Process Biochemistry 44 (2009) 1237–1244

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Exploring the potential of biopharmaceutical production by *Rigidoporus ulmarius*: Cultivation, chemistry, and bioactivity studies

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ARTICLE INFO

Article history:
Received 7 April 2009
Received in revised form 25 May 2009
Accepted 24 June 2009

Keywords:
Rigidoporus ulmarius
Polysaccharides
Nucleoside
Antiangiogenesis
Anti-inflammation

ABSTRACT

Rigidoporus ulmarius is used as a medicinal fungus in Asia. Three isolates (denoted #61, #62, and #63) of *R. ulmarius* were collected, and their biological activities were evaluated. Extracted polysaccharides from isolate #63 showed greater inhibition activity compared to isolates #61 and #62 in an *in vitro* endothelial cell tube formation assay, a standard evaluation of angiogenesis. The polysaccharides and ethanolic extract of isolate #63 dose-dependently suppressed the production of the interferon (IFN)-γ-induced inflammation marker, IP-10. Chemical analyses of the polysaccharides revealed that isolate #63 contained the highest value of fucose at a concentration of 59.1 ± 1.2 μmol/g polysaccharide. These results suggest that fucose-containing polysaccharides may play a role in the inhibitory effect. Isolate #63 showed the highest values of ADP among the three isolates in the ethanolic extract. These results suggest that different isolates from *R. ulmarius* exhibit different abilities to regulate antiangiogenic and anti-inflammatory processes.

1. Introduction

Mushrooms are well known and have been used for many years in Oriental populations as teas, nutritional foods, and even drugs. They exhibit special fragrances and textures [1]. Their biochemical composition consists mainly of proteins, carbohydrates, lipids, and vitamins. Fungal polysaccharides as biomaterials have found a wide range of applications including use in pharmaceutical therapy due to their unique physiological activities. Another advantage of obtaining bioactive polysaccharides from cultured mushrooms is the obvious potential for higher mycelial production in a compact space and in a shorter time with fewer chances for contamination. Basidiomycetes are the most used class as food due to their low toxicity to man, and they have extensively been studied. Despite their origin, the antitumor effects are generally attributed to polysaccharides with 1,3-β-glucan structures [2] or a β-glucan–protein complex [3]. However, the precise structure has not been fully characterized. Different physicochemical parameters of β-glucans, such as solubility, primary structure, molecular weight, extent of branching by side-chain substituents [4], and the charge on the polymer, all appear to influence their biological activities [5].

For many years, interest has concentrated on polysaccharides produced by mushrooms as potentially useful, biologically active ingredients for pharmaceutical applications as anticoagulants [6], antibiotics [7], and vaccines [8] due to a variety of biological activities, such as mitogenic activity, activation of alternative pathway complement (APC), an anti-hepatitis B surface antigen effect, anti-inflammation, plasma clotting activity, and tumor-suppressive effects. The mushroom, *Rigidoporus ulmarius*, of the Polyporaceae, is found mainly on broadleaf trees. The fruiting bodies are white, yellowish to rose pink color, and rigid after dryness. It has long been used as a traditional medicine in Asia. Our previous serial study showed that fungal polysaccharides play important roles in regulating angiogenesis [9]. Angiogenesis is a dynamic process of endothelial proliferation and differentiation. Tumors with high angiogenic activity have been correlated with poor patient survival [10–12]. Therefore, fungal polysaccharides can be a useful adjunct to conventional cancer therapies. Interferon-gamma (IFN-γ) has been demonstrated to be a key cytokine involved in inflammatory processes. IFN-γ induces the JAK/STAT1 signaling pathway in endothelial cells (ECs) followed by induction of chemokines which serve as chemotactic factors to recruit T cells to sites of inflammation then induce atherosclerotic lesions. CXCL10 (IP-10) is the major chemokine induced after IFN-γ release [13–15]. However, there is no other documentation of its biological functions or chemical constituents. It would be interesting and worthwhile to investigate

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doi:10.1016/j.procbio.2009.06.018

the chemical components and biological functions of *R. ulmarius*. In an attempt to assess the potential usage of *R. ulmarius*, *in vitro* experiments were first conducted with mycelial extracts of three *R. ulmarius* isolates to verify their biological functions. Therefore, in this study, we monitored the effects of endothelial tube formation to identify their actions on angiogenesis, IP-10 release to identify their actions on inflammation, and the biological correlations with the chemical components of the *R. ulmarius* extracts.

2. Materials and methods

2.1. Materials

R. ulmarius isolates #61 (TFRI #1047) and #62 (TFRI #1047 MG) were isolated from fruiting body collected from South of China (Fu-Jian) on April 2002, and #63 (TFRI #1058) was isolated from fruiting body collected from Northern Taiwan (Fu-Shan Research Station) on February 2002. High-performance liquid chromatographic (HPLC) standards, ADP (97%), cytidine (99%), adenosine (99%), inosine (99%), myo-inositol (99%), sorbitol (98%), fucose (99%), galactosamine (99%), glucosamine (99%), galactose (99%), glucose (99.5%), mannose (99%), and fructose (99%) were purchased from Sigma (Saint Louis, MO, USA).

