol precipitation, a fraction would resemble the polysaccharide–protein complex (Gan, Zhang, Liu, & Xu, 2003; Gan, et al., 2004), which was found to be different to the ePSP-Cv and ePSP-Cv-LBE in terms of simple sugar composition and protein contents. Additionally, the FTIR spectra of PSP-Lb were different from those of ePSP-Cv and ePSP-Cv-LBE. All of the PSPs mentioned above were subjected to the bioactivity assays including cell proliferation effect and productions of NO, TNF-α, IL-1β, and IL-6. The differences in immunostimulatory effects of these PSPs may due to differences in simple sugar compositions, protein contents, and β-glucans. It should be noted that since the interplays of the cytokines are not completely clear and that the *ex vivo* data might be not truly reflected in the *in vivo* models, further characterizations of the compounds are warranted. Nevertheless, we provide the first example for production of *C. versicolor* LH1 mycelial and extracellular PSPs cultured with herbal extracts that may pave new avenues for future development of dietary supplements based on *C. versicolor* LH1 polysaccharopeptides.

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