UDC 57.083.3:547.458:582.288
ISSN 1330-9862

original scientific paper

(FTB-1088)

Immunostimulatory Effects of Fungal Polysaccharides from *Ganoderma lucidum* Submerged Biomass Cultivation

Jožica Habijanič[1], Marin Berovič[1], Branka Wraber[2], Damjan Hodzar[3] and Bojana Boh[3]*

[1]National Institute of Chemistry, Hajdrihova 19, SI-1001 Ljubljana

[2]Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana

[3]Faculty of Science and Engineering, University of Ljubljana

*Department of Chem. Biochem. Engineering, University of Ljubljana, Slovenia

Received: April 6, 2001
Revised version: September 5, 2001
Accepted: November 8, 2001

Dedicated to the memory of Professor Vera Johanides

Summary

A Slovenian *Ganoderma lucidum* strain MZKI G97 was isolated and cultivated in a 10 L stirred tank reactor, on potato dextrose substrate. Biomass up to 15.2 g L^{-1} and fungal polysaccharides were produced. The extracellular polysaccharide fraction was obtained by the precipitation method with ethanol. Four intracellular polysaccharide fractions were obtained by the hot-water extraction and precipitation with ethanol, by ammonium oxalate extraction, and by extraction with sodium hydroxide, followed by precipitation with acetic acid and precipitation with ethanol. Immunostimulatory effects of isolates were tested on induction of cytokine (TNF-α, IFN-γ) synthesis in primary cultures of human mononuclear cells (PBMC) isolated from a buffy coat. Results have shown the potential of isolates to induce moderate amounts of TNF-α (max. 630 pg mL^{-1} of a culture supernatant), and IFN-γ in trace amounts (max. 11.5 pg mL^{-1}), respectively. The TNF-α inducing activity is comparable to romurtide, which has been used as a supporting therapy in cancer patients treated with radiotherapy and/or chemotherapy.

Key words: Ganoderma lucidum, submerged biomass cultivation, polysaccharides, cytokine assay, TNF-α, IFN-γ

Introduction

Many of the bioactive substances recently isolated from some higher *Basidiomycetes*, known as medicinal mushrooms, show antitumour, immunomodulating, antiviral and other promising effects. The inhibition of growth of different tumours was detected in about 200 species *(1)*. *Ganoderma lucidum* and its isolates have been known as a traditional remedy, used in Chinese and Japanese traditional medicine for treatment of several diseases, such as hepatitis, hypertension, hyperglycemia, chronic bronchitis, bronchial asthma, cancer and others (2).

Pharmaceutically active compounds from *Ganoderma lucidum* include triterpenoids, proteins, steroids, alkaloids, nucleotides, lactones and fatty acids. Polysaccha-

* Corresponding author; Phone: ++ 386 1 2419 510; Fax : ++ 386 14259 244; E-mail: marin.berovic@uni-lj.si

rides (especially β-D-glucanes) have been recognised as an effective anti-cancer drug *(1,3,4).* In *Ganoderma* polysaccharide research, special attention was paid to their immunomodulatory effects *(2,5).*

One of the important approaches to evaluate potential immunomodulating activity is the assessment of the capacity of particular substance to influence immune functions *in vivo*, *ex vivo* and *in vitro*, among them also cytokine synthesis. Cytokines are signalling molecules produced and secreted mainly by activated immune cells. They are essential for the maintenance of high-level functions of the organism and play an important role in controlling homeostasis of the whole organism by the surveillance of cell differentiation, proliferation and apoptosis, as well as defence functions such as immune responses and inflammatory reactions *(6–8).* In the case of antitumour activity of a potential immunomodulator, special attention is paid to the induction of tumour necrosis factor α (TNF-α). This is one of the proinflammatory cytokines with pleiotropic effects depending on concentration. At high concentrations it exerts vasculotoxic effects, which is probably the basis for its antitumour activity, but also for the majority of life threatening events in sepsis and septic shock. Its expression and regulation is affected by a variety of cytokines, among them also by interferon γ (IFN-γ) *(8–10).*

