Biosynthesis of Immunostimulatory Compounds from *Ganoderma sp.* from European Habitats by Submerged and Solid State Cultivation

MARIN BEROVIC[1], BOJANA BOH[2] & BRANKA WRABER[3]
[1]Department of Chemical, Biochemical & Ecology Engineering, University of Ljubljana, Askerceva 9, 1000 Ljubljana; [2]Faculty of Science and Engineering, University of Ljubljana, Vegova 4, 1000 Ljubljana; and [3]Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana, Zaloska 4, 1000 Ljubljana, Slovenia. E-mail: marin.berovic@fkkt.uni-lj.si

Abstract: An original strain of *Ganoderma lucidum* MZKI G97, was isolated from Slovenian forests was cultivated in a liquid substrate based on potato dextrose and olive oil in a 10L laboratory stirred tank bioreactor as well as in 15L solid state cultivation in a horizontal reactor in substrate based on beech sawdust and mineral media. In submerged cultivation, up to 17.0 gL^{-1} of dry fungal biomass was obtained in a fed batch process. From the mycelium biomass, 1.7 gL^{-1} of extracellular and 0.45 gL^{-1} of intracellular polysaccharides were isolated, which were mainly ß-D-glucans. Polysaccharide fractions, tested *in vitro* for the induction of cytokine synthesis in primary cultures of human mononuclear cells from a buffy coat of healthy donors, induced 3.0-630 pg mL^{-1} of TNF-α, and 1.23 - 2.18 pg mL^{-1} of IFN-γ which is comparable to the activity of romurtide, a supporting drug in cancer therapy.

Key words: Submerged and solid state biomass cultivation, polysaccharides, cytokine assay

1 Introduction

Basidiomycetes of various *Ganoderma* spp. and their wide range of pharmaceutically interesting products are, in the last decades, one of the most attractive groups of natural products in Asia and North America. Most of the research has been related to *G. lucidum*, a recognised traditional remedy used in Chinese and Japanese traditional medicine for treatment of several diseases since the earliest time. In Asian traditional medicine, the fruiting body of *G. lucidum*, called Ling-Zhi in Chinese and Reishi in Japanese, has been used for the treatment of a series of diseases including hepatitis, arthritis, nephritis, bronchitis, asthma, arteriosclerosis, hypertension, cancers and gastric ulcer.[1] Newer investigations report on its antiallergenic constituents,[2] immunomodulatory action[3, 4] and treatment of HIV infections,[5, 6] antitumor[7] and cardiovascular effects,[8] liver protection and detoxification and effects on nervous system.[9]

Pharmaceutically active compounds from *G. lucidum* include triterpenoids, proteins, steroids, alkaloids, nucleotides, lactones and fatty acids. Polysaccharides (especially ß-D-glucans) have been recognised as an effective anti-cancer drug.[10-12] In *Ganoderma* polysaccharide research, special attention was paid to their immunomodulatory effects.[13, 14] In 1977, Wang[15] reported an increased interleukin (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 *G. lucidum*.[16]

In 1971, Sasaki et al.[17] reported on antitumor polysaccharides from some Polyporaceae, including *G. applanatum*. In the 1990's it became evident that *Ganoderma* polysaccharides affect the immune system. A number of reports showed that polysaccharides stimulated immune functions both i*n vivo* and *in vitro*, and that macrophages were involved in this mechanisms.[18] Several authors reported on enhancement of cytokine production, both from macrophages and T lymphocytes, especially tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). The proliferation of cancer cells was not affected by *Ganoderma* polysaccharides alone, but were signifi

cantly inhibited by the conditioned medium from polysaccharide activated blood mononuclear cells.[19-21] Lei et al.[22] studied the antagonistic effects of *G. lucidum* polysaccharides on the immunosuppressive response induced by cyclosporin A, hydrocortisone and antitumor agents, and reported on enhancement of cell-mediated immune functions and augmentation of cytokine production. Other immunomodulation effects, i.e. enhancement of unspecific immune functions, were reported by Li et al.[23] where *Ganoderma* polysaccharides affected intracellular free calcium and oxygen free radicals in murine peritoneal macrophages.