2.2. Liquid culture

R. ulmarius was cultured in 100 ml of 24 g/L potato dextrose broth (PDB), with 20 g/L glucose pH 5.6 at 28 °C. A 35-day-old culture was harvested for polysaccharides and ethanolic extraction. PDB and glucose were purchased from Sigma (Saint Louis, MO, USA). LC-grade organic solvents were purchased from Merck (Darmstadt, Germany).

2.3. Preparation of polysaccharides and the ethanolic extract

Polysaccharides were isolated from lyophilized mycelia by hot water at 80 °C in a 1:100 (w/w) ratio for 6 h twice and cooled, and then four volumes of 95% ethanol were added and precipitated overnight at 4 °C. The precipitated polysaccharides were collected by spinning at 9000 × *g* for 20 min and lyophilized, resulting in a crude polysaccharide sample. The ethanolic supernatant was lyophilized following centrifugation and was denoted the ethanolic extract.

2.4. Endothelial cell culture

ECs were cultured under condition as described before [13]. Briefly, endothelial cells were maintained in DMEM medium (Life Technologies) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies) under standard culture conditions. The cell viability and cell numbers were determined by the trypan blue dye-exclusion method.

2.5. MTT assay

Measurement of cellular 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) reduction was performed as described previously [16]. The result of absorbance of MTT-formazan adducts is shown as the percentage of untreated control cells to indicate percentage of surviving cells.

2.6. Matrigel EC tube formation assays

In vitro Matrigel tube formation was performed as previously described [17]. The tube formation area, length, joints, and branches were expressed as percentages of the VEGF-treated controls using KURABO Angiogenesis Image Analysis Software.

2.7. Detection of IP-10 protein release

Levels of IP-10 secreted into the culture supernatant collected from ECs after IFN-γ stimulation with or without pretreatment with *R. ulmarius* polysaccharides or the ethanolic extract were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Concentrations of secreted IP-10 were determined using ELISA readers from BioSource (Camarillo, CA, USA).

2.8. Size-exclusion chromatography (SEC) of polysaccharides

SEC measurements were performed at room temperature on two SEC columns ($G4000PW_{XL}$ 7.8 mm × 300 mm and $G3000PW_{XL}$ 7.8 mm × 300 mm, Viscotek). The flow rate was 0.5 ml/min, with deionized water was used as the eluent. An RI detector (Viscotek model 301) was used. A polysaccharide solution in milli-Q water was diluted to give a concentration of 1 mg/ml. The sample injection volume was 0.1 ml. Calibration was performed against an authentic standard, Sodex P-82 series (Showa Denko, Mentor, OH) containing polymaltotriose with molecular weights of 788, 404, 212, 112, 47.3, 22.8, 11.8 and 5.9 kilodaltons (kDa).

2.9. Compositional analysis of polysaccharides

Acid hydrolysis of polysaccharides and determination of monosaccharides composition were carried out according to Cheng et al. [17]. Monosaccharides were identified by comparing retention times of sample peaks with those of standards. Quantification of each monosaccharide was carried out by integration of the chromatographic peak on a PeakNet system (Dionex, Sunnyvale, CA). Standards of monosaccharide were dissolved in Q-water. Myo-inositol, sorbitol, fucose, galactosamine, glucosamine, galactose, glucose, and mannose were obtained from Sigma (Saint Louis, MO, USA). Sodium hydroxide solution was obtained from Fisher Scientific (Pittsburg, PA, USA).

2.10. High-performance liquid chromatographic (HPLC) analysis of the ethanolic extract

Nucleoside and nucleotide were identified by comparing retention times and photodiode array spectra (200–400 nm) of sample peaks with those of standards. Quantification of each nucleoside and nucleotide was carried out by integration of the chromatographic peak. Separations were carried out according to Cheng et al. [17] and modified as follows: standards were dissolved in 0.02 M NaH_2PO_4, pH 7.0. Samples were dissolved in 50% methanol.

2.11. Statistical analysis

Statistical analysis was performed using Student's *t*-test. Data are presented as the mean ± SEM. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. The growth and polysaccharide production of R. ulmarius

Mycelia of *R. ulmarius* of isolate #61 cultured by PDB as carbon-sourced medium was shown (Fig. 1A). The culture period between 7 and 28 days was a linear phase. Beyond 28 days, the culture entered a senescence phase. The time-course study of the polysaccharide yield showed that at 21 days of culture, the highest value of 0.74 ± 0.07 g/L was achieved (Fig. 1B). Comparisons