Many potential immunomodulating substances have been synthesised and tested for modulation of cytokine response, such as synthetic muramyl dipeptide (MDP) analogues *(11–13)*, including a MDP analogue romurtide, which is an example of a registered immunostimulatory drug *(14,15).* In recent years, attention has been focused also on immunomodulatory active isolates from natural resources, well known in different national healing traditions. An example are potentially immunomodulatory active substances from *Ganoderma lucidum*. In 1977, Wang reported an increased interleukine (IL-1, IL-6), TNF-α and IFN-γ production by human macrophages and T-lymphocytes after incubation with polysaccharides from fresh fruiting bodies of *Ganoderma lucidum (16).*

Several products of *G. lucidum* have recently undergone clinical trials and became available as syrup, injection, tablets, tincture, bolus of powdered medicine and honey, both in solution and a mixture *(17).*

As *Ganoderma lucidum* is very rare in nature, the amount of wild mushroom is not sufficient for commercial exploitation. The main goals of this research work were to use submerged cultivation for the production of *Ganoderma lucidum* biomass, and to evaluate the potential immunostimulatory effects of polysaccharides produced by this method. Therefore, immunostimulatory effects of isolates were tested on the induction of cytokine (TNF-α, IFN-γ) synthesis in primary cultures of human mononuclear cells.

Materials and Methods

Microorganism

A Slovenian *Ganoderma lucidum* strain MZKI G97 was used in all experiments. The strain was maintained by reinoculation on potato dextrose agar (PDA) at 24 °C. The inoculum consisted of five 1 cm^2 cuts of a 7-day old culture cultivated on PDA. After inoculation of 100 mL of liquid substrate, the biomass was cultivated at a rotary shaker with frequency of 100 min^{-1} at 24 °C through 6 days.

Substrate

The mass of 3.0 kg of peeled potatoes were autoclaved (t = 121 °C, $p = 1.2 \cdot 10^5$ Pa) in 10 L of demineralised water for 20 minutes. A volume of 4.5 L of filtrate was filled with demineralised water up to 10 L. The concentration of 20 g L^{-1} of glucose and 2 % (volume fraction) of olive oil were added, and pH was adjusted to 5.8. The substrate was sterilised for 20 min in bioreactor (t = 121 °C, $p = 1.2 \cdot 10^5$ Pa) at a stirrer speed of 300 min^{-1}.

Bioreactor

All experiments were performed in a 10 L stirred tank reactor (STR), (Bioengineering AG, Switzerland), mixed by three Ruston turbines (d = 60 mm) with four baffles and a standard tank configuration. The cultivation conditions were: temperature of cultivation 30 °C, average oxygen partial pressure 70–80 %, redox potential 100–450 mV, stirrer speed of 300 min^{-1} and aeration 10 L min^{-1}.

Analytical methods

Glucose was controlled by Knauer HPLC. Total organic acids were determined titrimetrically by 0.1 M NaOH. The biomass was determined gravimetrically after filtration and drying for 24 hours at 104 °C.

Extraction and fractionation of polysaccharides

The method described earlier for the extraction and fractionation of antitumour polysaccharides from the fruiting bodies of *G. tsugae (19)* was adapted and used in this case for the extraction and fractionation of polysaccharides from the mycelium of *G. lucidum.* Mycelium was separated from the cultivation broth by vacuum filtration. Filtered cultivation medium was concentrated at 50 °C and a reduced pressure. Extracellular polysaccharides were precipitated from the concentrate by 96 % ethanol, filtered, washed with acetone and dried (fraction A). The mycelium was extracted with 85 % ethanol to eliminate low molecular components. After that the first fraction of intracellular polysaccharides was extracted with boiled water through 3 hours, then filtered, concentrated and precipitated by 96 % ethanol (fraction B). The mycelium was further extracted with boiled 1 % ammonium oxalate solution through 3 hours (fraction C), and with 5 % sodium hydroxide solution at 25 °C through 12 hours, from which polysaccharides were precipitated by acetic acid (fraction D), and from the remaining solution by ethanol (fraction E). Samples of fractions A – E were used in cytokine assays.

