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Chemical composition and cytotoxic activity of the polysaccharide fractions in *Sarcodon imbricatus* (Basidiomycota)

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The aim of the study was chemical analysis of polysaccharide fractions from sporocarps of *Sarcodon imbricatus* collected in natural sites and from the mycelium of *in vitro* cultures. Three polysaccharide fractions (F_OI, F_OII, F_OIII) were isolated from sporocarps and two (F_KI, F_KII) from *in vitro* cultures. Qualitative analysis by HPLC method showed that they are composed of galactose and fucose (F_OI, F_KI) or glucose and fucose (F_OII, F_KII). F_OIII fraction of the sporocarps consisted of glucose only. Molecular weights of isolated fractions ranged from 3.8 to 16.3 kDa for fractions from the sporocarps and from 5.8 to 14.7 kDa for that ones isolated from *in vitro* culture. The total percentage of sugar content for all fractions ranged from 97.8% to 99.1%. The percentage of uronic acids contents in acidic fractions was 2.6% and 2.7% for the F_OI and F_KI respectively.

The work included also an assessment of cytotoxic activity of polysaccharide fractions in relation to tumor cell lines of human breast cancer MCV-7. F_OI polysaccharide fraction of the sporocarps inhibited the growth of cancer cells in 50% compared to the control at a concentration of 0.0125%, while the polysaccharide fraction F_KI from *in vitro* cultures inhibited cell growth in a concentration of 0.016%.

Key words: *Basidiomycota,* polysaccharide fractions, cytotoxicity, breast cancer MCV-7

INTRODUCTION

Basidiomycota species are an important source of biologically active compounds. Substances with antiviral, antibacterial, cytotoxic and anticancer activity were detected in the sporocarps of this taxon (Wasser 2002). The best-studied fungal metabolites, exhibiting therapeutic properties are polysaccharides. Due this reason more and more studies are undertaken, not only on the chemical composition of the sporocarps but also *in vitro* mycelial cultures (Sułkowska-Ziaja et al. 2005). Fungal polysaccharides include mostly

glucans, mannans and galactans. Biological activity is characteristic mainly for β-glucans in contrast to α-glucans which are rarely active and is determined by their chemical structure, particularly the type of glycoside bond (α or β) and spatial structure of polysaccharide molecule. Polysaccharides with linear structure and without long side chains show the highest activity. It is connected with their better solubility in water and thereby easier bioavailability by the cells. Therapeutically important polysaccharides have been found in microscopic as well as higher fungi. In clinical practice, amongst many pharmacologically active fungal polysaccharides, lentinan and krestin (so-called PSK) are used (Chihara et al. 1970).

The object of our study was *Sarcodon imbricatus* (L.: Fr.) P. Karst. occurring in spruce forests. This species is covered with strict protection in Poland.

The main goal of the study was examination of chemical composition and cytotoxic activity of the polysaccharide fractions from *in vitro* cultures and from sporocarps.

MATERIAL AND METHODS

Samples of sporocarps. Mature sporocarps of *Sarcodon imbricatus* were collected in September 2008 in spruce forests in Southern Poland. After taxonomic identification according to Hrouda (2005a, b) sporocarps were cut to small pieces and dried at 40ºC.

***In vitro* culture of *Sarcodon imbricatus*.** Initial cultures were derived from explants originated from the hymenial part of sporocarps which were sterilized with 70% ethyl alcohol and placed on Petri dishes with solid medium according to Lubiński with modifications (Turło et al. 2004). Cultures were incubated at a temperature 25±2ºC C under 12-h light (900 lx)/12 dark cycle and were subcultured every three week. Experimental cultures were maintained as agitating ones in Erlenmayer flask (500 mL) containing 250 mL medium under the same conditions as initial culture and were subcultured also every three weeks.

Isolation of crude polysaccharides. Isolation of polysaccharides was performed according to Mizuno method with modifications (Mizuno 1999). Dried sporocarps (50g) and lyophilized mycelium (25g) were refluxed with petroleum ether (1L) for 5 h to remove liposoluble constituents and then extracted with boiling water (1L) for next 5 h. The extraction process was repeated three times. The extracts were mixed, filtered, concentrated and centrifuged. The Sevag method (Darvill et al. 1985) was used to remove protein. Obtained supernatant was added to absolute ethanol and kept overnight. The precipitate was collected and washed with absolute ethanol and acetone, then dried by lyophilization, yielding crude polysaccharide.