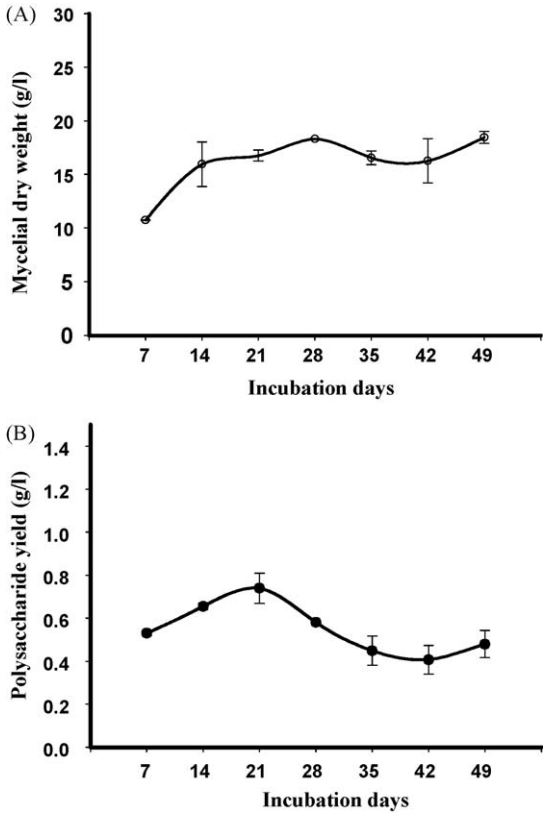


Fig. 1. Time course of growth and polysaccharide production in a mycelial culture of *Rigidoporus ulmarius* isolate #61. (A) Growth; (B) polysaccharide yield. Data are presented as the mean ± S.E. from three independent experiments.

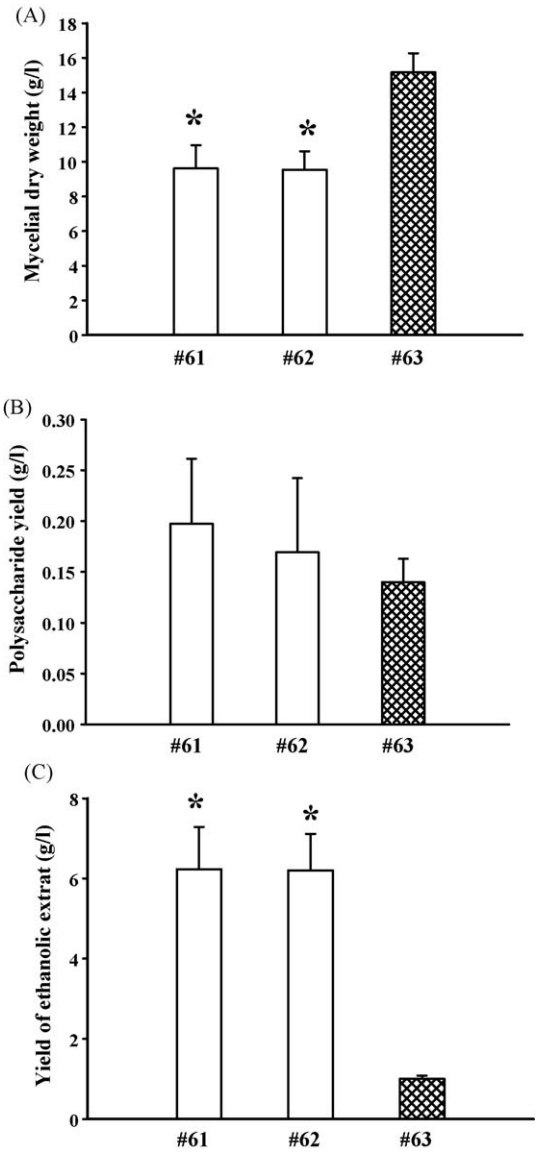


Fig. 2. Comparisons of yield of growth, polysaccharide and ethanolic extract production in a mycelial culture of among three isolates of *Rigidoporus ulmarius*. (A) Dry weight; (B) polysaccharides; (C) ethanolic extract. Data are presented as the mean ± S.E. from three independent experiments. $^{*}p < 0.05$ vs. isolate #63.

were made of the dry weight (Fig. 2A), polysaccharides (Fig. 2B), and ethanolic extract production (Fig. 2C) of 35-day cultures among the three isolates. Isolates #63 showed the highest dry-mass accumulation and the lowest ethanolic extract production among the three isolates. Isolates #61 and #62 showed no significant differences in growth, polysaccharides, or ethanolic extract production.

3.2. Toxicity of R. ulmarius

MTT assay was performed to evaluate the toxicity of *R. ulmarius* polysaccharides and the ethanolic extract toward EC. Confluent ECs was changed to serum-free medium and a serial dilution of polysaccharides and ethanolic extract were used to evaluate the toxicity toward ECs for 24 h. Neither the polysaccharides nor the ethanolic extract showed any toxicity toward ECs up to a concentration of 1000 μg/ml, except for the ethanolic extract of #63 at the concentration of 1000 μg/ml showed slightly degree of cytotoxicity (Table 1).