One of the important approaches to evaluate potential immunomodulating activity is the assessment of the capacity of the 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.[2-4]

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 pro-inflammatory 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).[24-26]

Although many potential immunomodulating substances were being synthesised and tested for modulation of cytokine response, such as synthetic muramyl dipeptide (MDP) analogues,[27-29] including a MDP analogue romurtide, an example of a registered immunostimulatory drug,[2, 30] in recent years, much of attention has been focused also on immunomodulatory active isolates from natural resources, well known in different national healing traditions

A successful artificial cultivation has been reported on solid substrates, utilising e.g. sawdust and agricultural wastes as the main media components[31] as well as submerged cultivation in liquid media.[32] The quality and content of physiologically active substances vary from strain to strain and also depends on location, culture conditions[19] and growth of the mushroom.[33] As *G. lucidum* is very rare in nature, the amount of wild mushroom is not sufficient for commercial exploitation. A systematic research of its pharmacological effects started only about twenty years ago. As *G. lucidum* is scarce in nature, the amount of wild mushroom is not sufficient for commercial exploitation. Its cultivation on solid substrates, stationary liquid medium or, in the last time, by submerged cultivation has become essential to meet the increasing demands on the international markets.

The main goals of this research were to test the ability of submerged cultivation for the production of *G. lucidum* biomass, and to evaluate the potential immunostimulatory effects of polysaccharides tested on the induction of cytokine (TNF-α, IFN-γ) synthesis in primary cultures of human mononuclear cells.

2 Materials and Methods

2.1 Microorganism

G. lucidum strain MZKI G97, originally isolated from the Slovenian forest, was used in all of the experiments.

2.2 Submerged cultivation

2.2.1 Inoculum

The strain was maintained on potato dextrose agar (PDA) at 24°C. The inoculum consisted of five 1 cm^2 cuts of a 7-days old culture from PDA. After a sterile inoculation of 100 mL of liquid substrate, the biomass was cultivated at a rotary shaker at 100 rpm and 24 °C. Inoculum concentrations 14, 17 and 20 % (wet weight) were used.

2.2.2 Substrate

Peeled potatoes (3.0 kg) were autoclaved (121 °C, 1.2×10^5 Pa) in 10 L of demineralised water for 20 minutes. Filtrate (4.5 L) was made up to 10L with demineralised water. Glucose (20 gL^{-1}) and 2% (v/v) of olive oil were added, and the pH adjusted to 5.8. The substra[e was sterilised for 20 min in the bioreactor(121°C,1.2·105 Pa) at a stirrer speed of 300 rpm.

2.2.3 Bioreactor

All experiments were performed in 10L stirred tank reactor (STR), Bioengineering AG, Switzerland mixed by three Ruston turbines (d = 60 mm) with four baffles and standard tank configuration. The oxygen partial pressure was measured by a polarographic sensor IL MGF 509, the redox potential by a Ingold Pt 4865, and the pH by a Ingold 465-35 k1 sensor.

2.2.4 Cultivation conditions

Cultivation conditions were as follows: temperature of cultivation = 30°C, mixing N = 300 min^{-1} and aeration Q_g = 10 L min^{-1}, average values of : pH = 5.8 - 4.2 , oxygen partial pressure = 70-80%, and redox potential Eh = 300-400 mV. Number of cut filaments was calculated out from 1.0 ml of sample as a ratio of the number of cut hyphae related to the number of all the hyphae observed in hemocytometer measuring cell. All of the experiments were performed in six runs, the average values were calculated from the sum of the data. Typical courses of cultivation were presented for each run.

2.2.5 Biomass determination

Biomass was determined gravimetrically after filtration and drying for 24 hours at 104 °C.