Fractionation of crude polysaccharides by ion-exchange column chromatography. Crude polysaccharide fractions (1g) was dissolved in 100 mL of distilled water and loaded on DEAE-Sephacel column. The column was at first washed with water, then with phosphate buffer pH 6.0 with increasing ionic strength and finally with aqueous NaOH solution (0.2 M). Two-milliliter fractions were collected. In order to detect polysaccharides, a 0.2 mL sample was taken from each eluted fraction that was

mixed with sulfuric acid and phenol to yield color reaction (Dubois et al. 1956). Fractions containing polysaccharides were mixed, concentrated under vacuum, dialyzed and lyophilized.

Monosaccharide composition analysis. Polysaccharide fractions (1g) were hydrolyzed in 1M trifluoroacetic acid for 10 h at 100°C in sealed glass tube. The monosaccharide compositions of the obtained solution were determined using HPLC method. Identification of the monosaccharides was carried out by comparing their retention times with those of standards under the same HPLC conditions. Briefly, the analytical conditions were as follows: HPLC apparatus: type La Chrom Hitachi (MERCK); pump: L-7100; column: Supelcosil LC-NH2 (250x4.6mm, 5μm); solvent system: acetonitryl : water 8:2 (v/v); flow rate: 1.3mL/min; detector: refractometric: L-74800; standards: L(+) arabinose, D(-) fructose, D(+) galactose, D(+) glucose, D(+) xylose, D(+) mannose, L(+) rhamnose (MERCK).

Determinations of moleculars weight. The molecular weight of polysaccharide fractions (1g) was determined by a gel filtration technique (RodriguezVanderwieles 1979). Standard dextrans T-200, T-70, T-40, and T-10 were passed through a Sepharose CL-4B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights.

Determinations of the chemical character of the polysaccharide fractions. Total sugar content in all the received fractions was determined by the phenol-sulfuric acid colorimetric method using glucose as the standard (Dubois et al. 1956). Total uronic acid content in all the received fractions was determined by *m*-hydroxydiphenyl method using galacturonic acid as the standard (Filisetti-Cozzi, Carpita 1991).

The examination of the cytotoxicity of polysaccharide fractions in the resazurin test. The influence of polysaccharide fractions on the metabolism of breast cancer tumor cells MCF-7 was examined with colorimetric method based on the reduction of the resazurin (sodium salt 10-oxide 7-hydroxy-3H-phenoxazin-3-one). Resazurin is a metabolic activity indicator that in the oxidized form is a deep purple and when reduced turns to a light pink. Spectrophotometric measurement of the absorbance for resazurin and resorufin performed at the wavelength $\lambda=600$ and 570 nm respectively (Reddy et al. 1997).

Statistical analysis. Obtained results were analyzed using non-parametric Mann-Whitney U test ($n=3$). Differences with $p<0.05$ were considered to be statistically significant. The results were expressed as the mean values ± SD.

RESULTS

***In vitro* culture of *Sarcodon imbricatus*.** In agitated culture of *S. imbricatus* there was a 22-fold increase in fresh biomass within 3-week growth cycles. Obtained biomass was used as a source of polysaccharide fractions.

Isolation and fractionation of polysaccharide fractions. During isolation process 15.1 g and 2.1 g of polysaccharides from the sporocarps and mycelium cultured *in vitro* were obtained respectively. Efficiency of the isolation process in the case of sporocarps was 3.16%, while in the mycelium from *in vitro* cultures was 8.3%.