3.3. Influences of R. ulmarius on EC tube formation

In vitro Matrigel tube formation model for evaluation of angiogenesis were performed to study the effects of fungal polysaccharides and the ethanolic extract. Several parameters for determining antiangiogenic efficiency was checked for EC tube-like structures by calculating number of pixels in presenting total vascular area ratio, vascular length, and branching numbers. The area ratio was calculated by the area of tube formation/the field of vision. The smaller the value is, the lesser the degree of tube formation (the higher degree of inhibition of angiogenesis). Serial dilutions were used to study for their effects on VEGF-induced Matrigel tube formation (Fig. 3). The tube formation area ratio, and the ratio of tube length, joints, and branches all indicated a dose-dependent effect of inhibiting angiogenesis with polysaccharide pretreatment. Among the three fungal polysaccharides, #61 and #62 showed moderate inhibition of tube formation. The fungal polysaccharides of #63 showed the highest degree of inhibition of endothelial tube formation. These results indicate that polysaccharides of isolate #63 can be an angiogenesis inhibitor.

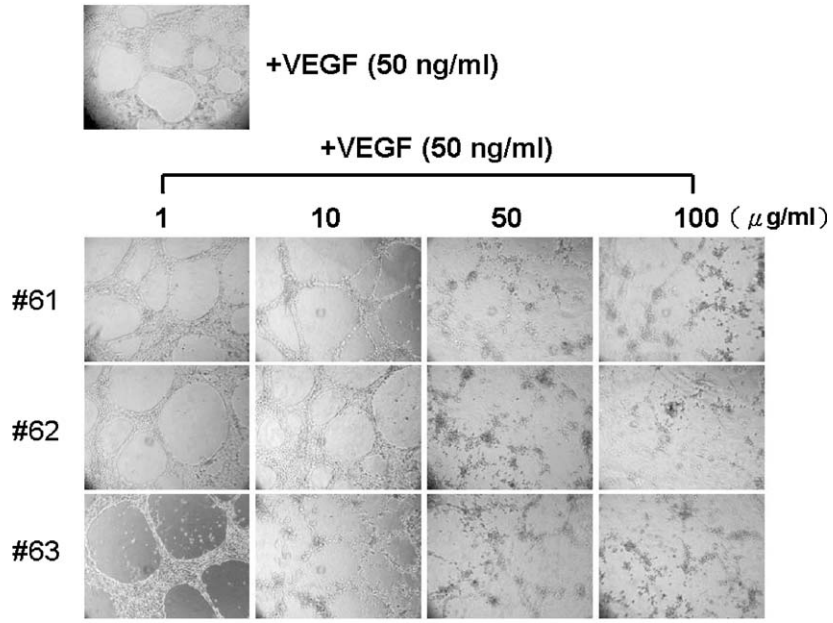
3.4. Influences of R. ulmarius on IFN-γ-induced IP-10 secretion

Pretreatment of polysaccharides or the ethanolic extract isolated from *R. ulmarius* were evaluated for their inhibitory effects on IP-10 protein release induced by IFN-γ. As shown in Fig. 4, IFN-γ treatment increased IP-10 protein release on ECs. Pretreatment of ECs with polysaccharides of isolate #63 suppressed IFN-γ-induced IP-10 protein release in a dose-dependent manner. However, pretreatment with the ethanolic extracts of #61 and #62 showed no effects on IFN-γ-induced IP-10 protein release. Only pretreatment of ECs with the #63 ethanolic extract suppressed IFN-γ-induced IP-10 protein release in a dose-dependent manner. This result indicates that the different isolates

Table 1
Toxicity of *R. ulmarius* to endothelial cells (ECs).

	Cell viability (% of control)				
	20[a]	100[a]	250[a]	500[a]	1000[a]
Polysaccharides					
#61	118.65 ± 6.19	111.31 ± 8.75	121.14 ± 12.50	121.59 ± 12.08	123.28 ± 12.71
#62	118.05 ± 8.49	108.03 ± 9.37	126.49 ± 11.93	133.71 ± 18.49	114.56 ± 9.39
#63	116.00 ± 11.44	103.85 ± 7.90	134.42 ± 13.91	138.10 ± 21.04	115.99 ± 9.73
Ethanolic extract					
#61	103.90 ± 6.60	113.23 ± 3.58	117.82 ± 3.09	117.83 ± 8.00	127.20 ± 14.78
#62	118.18 ± 8.55	122.96 ± 7.26	137.55 ± 24.07	131.77 ± 14.08	119.05 ± 13.81
#63	112.96 ± 9.67	117.47 ± 12.01	134.69 ± 8.61	122.57 ± 8.26	87.11 ± 4.59

A serial dilution of each fungal polysaccharide was applied to ECs for 24 h. Cell viability was evaluated using the MTT test. Cell viability was calculated as a percentage of the control from three separate experiments.
[a] Concentration (μg/ml).