2.3 Solid-state cultivation

2.3.1 Inoculum

Vegetative inoculum was obtained in three steps. The mycelium from agar plate was first transferred to liquid medium by cutting out 5 rolls (5 mm diameter) of mycelium with agar and transferring to a 500 ml baffled erlenmeyer flask containing 100 ml of a medium A. Flasks were incubated with shaking for 14 days at 30°C and 100 rpm and then used to inoculate a batch bioreactor. The volume of inoculum was varied from 10% (v/v) in 8L of the same medium (medium A). At this stage, the cultivation conditions in the submerged bioreactor were 30°C, pH 5.8, partial oxygen pressure 70-80 %, redox potential 300-400 mV, mixing 300 rpm, and vvm 10 L/min.[34] To obtain higher concentrations of vegetative inoculum, the culture was fed after 8 days with the same medium. At maximum biomass concentration after a few days, the culture was prepared to inoculate the solid medium for solid-state cultivation. The volume of liquid inoculum was 2L.

2.3.2 Medium

The medium for the cell propagation (medium A):water from cooked peeled potatoes 300 g/l, glucose 20 g/l, olive oil 2 %, distilled water.[34] The medium for the solid-state cultivation was (medium B): beech saw-dust 800 g, olive oil 20 ml, $(NH_4)_2SO_4$ 50 mg, KH_2PO_4 200 mg, $CaCl_2.2H_2O$ 50 mg, $MgSO_4.7H_2O$ 50 mg, $FeSO_4.7H_2O$ 150 mg, distilled water. Distilled water (1.5L) with olive oil and trace elements was used to moisten the

saw-dust.

2.3.3 Bioreactor

Experiments were carried out in a horizontal stirred tank reactor (HSSR) of our own construction and design. The cultivation was carried out at 30°C and airflow 2 L/min.[35] Periodic mixing of 80 rpm per 2 minutes (in the first 7 days every second day, and 2 minutes every day in the last part of cultivation) was used. After one week of cultivation, a gas washing vessel with sterilized distilled water was connected to the air pump to moisturize the substrate (Figure1)

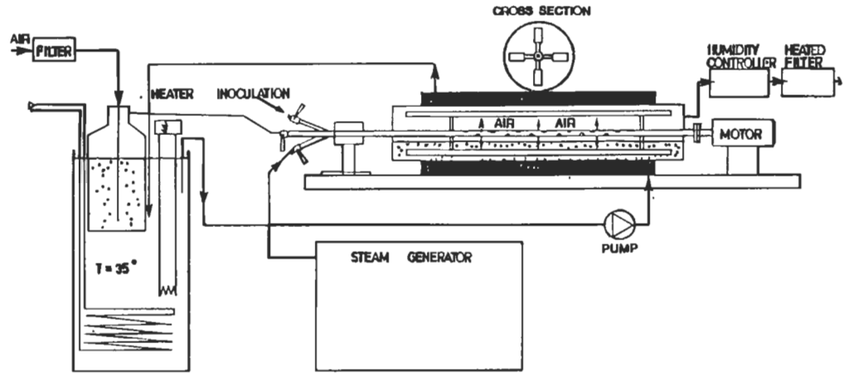

Figure 1.Horizontal stirred tank reactor and equipment

2.4 Analytical methods

For biomass concentration determination on particles by measuring glucosamine (chitin) contents[36] and glucosamine assay with 3-methyl-2-benzothiazole hydrazone were used.[36] Polysaccharides were determined by disintegration and extraction of 15 g sample (mycelium with sawdust) with boiling water for 5 hours, filtration the suspension to remove the insoluble matter and precipitation of polysaccharides by adding 3 volumes of 96% ethanol. The precipitate was then freeze-dried.[6]

2.5 Isolation and separation of fractions

Crude polysaccharides were suspended in water with stirring. The insoluble polysaccharides were removed by filtration. The masses of polysaccharide fractions were: soluble extracellular 690 mg, insoluble extracellular 320 mg, soluble intracellular 180 mg, insoluble intracellular 120 mg. Soluble fractions were separated by ion-exchange chromatography on DEAE-cellulose (20 × 3 cm). Water-soluble fractions of extracellular polysaccharides were eluted with 150 mL of water (fraction A1), 200 mL of 0.1M $NaHCO_3$ (fraction A2), 200 mL of 0.3M $NaHCO_3$ (fraction A3), 200 mL of 0.5M $NaHCO_3$ (fraction A4) and 200 mL of 0.1M NaOH (fraction A5) Water soluble fraction of intracellular polysaccharides was eluted with 200 mL of water (fraction B1), 180 mL of 0.1M $NaHCO_3$ (fraction B2), 150 mL of 0.3M $NaHCO_3$ (fraction B3), 150 mL of 0.5M $NaHCO_3$ (fraction B4) and 200 mL of 0.1M NaOH (fraction B5)
Polysaccharide fractions were separately dialysed, polysaccharides were precipitated with ethanol, precipitates were collected by centrifugation and washed with ether. Fraction A1 (160 mg), fraction B1 (75 mg) and fraction