Evaluation of cytokine inducing capacity

Human peripheral blood mononuclear cells (PBMC) from the buffy coat of a healthy blood donor were isolated by a density gradient centrifugation with Ficoll-Paque (Pharmacia, Sweden). The cells were cultured in a tissue culture medium RPMI 1640 (Sigma, USA) supple-

mented with 100 U mL^{-1} penicillin (Sigma, USA), 100 μg mL^{-1} streptomycin (Sigma, USA), 20 mM Hepes (Sigma, USA) and 10 % heat-inactivated AB normal human serum (Sigma, USA). The aliquotes of 10^6 cells (final culture volume 1.5 mL) were plated in 24-well culture plates (Nunc, Denmark) with each of five fractions alone in different concentrations (3.25, 12.5, 50, 100, 400 μg mL^{-1}), at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Cultures of untreated cells in tissue culture medium without active substances were considered as negative control. To rule out a possible contamination by the endotoxin – a lipopolysaccharide from the Gram negative bacterial cell wall (LPS) of our polysaccharide samples, the samples with polysaccharide concentrations of 12.5, 100, 400 μg mL^{-1} with added polymyxin B (Sigma, USA) in concentration 10 μg mL^{-1}, *(18)* were tested parallelly. The cell-free supernatants were collected after 4-hour incubation for TNF-α and after 72-hour incubation for IFN-γ measurement, and stored at –70 °C before being evaluated for cytokines.

Measurement of cytokines

The concentration of cytokines (pg mL^{-1}) in PBMC culture supernatant was measured by commercially available ELISA kits, TNF-α from DPC (USA) and IFN-γ from Endogen (USA), according to the manufacturer instructions. The detection limit for TNF-α was 15.0 pg mL^{-1} and for IFN-γ 1.0 pg mL^{-1}, respectively.

Results and Discussion

Submerged production of *Ganoderma lucidum* biomass proceeded in the first phase, up to 200 hours, as batch cultivation using 6-day old vegetative inoculum from shaking flask cultures in concentrations of 17 % (wet weight). This concentration of vegetative inoculum was effective for production of starting extracellular activators and growth factors needed to support fast fungal growth. In the first stage of cultivation, after 55 hours and an average biomass concentration of 3.0 g L^{-1} a pH drop from 5.5 to 3.6 was detected. Decrease of pH was probably related to the secretion of various acids. It stayed unchanged until the end of cultivation. After 200 hours when biomass concentration reached 5.4 g L^{-1}, seven litres of cultivation broth were pumped out of the bioreactor and substituted with fresh sterile medium. After feeding, the biomass concentration dropped to 4.0 g L^{-1} and after 365 hours of cultivation it reached 9.6 g L^{-1} (Fig.1).

After cultivation, the biomass was filtered and washed. Extracellular and intracellular polysaccharides were fractionated into five fractions (Table 1).

In our previous immunological research, the attention was focused on the synthesis and evaluation of potential immunomodulating substances – desmuramyl analogues of MDP *(20,21)*. *Ex vivo* as well as *in vitro* models for the evaluation of the immunomodulating activity were developed, among them also a cytokine secretion with special regard to pro-inflammatory response on one side and T-cell response on the other side *(12,13)*. Recently emerging interest for the scientific evaluation of the well known remedies from different national

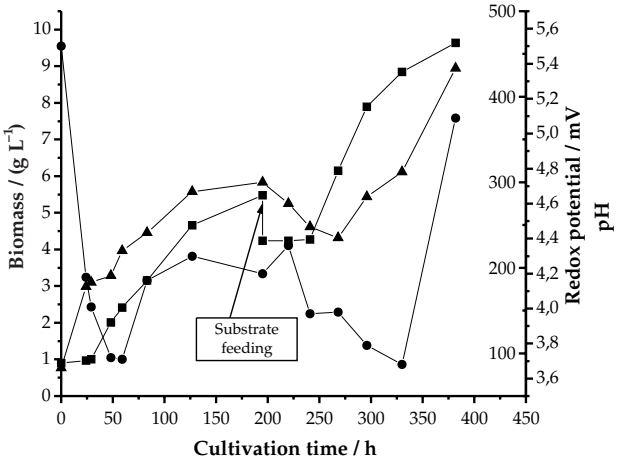

Fig. 1. The time course of *Ganoderma lucidum* submerged cultivation; ● pH; ■ biomass; ▲ redox potential