Isolate	Concentration	Area ratio	Length ratio	Joints ratio	Branches ratio
#61	1	0.23	0.95	0.99	0.99
	10	0.21	0.39	0.16	0.20
	50	0.10	0.34	0.29	0.29
	100	0.06	0.10	0.01	0.03
#62	1	0.22	0.82	0.71	0.72
	10	0.22	0.49	0.17	0.23
	50	0.05	0.09	0.02	0.04
	100	0.04	0.03	0.01	0.05
#63	1	0.24	0.61	0.31	0.35
	10	0.10	0.30	0.21	0.24
	50	0.06	0.18	0.13	0.13
	100	0.03	0.08	0.04	0.04

Fig. 3. Effects of *Rigidoporus ulmarius* on endothelial cell (EC) tube formation. ECs were seeded onto Matrigel and cultured for 24 h under VEGF stimulation with or without (negative control) pretreatment with a serial dilution of polysaccharides for 1 h. Capillary tube formation on Matrigel was visualized with an inverted Zeiss microscope at a magnification of 10×. The ratio of the tube formation area and percentages of tube length, joints, and branches were calculated as a percentage of VEGF-treated ECs using KURABO Angiogenesis Image Analysis Software as indicated in the lower panel.

of *R. ulmarius* or different extracts of the same isolate may exhibit different levels of anti-inflammatory effects.

3.5. Characteristics of polysaccharides

To study the relationships between the structure and biological function of these polysaccharides, the polysaccharides were characterized according to their molecular size distributions and sugar compositions. The molecular weight distribution of the lyophilized polysaccharide-containing preparation was determined by size-exclusion column chromatography (Fig. 5). A calibration curve was constructed using a series of standards containing polymaltotriose with molecular weights of 788, 404, 212, 112, 47.3, 22.8, 11.8 and 5.9 kilodaltons (kDa). The regression equation was made between the log[Mw] (Y) and the fraction number (X) as $Y = 9.34 - 0.24X$; $R^2 = 0.99419$. The molecular weight distributions were characterized as very high molecular weight (>2000 kDa, peak 1), high molecular weight (>400 kDa, peak 2), medium high molecular weight (>100 kDa, peaks 3), low molecular weight (>7 kDa, peaks 4), and very low molecular weight (<1 kDa, peak 5). The results showed that the very high molecular weight (peak 1) and very low molecular weight (peak 5) contributed equally and represented the major species in the total polysaccharides of isolates #61 and #62. The molecular weight distribution of #63 differed from those of the others in the lack of the very high molecular weight (peak 1) of 2300 kDa, which accounted for 55.2% of total polysaccharides, and of very low molecular weight (peak 5) polysaccharides.

The chemical compositional of polysaccharides was obtained after the polysaccharide fraction had been completely hydrolyzed. The carbohydrate chromatogram (Fig. 6) and composition is presented in Table 2. The results showed that fucose, glucose, mannose, and fructose were the dominant sugars in the polysaccharides. Comparisons were made among the three isolates. Isolate #63 contained the highest value of fucose at a concentration of 59.1 ± 1.2 μmol/g polysaccharide.

3.6. Chemical composition of the ethanolic extracts from R. ulmarius

The *R. ulmarius* ethanolic extracts were separated by HPLC. Components were eluted from the column with mixtures of acetonitrile and NaH_2PO_4 and analyzed by UV detection at 260 nm.