B2 (15 mg) were dissolved in water and separated by gel chromatography on Sepharose 4B (70 × 1.2 cm), eluted with water. Fraction A1-2 (35-80 mL) and fraction B1-2 (38-80 mL) were dialysed, concentrated under reduced pressure and further separated by affinity chromatography on Concanavalin A -Sepharose 4B(20 × 1.0 cm). ß-Polysaccharide fractions (fraction A1-2-ß, 11mg, and fraction B1-2-ß,2mg) were eluted with 75 mL 0.1 M phosphate buffer (pH 7.0) in 1M NaCl, (Figure 7) and α-polysaccharide fractions (fraction A1-2-α, 2 mg, and fraction I1-2-α, 0.5 mg) with 150 mL 0.1 M glucose in 1M NaCl.
Glucose was determined as the main monosaccharide by thin layer chromatography of extracellular and intracellular polysaccharide hydrolysates on silica gel plates,.

2.6 Extraction and fractionation of polysaccharides

Mycelium was separated from the cultivation broth by vacuum filtration. Filtered cultivation medium was concentrated at 50°C and 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. Then, the first fraction of intracellular polysaccharides was extracted with hot water (100°C, 3 hours), filtered, concentrated and precipitated by 96 % ethanol (fraction B). The mycelium was further extracted with 1% ammonium oxalate solution (98°C, 3 hours) (fraction C), and with 5 % sodium hydroxide solution (25°C, 12 hours), from which polysaccharides were precipitated by acetic acid (fraction D), and from the remaining solution by ethanol (fraction E). Extracellular and intracellular polysaccharides were fractionated into five fractions (Table 1). Samples of fractions A - E were used in cytokine assays.
Polysaccharide fractions A and B were further fractionated and purified by ion-exchange chromatography on DEAE-cellulose (column 20 × 3.0 cm, elution with water, 0.1M $NaHCO_3$, 0.3M $NaHCO_3$, 0.5M $NaHCO_3$ and 0.1M NaOH), gel filtration on Sepharose 4B (column 70 × 1.2 cm, elution with water), and affinity chromatography on Concavalin A-Sepharose 4B (column 20 × 1.2 cm). For ß-polysaccharides, the column was eluted with 0.1 M phosphate buffer (pH 7.0) in 1M NaCl, and for α-polysaccharides with 0.1 M glucose in 1M NaCl (19). Extracellular and intracellular polysaccharides were fractionated into five fractions (Table 1).

2.7 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) supplemented 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). Cells (1×10^6) (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 µgm L^{-1}), at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures of untreated cells in RPMI 1640 without active substances were considered as a 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 µgmL^{-1}, (18) were tested in parallel. 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.

2.8 Measurement of cytokines

The concentration of cytokines (pg mL^{-1}) in PBMC culture supernatant was measured by commercially avail

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% NaOH solution extract, precipitated with acetic acid	2068	1,75
E	intracellular polysaccharides, 5% NaOH solution extract, precipitated with 96% ethanol	650	0,55

able 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 pgmL^{-1} and for IFN-γ 1.0 pg mL^{-1}, respectively.

3 Results and Discussion

The main goal of our research work was to find out if a natural *G. lucidum* strain MZKI G97, isolated from the Slovenian forest, is able to produce in submerged and solid state cultivation its fungal polysaccharides with expressed immunostimulatory activities. Both cultivation processes could represent an effective and fast technological process for large-scale production of fungal biomass and its active compounds.