Table 1. Characteristics and yields of polysaccharide fractions from *G. lucidum* mycelium, produced by submerged biomass cultivation, further used for the evaluation of cytokine inducing capacity

Fraction	Properties	Mass / mg	Yield / %
A	extracellular polysaccharides, water soluble, precipitated with 96 % ethanol	1616	1,37
B	intracellular polysaccharides, hot water extract, precipitated with 96 % ethanol	2414	2,04
C	intracellular polysaccharides, 1 % ammonium oxalate solution extract, precipitated with 96 % ethanol	1183	1,002
D	intracellular polysaccharides, 5 % sodium hydroxide solution extract, precipitated with acetic acid	2068	1,75
E	intracellular polysaccharides, 5 % sodium hydroxide solution extract, precipitated with 96 % ethanol	650	0,55

healing traditions *(22-24)* attracted our attention. Our experiences with the *Ganoderma lucidum* cultivation *(25)* led us to the isolation of potentially active polysaccharide fractions and to the evaluation of their immunostimulatory activity.

The results of the TNF-α inducing capacity of isolated polysaccharide fractions from a biocultivated mycelium (fractions A,B,C,D,E) are presented in Figs. 2–3. Following the stimulation of PBMC with different concentrations of polysaccharide fractions with or without polymyxin, the supernatants were screened for the content of TNF-α after a 4-hour incubation.

From the results it is evident that the polysaccharide fractions from a biocultivated mycelium induce moderate amounts of TNF-α in the extent of < 3.0 to 630 pg

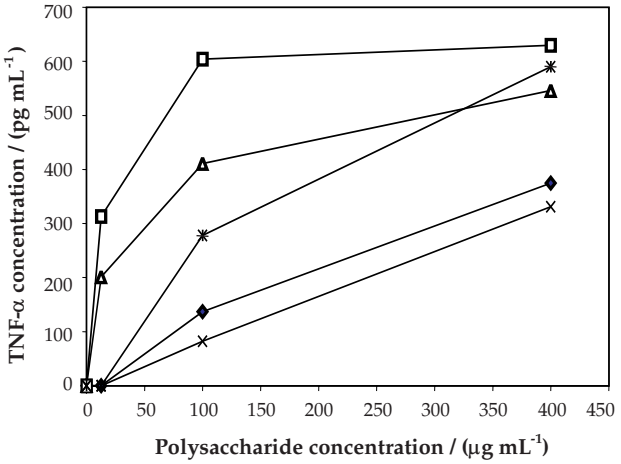

Fig. 2. The comparison of TNF-α inducing capacity of five polysaccharide fractions from biocultivated *Ganoderma lucidum* mycelium in human PBMC cultures after 4 hours of incubation in the presence of polymixin B; ◆ fraction A; □ fraction B; Δ fraction C; × fraction D; ✳ fraction E

mL^{-1} of a culture supernatant. This activity is not affected by the addition of polymyxin B to the cultures in the case of polysaccharide fraction B and only slightly affected in the case of polysaccharide fraction C. Other fractions seem to be more contaminated with the LPS (Fig. 3).

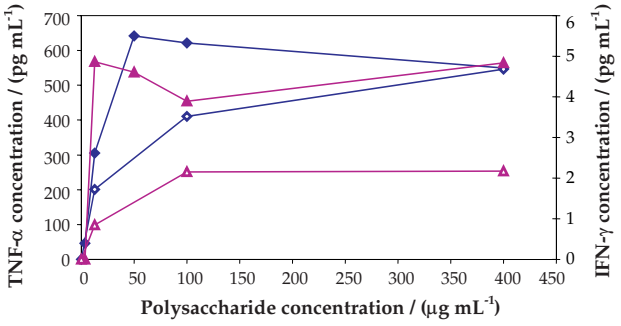

Fig. 3. Induction of TNF-α and IFN-γ production by sample C – polysaccharides from ammonium-oxalate extract, in the presence of polymyxin and without polymyxin; ◆ TNF-α; ◊ TNF-α + polymixin; Δ IFN-γ; ▲ IFN-γ + polymixin