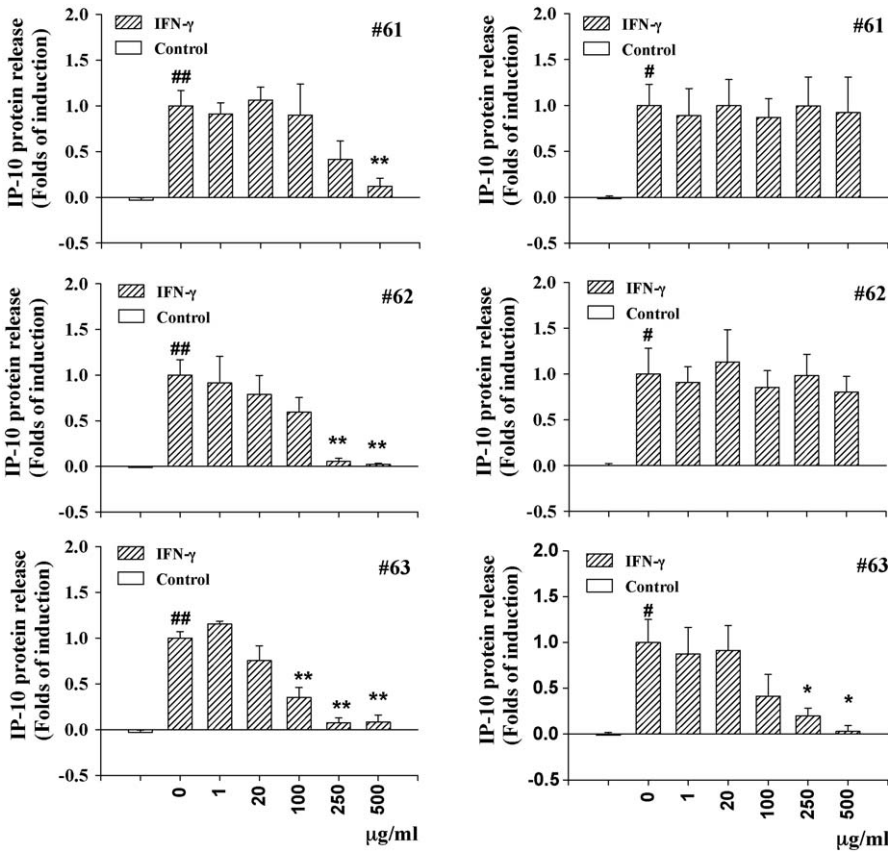


Fig. 4. Effects of *Rigidoporus ulmarius* on interferon (IFN)-γ-induced IP-10 protein release from endothelial cells (ECs). ECs were pretreated with (A) polysaccharides and the (B) ethanolic extract from *R. ulmarius* isolates #61, #62, and #63, and the IP-10 protein released from ECs at the indicated concentrations for 1 h followed by IFN-γ (10 ng/ml) stimulation for 24 h was detected. After treatment, conditioned medium was collected for the ELISA analysis. IP-10 protein release is expressed as pg/mg of total protein and is presented as the mean ± S.E. from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. IFN-γ treatment; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the control.

The separation chromatogram (Fig. 7) and the data are shown in Table 3. Three nucleoside-type compounds were identified. Isolate #63 had the highest values of ADP at 6.61 ± 0.19 mg/g ethanolic extract.

4. Discussion

Polysaccharides are of great interest from both an ecological perspective and human health demands. They also play important roles for the fungus itself as primary metabolites. There is growing interest in their use as pharmaceuticals. It is interesting to use an *in vitro* system to improve the production of these compounds. Also in the middle of the 20th century, improvements in analytical techniques such as chromatography allowed the successful identification of these macromolecules. Polysaccharides of the mushroom, *R. ulmarius*, used in this study were previously shown to exhibit antiangiogenic activity [16]. Different sources of mushrooms from nature are expected to produce different isolates with different chemical texture, for example, polysaccharides differing somewhat in structures, and compositions [18,19]. These different chemical compositions may lead to different bio-functions of this fungus. Therefore, this article describes the characteristics of different isolates of *R. ulmarius* and its bioactivities so that it can be developed as a potential food supplement and for therapeutic uses.

Due to the difficulty in collecting sufficient quantities of the remedy in the wild, we successfully used established liquid culture methods to acquire mass-produced cellular material from cultured mycelia. These mycelia cultures allowed us to evaluate the possible mechanisms underlying the antiangiogenic, and anti-inflammatory effect. According to our results, myo-inositol, fucose, glucose, mannose, and fructose were neutral sugars in polysaccharides of *R. ulmarius*. Isolate #63 exhibited a higher amount of fucose. In this study, the antiangiogenic activity of isolate #63 was much higher fucose compared those of the other isolates. The amount equaled to fucose in total polysaccharides of #63 was added and showed no significant inhibition on Matrigel tube formation (data not shown). Same observation showed that the content of fucose in the fucoidans from brown seaweeds did not significantly affect its antiangiogenic activity [20]. Although fucose was documented to be a major component involved in antiangiogenic, anti-inflammatory, anticoagulant, and anti-adhesive activities [20,21]. Mourão et al. [22] observed that sulfated fucose branches are also necessary for the anticoagulant and antithrombotic [23,24] activities of this glycosaminoglycan. In the structure feature of polysaccharides, fucose was one of the sugar components. The biological activity of polysaccharides of #63 could be due to fucose-containing linkage in structure which may important for its activity, but not the fucose itself. Those results suggested that different bioactivities may result from different levels of conjugation from monosaccharides to polysaccharides, including the composition of the side chain, the length of the side chain, and the number of repeated individual units.

Our findings indicate that polysaccharides and the ethanolic extract of isolate #63 showed the most potent anti-inflammatory activities. The very high molecular weight (peak 1) of 2300 kDa, accounted for 55.2% of the #63 polysaccharides. This suggests that the proportion of polysaccharides with a high molecular weight