Table 1
Characteristic of polysaccharide fractions of *Sarcodon imbricatus*

Polysaccharide fraction	Molecular weight [kDa]	Monosacharide composition	Total sugars content [%]	Uronic acids [%]
Polysaccharide fractions from sporocarps				
F_OI	9.7±1.6	Gal, Fuc	99.8±1.8	27±1.5
F_OII	16.3±1.5	Glu, Fuc	99.1±1,6	-*
F_OIII	3.8±0.9	Glu	99.1±2.1	-*
Polysaccharide fractions from *in vitro* cultures				
F_KI	14.7±1.4	Gal, Fuc	98.5±2.2	2.8±0.8
F_KII	5.8±1.5	Glu, Fuc	97.8±1.8	-*

Data presented as mean of 3 series ±SD; Gal=galactose; Glu=glucose; Fuc=fucose; * not detected

Fractionation of crude polysaccharide fractions was performed using DEAE ion exchange column chromatography. Three polysaccharide fractions (F_OI, F_OII, F_OIII) were received from sporocarps and two (F_KI, F_KII) from *in vitro* cultures. Fractionation efficiency of the process amounted to 8.1%, 9.3%, 2.5% for the fraction of the sporocarps and 6.4% and 4.5% for the *in vitro* cultures.

Chemical analysis of polysaccharide fractions. Monosaccharides composition, molecular weights, the total sugar and uronic acids contents of the polysaccharide fractions are presented in Table1. Qualitative analysis by HPLC method showed that they are composed of galactose and fucose (F_OI, F_KI) or glucose and fucose (F_OII, F_KII). Fraction F_OIII consisted of only glucose. The average molecular weight of isolated fractions was estimated by reference to the calibration curve with standard dextrans and ranged from 3.8 to 16.3 kDa for fractions from the sporocarps and from 5.8 to 14.7 kDa for the polysaccharides isolated from *in vitro* culture. The total percentage of sugar content for all fractions ranged from 97.8% to 99.1%. The uronic acid contents were evaluated only in F_OI and F_KI - 2.6 and 2.7% respectively. Other examined fractions (F_OII, F_OIII and F_KII) not revealed the presence of uronic acid.

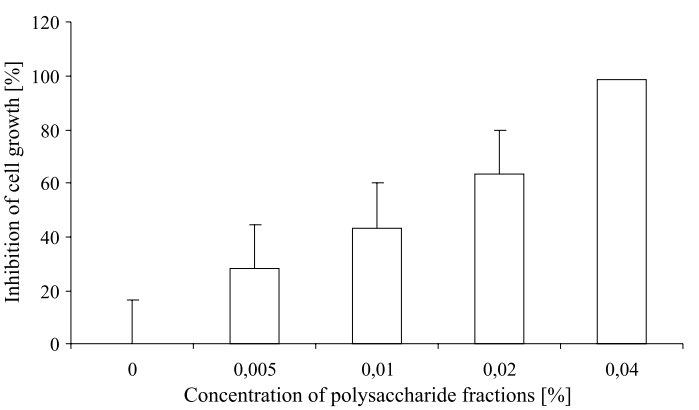

Fig. 1. The degree of tumor cell inhibition of breast cancer lines MCV-7 by the polysaccharide fractions F_OI from sporocarps of *Sarcodon imbricatus*.

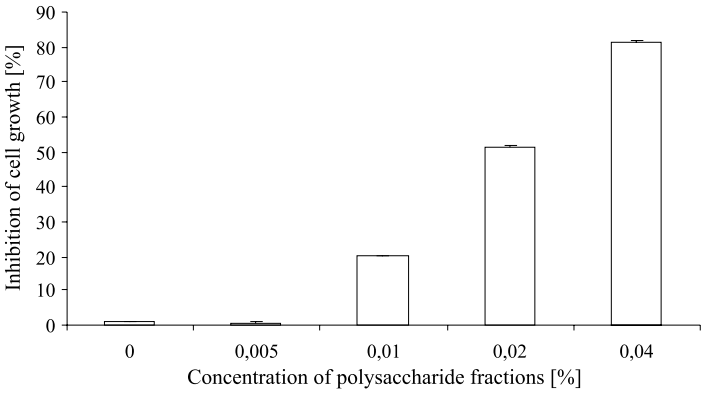

Fig. 2. The degree of tumor cell inhibition of breast cancer lines MCV-7 by the polysaccharide fractions $F_K I$ from *in vitro* cultures of *Sarcodon imbricatus.*

The study of cytotoxicity of the polysaccharide fraction against human tumor cells. Assessment of cytotoxic activity of polysaccharide fractions in relation to tumor cell lines of human breast cancer MCV-7 was made. Polysaccharide fraction $F_O I$ from the sporocarps inhibited the growth of 50% cancer cells in comparison with the control at a concentration of 0.0125 % (Fig. 1), while the polysaccharide fraction $F_K I$ from *in vitro* cultures inhibited cell growth in a concentration of 0.016 % (Fig. 2). Other fractions not revealed antitumor activity in this test.