The extent of TNF-α induction is comparable to the one achieved by Wang with the water-soluble polysaccharide-enriched fraction in mononuclear cell cultures of healthy donors *(16).* The induced amount of TNF-α is also comparable to our previous results. Applying the same experimental model, a similar TNF-α inducing activity was found in the case of romurtide (*12*), which is used as a supporting therapy in cancer patients treated with radiotherapy and/or chemotherapy (*14*). The TNF-α secretion in untreated PBMC cultures was under the detection limit in both, our recent and previous experiments.

The IFN-γ inducing capacity of our polysaccharide fractions differs from the capacity of polysaccharide enriched preparation reported by Wang (*16*). Only fractions B and C are able to induce slight amounts of IFN-γ (Table 2).

Table 2. The concentration of IFN-γ in culture supernatants of human PBMC incubated for 72 hours with two polysaccharide fractions showing the strongest TNF-α inducing capacity (B and C), with polymixin B

Concentration of polysaccharide fraction/ (μg/mL)	Concentration of IFN-γ / (pg/mL)	
	Polysaccharide fraction B	Polysaccharide fraction C
12.5	<1	<1
100	1.23	2.15
400	1.39	2.18

For other polysaccharide fractions, the capacity of IFN-γ induction is under the detection limit of 1.0 pg mL^{-1} in ELISA measurement. Untreated cells do not secrete IFN-γ. This is in accordance with our previous results, showing that the T-cell cytokine synthesis is much more tightly under control than in the case of pro-inflammatory cytokines secreted mainly by monocytes/macrophages. The immunomodulatory substance might in the first place modulate antigenically or polyclonally evoked T-cell cytokine synthesis, rather than induce it *de novo* (*13*).

Conclusions

Polysaccharide fractions from biocultivated mycelium of the Slovenian strain *Ganoderma lucidum* strain MZKI G97 were proved to be inducers of moderate amounts of TNF-α in the extent of < 3.0 to 630 pg mL^{-1} of a culture supernatant. This is comparable to the TNF-α inducing activity of romurtide, which is used as a supporting therapy in cancer patients treated with radiotherapy and/or chemotherapy. Consequently, the polysaccharides isolated from the Slovenian *Ganoderma lucidum* strain represent a potential and promising natural immunomodulatory substance, which could be efficiently and economically produced by submerged cultivation and production of *Ganoderma lucidum* biomass.

Acknowledgements

The project was co-financed by the Ministry of Science and Technology of the Republic of Slovenia.

References

1. S. P. Wasser, A. L. Weiss, *Crit. Rev. Immunol. 19* (1999) 65–96.
2. T. K. Yun, *Ann. NY Acad. Sci. 889* (1999) 157–92.
3. H. S. Kim, S. Kacew, M. B. Lee, *Carcinogenesis, 20* (1999) 1637–1640.
4. Y. Mizushina, N. Takahashi, L. Hanashina, H. Koshino, Y. Esumi, J. Uzawa, F. Sugawara, K. Sakaguchi, *Bioorg. Med. Chem. 7* (1999) 2047–2052.
5. R. S. Kim, H. W. Kim, B. K. Kim, *Mol. Cells*, *7* (1997) 52–57.
6. H. Ibelgaufts: *Dictionary of Cytokines*, VHC, Weinheim (1995).
7. J. W. M. Van der Meer, M. T. E. Vogels, M. G. Netea, B. J. Kullberg, *Ann. NY Acad. Sci. 856* (1998) 243–251.