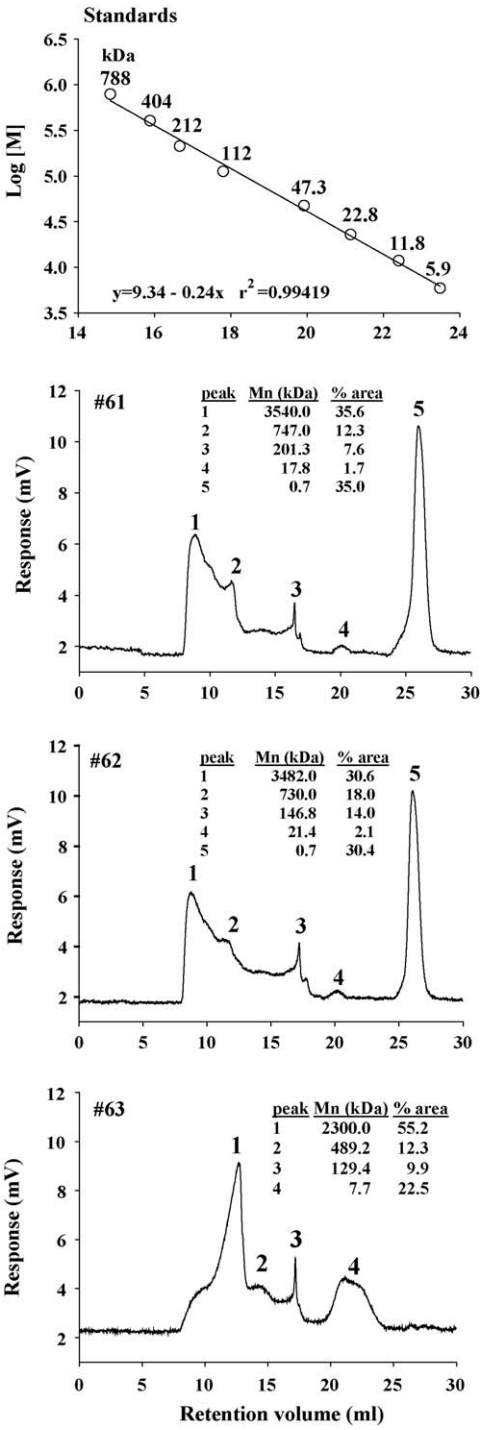


Fig. 5. Size-exclusion chromatography (SEC) profile of *Rigidoporus ulmarius* polysaccharides. SEC was performed using a ViscoTek model TDA-3-1 relative viscometer (Viscotek). A polysaccharide solution in milli-Q water was diluted to give a final concentration of 1 mg/ml for the determination.

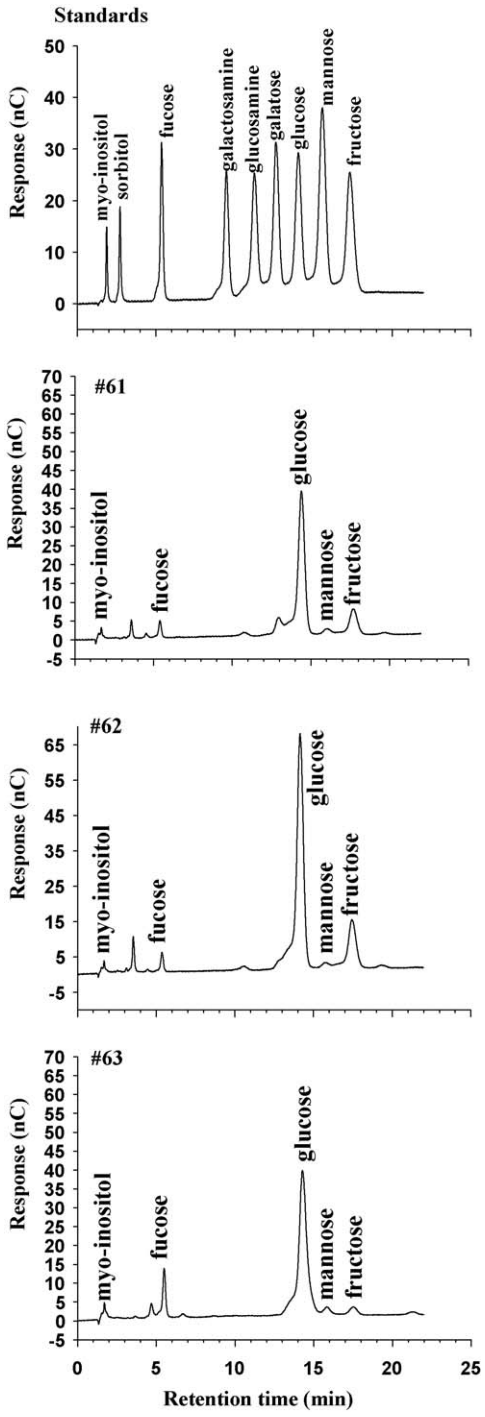


Fig. 6. High-performance anion-exchange chromatography (HPAEC) of *R. ulmarius* polysaccharide hydrolysates. The HPAEC analysis was carried out in 18 mM NaOH for 20 min at ambient temperature.