Statistical analysis revealed significant differences between control and all used concentrations ($p<0.01$) for fractions $F_O I$ from sporocarps and for concentrations ranged from 0.01 to 0.04 for fraction $F_K I$ from *in vitro* cultures.

DISCUSSION

One of the major groups of metabolites of medicinal importance are polysaccharides derived from Basidiomycota (Kohlmünzer et al. 1992; Wasser 2002). Polysaccharides from the sporocarps of *Sarcodon imbricatus* were separated into three fractions: two neutral and one acidic, and from *in vitro* cultures into two: an acidic and neutral. All fractions were white, contained the 97.8 - 99.1% sugars. The polysaccharide fractions from sporocarps had higher total carbohydrate content and did not contain phosphorus, sulfur, nitrogen, protein or free amino acids. Molecular weights of polysaccharides were ranged from 3.8 to 16.3 kDa for sporocarps and from 5.8 to 14.7 kDa for mycelial cultures. HPLC analysis of acid hydrolysis products showed that they are composed of galactose, fucose and glucose. The obtained results indicated that glucose was the dominant monosaccharide in all the fractions. Biologically active fungal polysaccharides are represented mainly by glucans, but also by galactans, mannans or fucogalactans (Wasser, Weis 1999).

Other studies on related species i.e. *Sarcodon aspratus* revealed the presence of polysaccharide with structure known as fucogalactan (Maruyama et al. 1989).

Fucogalactans are also present in sporocarps of other Basidiomycota species e.g., *Coprinus comatus* – (molecular weight of 1.03 kDa) (Fan et al. 2006) and sporocarps of *Ganoderma lucidum* (molecular weight 2.8 kDa) (Bao et al. 2001; Ye et al. 2008).

The chemical composition of polysaccharides isolated from *in vitro* cultures is rare similar to polysaccharides isolated from sporocarps. A good example is a glucan from sporocarps of *Tylopilus felleus* named tylopilan isolated at the Department of Pharmaceutical Botany, Jagiellonian University Collegium Medicum (Defaye et al. 1988). Tylopilan showed antitumor activity against transplantable Sarcoma-180 cells in mice (more than 98% inhibition) and glioma cells (Kohlmünzer et al. 1980). Studies on the polysaccharides isolated from *in vitro* cultures of these species showed differences in their structure and biological properties.

Our biological studies have proved that both polysaccharide fractions from *Sarcodon imbricatus* had inhibitory effects on breast cancer cells of MCV-7. Polysaccharide fraction F_OI of sporocarps inhibited the growth of cancer cells in 50% compared to the control sample at a concentration of 0.0125%, while the polysaccharide fraction F_KI from *in vitro* cultures inhibited cell growth in a concentration of 0.016%.

Our earlier studies on the biological activity of polysaccharide fractions isolated from mycelium of *Sarcodon imbricatus* cultured *in vitro* indicated very interesting antibacterial and antiviral (HPV) activity which prompts to continue studies of biological activity and also to compare the activity of fractions isolated from sporocarps (Sułkowska-Ziaja et al. 2011). Acidic fraction contained galactose and fucose (F_KI) showed a higher microbial as well as cytotoxic activity. Also the fraction from the sporocarps of a similar chemical nature showed cytotoxic activity (F_OI).

Literature data of genus *Sarcodon* describe potential therapeutic effect of their metabolites and polysaccharide fractions can be considered as responsible for the cytotoxic activity. Fucogalactan from sporocarps of *Sarcodon aspratus* leads to the release of tumor necrosis factor-alpha (TNF-alpha) and nitric oxide in macrophages of mice *in vitro*. TNF-alpha production induced with 50 μg/ml of fucogalactan was significantly higher than induced by lentinan, the most active fungal polysaccharide with anticancer activity (Mizuno et al. 2000). Another example of a polysaccharide that shows cytotoxic activity against tumor cells Sarcoma - 180 is complex isolated from *Trametes versicolor* (Zjawiony 2004). In turn, polysaccharides isolated from *Pyrofomes demidoffi* have cytotoxic influence against murine L929 fibroblastoma cells (Zjawiony 2004). Polysaccharides isolated from *in vitro* cultures of *Poria cocos* are also good examples of cytotoxicity against the cells of Sarcoma 180 *in vivo* (Jin et al. 2003).