8. A. K. Abbas, A. H. Lichtman, J. S. Pober: *Cellular and Molecular Immunology*, (4th ed.). WB Saunders Company, Philadelphia (2000) 235–269.
9. A. Van Der Veen, T. L. ten Hagen, J. H. de Wilt, M. G. van Ijken, A. M. Eggermont, *Anticancer Res. 20* (2000) 3467–3474.
10. C. A. Dinarello, *Chest*, *118* (2000) 503–508.
11. M. Parant, *Int J. Immunopharmacol. 16* (1994) 445–449.
12. S. Simčič, B. Wraber, M. Sollner, U. Urleb, S. Gobec, *Pflügers Arch. – Eur. J. Physiol. 440* (suppl.) (2000) R64–R66.
13. S. Gobec, U. Urleb, S. Simčič, B. Wraber, *Pharmazie, 56* (2001) 523–526.
14. I. Azuma, *Trends Pharmacol. Sci. 13* (1992) 425–428.
15. K. Kino, T. Sone, J. Watanabe, A. Yamashita, H. Tsuboi, H. Miyajima, H. Tsunoo, *Int. J. Immunopharmacol. 13* (1991) 1109–1115.
16. S. Y. Wang, M. L. Hsu, H. C. Hsu, C. H. Tzeng, S. S. Lee, M. S. Shiao, C. K. Ho, *J. Int. Cancer, 70* (1997) 699–705.
17. S. P. Wasser, A. L. Weiss, *Medicinal Mushrooms – G. lucidum, Reishi Mushroom*, Pedeifus Publishing House, Haifa (1997).
18. M. Cinco, E. Vecile, R. Murgia, P. Dobrina, A. Dobrina, *FEMS Microbiol. Lett. 138* (1996) 211–214.
19. T. Mizuno, G. Wang, J. Zhang, H. Kawagishi, T. Nishitoba, J. Li, *Food Rev. Int. 11* (1995) 151–166.
20. M. Sollner, V. Kotnik, S. Pečar, A. Štalc, S. Simčič, L. Povšič, B. Herzog-Wraber, L. Klampfer, A. Ihan, P. Grosman, *Agents Actions, 38* (1993) 273–80.
21. M. Sollner, A. Štalc, S. Simčič, B. Wraber, V. Kotnik, L. Povšič, U. Urleb, *Acta Pharm.* (2001) (in press).
22. J. N. Dhuley, *J. Ethnopharmacol. 58* (1997) 15–20.
23. A. Bussing, K. Schweizer, *J. Ethnopharmacol. 59* (1998) 139–146.
24. E. Yesilada, D. Deliorman, F. Ergun, Y. Takaishi, Y. Ono, *J. Ethnopharmacol. 61* (1998) 195–200.
25. J. Habijanič, M. Berovič, *Food Technol. Biotechnol. 38* (2000) 225–228.

Imunostimulatorno djelovanje fungalnih polisaharida iz biomase *Ganoderma lucidum* dobivene submerznim uzgojem

Sažetak

Izoliran je soj *Ganoderma lucidum* MZKI G97 iz Slovenije te uzgojen na dekstrozi iz krumpira u reaktoru od 10 L s miješalicom. Proizvedeno je do 15,2 g/L biomase koja je sadržavala polisaharide. Ekstracelularna polisaharidna frakcija dobivena je postupkom taloženja s etanolom. Četiri intracelularne polisaharidne frakcije dobivene su ekstrakcijom vrućom vodom i taloženjem s etanolom, ekstrakcijom s amonijevim oksalatom te ekstrakcijom s natrijevim hidroksidom uz naknadno taloženje s octenom kiselinom i taloženjem s etanolom. Imunostimulatorno djelovanje izolata ispitano je na indukciju sinteze citokina (TNF-α, IFN-γ) u primarnim kulturama ljudskih mononuklearnih stanica (PBMC) izoliranih iz volovske kože. Rezultati su pokazali mogućnost izolata da induciraju umjerene količine TNF-α (maksimalno do 630 pg/mL u supernatantu) i IFN-γ u tragovima (maksimalno 11,5 pg/mL). Indukcijska aktivnost TNF-α može se usporediti s romurtidom koji se koristi kao dodatak u terapiji kanceroznih bolesnika što su obrađeni radioterapijom i/ili kemoterapijom.