3.1 Submerged cultivation

3.1.1 Batch cultivation experiments

In a series of experiments *G lucidum* cultivation 144 hours (6 days) old vegetative inoculum from shaking cultures in concentrations of 14, 17 and 20 % (wet weight) were studied in batch culture. In the first experiments using 14% inoculum, it was evident that the starting concentration of extracellular activators and growth factors produced by biomass was too low to support the growth intensively. Growth factors had to be synthesised first, and then the growth of fungal biomass started rapidly. This caused a time delay in biomass production. Decrease of pH was related to the secretion of various ganoderic, ganoderenic and lucidenic acids. Furthermore, 17 % inoculum seemed to be the most suitable choice, while the 20% inoculum concentration was too high and induced a competition in substrate consumption. In this case, higher substrate concentrations for faster biomass development were needed.

3.1.2 Fed batch experiments

Out of batch experiments three groups of fed batch experiments using 17% inoculum (wet weight) were performed. In the first one, the start of substrate feeding occurred at 90 hours, at biomass concentration of 5.2 gL^{-1}. At oxygen partial pressure of 80% the final biomass concentration was 8.2 gL^{-1} In the second experiment, the substrate feeding started at 200 hours and at a biomass concentration of 7.3 gL^{-1}. The final biomass concentration was 15.2 gL^{-1} at the oxygen partial pressure of 80 %.

3.2 Solid state cultivation

Experiments were carried out in a horizontal stirring bioreactor of our own construction and design. The cultivation was carried out at 30°C and airflow of 2 L/min. Mixing was done at 80 rpm for 2 minutes every second day in the first week, and 2 minutes every day in the last part of cultivation. After a week of cultivation, the vessel with sterilized and distilled water was connected to the air pump to moisturize the solid substrate. After

cultivation, the biomass was filtered and washed. Extracellular and intracellular polysaccharides were isolated. Biomass concentration on solid particles was determined by measuring glucosamine (chitin) contents and by glucosamine assay with 3 methyl-2-benzothiazole hydrazone.[36] Polysaccharides were determined by disintegration and extraction of 15 g sample (mycelium with saw-dust) with boiling water for 5 hours, filtration the suspension to remove the insoluble matter and precipitation by adding 3 volumes of 96% ethanol. The precipitate was freeze-dried.[6]

3.3 Isolation of polysaccharides

Extracellular (1,7 gl^{-1}) and intracellular (0.45 gl^{-1}) polysaccharide fractions were isolated. Polysaccharides were further separated by ion-exchange, gel and affinity chromatography. The isolated polysaccharides were proved to be mainly-D-glucans (Figures 2-5).

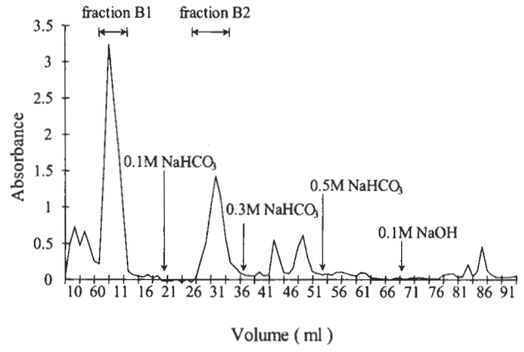

Figure 2. Ion-exchange chromatography of a water-soluble fraction of extracellular polysaccharides from *G. lucidum* cultivated in a submerged stirred tank reactor (fraction A1 was eluted with water)

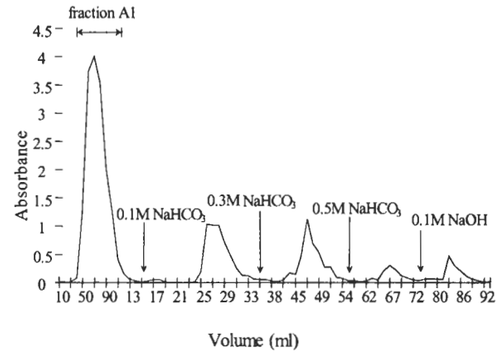

Figure 3. Ion-exchange chromatography of a water-soluble fraction of intracellular polysaccharides from *G. lucidum* cultivated in a submerged stirred tank reactor (fraction B1 was eluted with water)