(>2000 kDa) affects the anti-inflammatory properties. Observations of the anti-inflammatory effect of isolate #63 suggest that ADP in the ethanolic extract, which were much higher than those of isolates #61 and #62, might play roles in anti-inflammation. ADP and related nucleoside-based compounds are well-known intracellular constituents, intimately involved in all aspects of cell functions and act as enzyme cofactors, sources of energy, and building blocks for DNA. Currently, purinoreceptors can be subdivided into P1 receptors, which bind adenosine as natural ligands and P2 receptors which bind ATP and ADP [25]. At present,

Table 2
Sugar composition of *R. ulmarius* polysaccharides.

Isolates	μmol/g polysaccharide				
	Myo-inositol	Fucose	Glucose	Mannose	Fructose
#61	9.4 ± 2.4	20.0 ± 3.4	251.7 ± 11.7	23.1 ± 7.9	98.1 ± 15.6
#62	13.1 ± 5.3	22.5 ± 1.8	453.6 ± 9.6	30.4 ± 2.2	234.7 ± 17.2
#63	8.4 ± 0.9	59.1 ± 1.2	351.2 ± 9.1	18.8 ± 0.5	30.6 ± 0.2

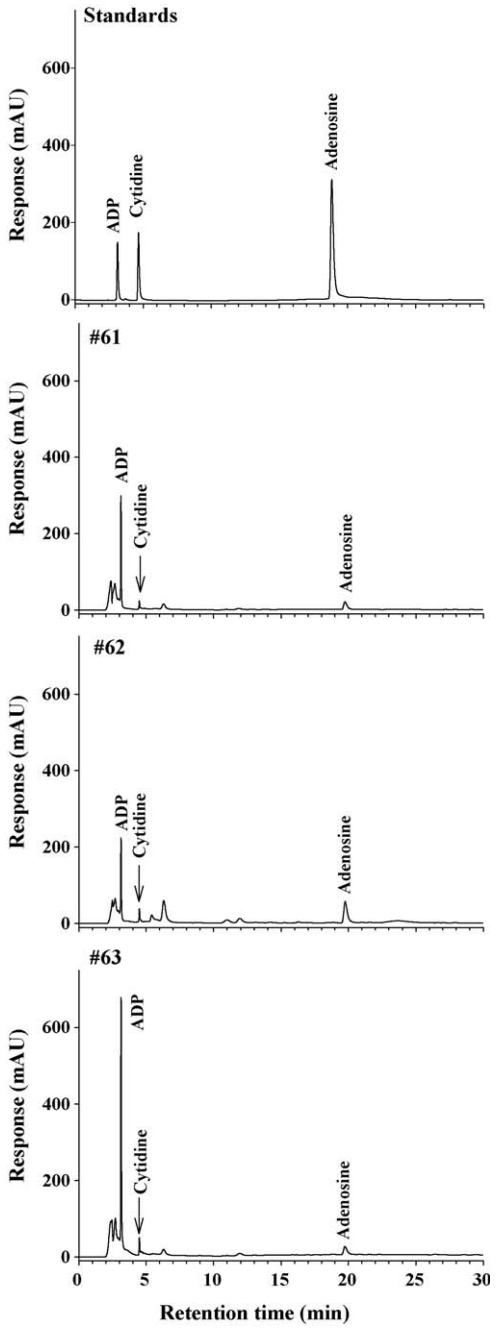


Fig. 7. High-performance liquid chromatography of *R. ulmarius* ethanolic extract.

these purinoreceptors are known to play important roles in regulating diverse physiological and pathological functions [26]. Furthermore, in comparison with our previous study that extracts from *Fomitopsis pinicola* [17], polysaccharides of three isolated of *R. ulmarius* in this study showed more potency than that of *F. pinicola* both in Matrigel tube formation and IP-10 release inhibition.

In conclusion, *R. ulmarius* have antiangiogenic and anti-inflammation properties. We observe that different isolates of this fungal species possess different degrees of anti-inflammatory and antiangiogenic effects. To date, no reports are available in the literature regarding the cultivation, chemical constituents, or their biological functions. If, as in studies of some other mushrooms, living cultures will be of great interest for future studies to establish a scaleable method for commercial production.

Table 3
Composition of ethanolic extracts isolated from *R. ulmarius*.

Ethanolic extract (mg/g)	#61	#62	#63
Adenosine	0.35 ± 0.02	1.05 ± 0.01	0.37 ± 0.01
ADP	2.19 ± 0.03	1.73 ± 0.04	6.61 ± 0.19
Cytidine	0.27 ± 0.01	0.41 ± 0.02	0.48 ± 0.01

Acknowledgements

We thank Dr. Tun-Tschu Chang, Division of Forest Protection, Taiwan Forestry Research Institute, Taipei, Taiwan for kindly supplying the isolates of *R. ulmarius* and Mr. D.P. Chamberlin for critically reading the manuscript. This work was supported by grants NSC96-2313-B-077-001-MY3 to MKL, and NSC97-2323-B-077-002, NSC94-2320-B-077-011 to JJC from the National Science Council, Taiwan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2009.06.018.

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