CONCLUSION

The analysis performed during this study allowed to more specific identification of the metabolite composition in sporocarps of *Sarcodon imbricatus* and determination of the biosynthetic abilities of the *in vitro* mycelium cultures. It has been demonstrated that *in vitro* cultures maintain ability to synthesize a range of metabolites occurring in the sporocarps like for example polysaccharides. Results of the performed

biological activity analyses lead to the conclusion that *Sarcodon imbricatus* may be qualified as a species with potential therapeutic properties and the polysaccharide fractions isolated from both the sporocarps and *in vitro* cultures may be deemed as compounds responsible for therapeutic effects.

The results of this study have not only cognitive importance but also have potential of practical application. It has demonstrated that *in vitro* cultures may be an alternative, high-yield source of a variety of biologically active metabolites.

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Skład chemiczny i aktywność cytotoksyczna frakcji polisacharydowych *Sarcodon imbricatus* (Basidiomycota)

Streszczenie

Przedstawiciele gromady Basidiomycota – której reprezentantem jest *Sarcodon imbricatus* (L.: Fr.) P. Karst., są ważnym źródłem związków aktywnych biologicznie. W ich owocnikach wykryto m.in. związki o działaniu przeciwwirusowym, przeciwbakteryjnym, fungistatycznym, a także przeciwnowotworowym. Najlepiej poznanymi metabolitami grzybowymi są polisacharydy. Coraz częściej podejmowane są badania nad składem chemicznym nie tylko owocników, ale także grzybni pozyskanej z kultur *in vitro.* W badaniach z tego zakresu dowiedziono istnienia jakościowych i ilościowych różnic w produkcji niektórych grup związków chemicznych przez owocniki i grzybnię z kultur *in vitro*.

Obiektem przeprowadzonych badań były owocniki *Sarcodon imbricatus* zebrane na terenie lasów świerkowych w roku 2008. Z warstwy hymenialnej owocnika wyprowadzono kultury *in vitro*, które prowadzono, jako płynne, wytrząsane. W ramach przeprowadzonych badań wyizolowano 3 frakcje polisacharydowe (F_OI, F_OII, F_OIII) z owocników i 2 (F_KI, F_KII) z mycelium z kultur *in vitro.* Analiza jakościowa z wykorzystaniem metody HPLC wykazała, że składają się one z galaktozy i fruktozy (F_OI, F_KI) oraz glukozy i fruktozy (F_OII, F_KII). Frakcja F_OIII z owocników składała się wyłącznie z glukozy. Oznaczono masy cząsteczkowe wyizolowanych frakcji. Wynosiły one od 3.8 do 16.3 kDa dla frakcji z owocników i od 5.8 do 14.7 kDa dla frakcji z kultur *in vitro*. Całkowita procentowa zawartość cukrów dla wszystkich frakcji mieściła się w przedziale od 97.8% do 99.1%, natomiast procentowa zawartość kwasów uronowych we frakcjach o charakterze kwaśnym wynosiła 2.6% dla frakcji F_OI z owocników i 2.7% dla frakcji F_KI z kultur *in vitro*.

Druga część pracy obejmowała ocenę aktywności cytotoksycznej wybranych frakcji polisacharydowych w stosunku do linii komórek nowotworowych ludzkich raka sutka MCV-7 w teście *in vitro*. Frakcje polisacharydowe F_OI i F_KI wykazywały działanie hamujące na komórki nowotworowe. Frakcja polisacharydowa F_OI z owocników hamowała wzrost komórek nowotworowych w 50% w stosunku do próby kontrolnej w stężeniu 0.0125%, natomiast frakcja polisacharydowa F_KI z kultur *in vitro* hamowała wzrost komórek w stężeniu 0.016%.