3.4 Immunomodulatory effects of *G. lucidum* polysaccharides

The results of testing immunomodulatory effects of polysaccharide isolates on the induction of cytokine (TNFα, IFNγ) synthesis in primary cultures of human peripheral blood mononuclear cells (PBMC) isolated from a buffy coat of healthy donors are presented in Figures 6-7. 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 4-hours incubation. The results showed that the polysaccharide fractions from a biocultivated mycelium induced moderate amounts of TNF-α in the extent of <3.0 pg mL^{-1} to 630 pg mL^{-1} of a culture supernatant. This activity was 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.
The extent of TNF-α induction was comparable to the one achieved by Wang et al.[15] with the water-soluble polysaccharide-enriched fraction in mononuclear cell cultures of healthy donors. The induced amount of TNF-α was also comparable to our previous results. Applying the same experimental model, a similar TNF-α inducing activity was found in the case of romurtide, which is being used as a supporting therapy in cancer patients treated with radiotherapy and/or chemotherapy. 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 et. al.[15] Only fractions B and C were able to induce slight amounts of IFN-γ (Table 2).

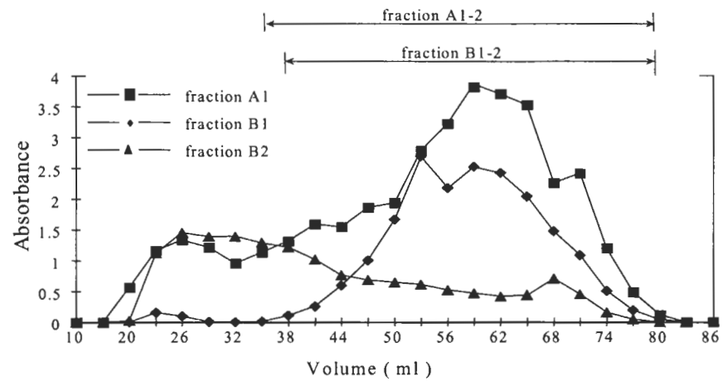

Figure 4. Gel filtration on Sepharose 4B: elution chromatograms of polysaccharide fractions A1, B1 and B2
■ Fraction A1 (largest extracellular polysaccharide fraction from ion exchange chromatography) ◆ Fraction B1, ▲ Fraction B2 (two largest intracellular polysaccharide fractions from ion-exchange chromatography)

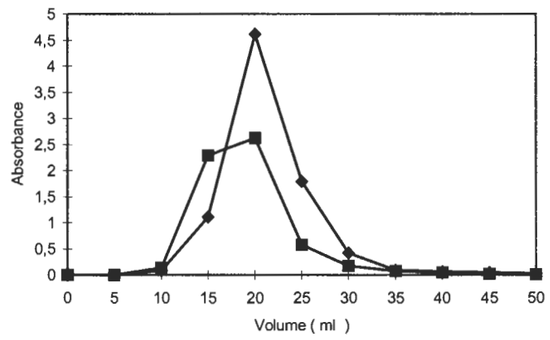

Figure 5. Affinity chromatography on Concanavalin A- Sepharose 4B
Elution chromatograms of ß-polysaccharide fractions (elution with 0.1 M phosphate buffer, pH 7.0, in 1M NaCl). ◆Fraction A1-2 (extracellular ß-polysaccharide), ■ Fraction B1-2 (intracellular ß- polysaccharide)

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

4 Conclusions

Experimental results of submerged cultivation of *G. lucidum* strain isolated from the Slovenian forest prove that the fungus is able to produce fungal polysaccharides with active immunostimulatory abilities. Experiments in batch and fed batch cultivation favoured fed batch cultivation using average oxygen partial pressure of 80 %. Vegetative inoculum (17 %), 200 hours old, has to be applied at a concentration of 7.3 gl^{-1}. For a low shear field application, mixing intensity of up to 300 min^{-1} was required. By optimised submerged fed batch cultivation up to 15.2 gl^{-1} of dry fungal biomass was obtained.

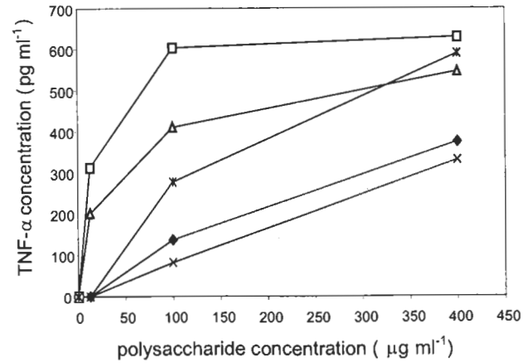

Figure 6. *In vivo* testing of immunomodulatory effects

Comparison of TNF-α inducing capacity of five polysaccharide fractions from biocultivated *G. lucidum* mycelium in human PBMC cultures after 4 hours of incubation in the presence of polymyxin B. ◆ Fraction A (extracellular polysaccharides, water soluble, precipitated with 96% ethanol), □Fraction B (intracellular polysaccharides, hot water extract, precipitated with 96% ethanol), Δ Fraction C (intracellular polysaccharides, 1% ammonium oxalate solution extract, precipitated with 96% ethanol), × Fraction D (intracellular polysaccharides, 5% sodium hydroxide solution extract, precipitated with acetic acid), * Fraction E (intracellular polysaccharides, 5% sodium hydroxide solution extract, precipitated with 96% ethanol)

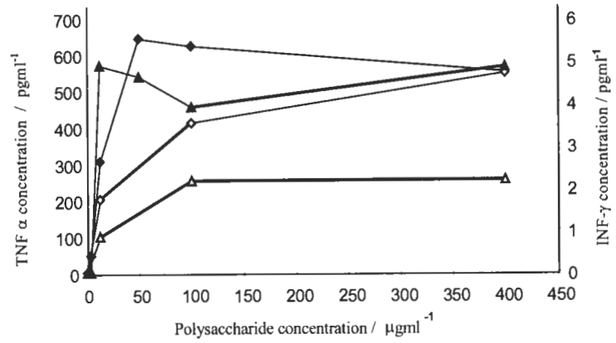

Figure 7. Induction of TNF-α and IFN-γ production by sample C -intracellular polysaccharides from ammonium oxalate extract, precipitated with 96% ethanol, in the presence of polymyxin and without polymyxin
◆ TNF-α ; ◇ TNF-α + polymyxin ; Δ IFN- γ ; ▲ IFN- γ + polymyxin

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

Concentration of polysaccharide fraction (µg/mL)	Concentration of IFN-γ (pg/mL)	
	Polysaccharide fraction B (intracellular, hot water extract, precipitated with 96% ethanol)	Polysaccharide fraction C (intracellular, 1% ammonium oxalate solution extract, precipitated with 96% ethanol)
12.5	<1	<1
100	1.23	2.15
400	1.39	2.18

The experiments have also shown that *G. lucidum* biomass production by submerged cultivation in a bioreactor enables the production of fungal polysaccharides. The yield of extracellular polysaccharides was 9.6 gl^{-1}, while the yield of intracellular polysaccharides was 6.3 gl^{-1}. Water-soluble polysaccharide fractions, separated by the ion-exchange, gel and affinity chromatography, were proved to be mainly ß-D-glucans, which are known from literature as the main immunomodulatory substances of *G. lucidum*.

In vitro testing of immunomodulatory effects of polysaccharide fractions from *G. lucidum* mycelium proved the induction of moderate amounts of TNF-α in the extent of < 3.0 pg mL^{-1} to 630 pg ml^{-1} of a culture supernatant. This is comparable to the TNF-α inducing activity of romurtide, which has been used as a supporting drug in therapy of cancer patients, treated with radiotherapy and/or chemotherapy.

Consequently, the polysaccharides isolated from the Slovenian *G. lucidum* strain represent a potential and promising natural immunomodulatory substance, which could be efficiently and economically produced by submerged cultivation of *G. lucidum* biomass in a bioreactor.

The reported results are a contribution to the extended research of this subject in the Far East countries, and represent a valuable new information on active fungal polysaccharides produced and isolated from *Ganoderma spp*. from the European habitats.

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