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ISOLATION AND BIOLOGICAL ACTIVITIES OF POLYSACCHARIDE FRACTIONS FROM MYCELIUM OF *SARCODON IMBRICATUS* L. P. KARST. (BASIDIOMYCOTA) CULTURED *IN VITRO*

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About 200 species of fungi called as "medicinal mushrooms" containing biologically activity substances, are currently known (1, 2). The greatest interest in mycochemistry is focused on polysaccharides, e.g., β-glucans, due to their immunomodulating, anti-inflammatory and anticancer properties. Some of them possess antibacterial and antiviral activity. These compounds have been isolated from fruit bodies collected in natural environment or from *in vitro* cultures (3, 4).

The genus *Sarcodon* (Basidiomycota) comprises some species with therapeutic potential among other things *Sarcodon aspratus* with antitumor (5–7) and *Sarcodon scabrosus* with anti-inflammatory activity (8).

The present studies were conducted on a mycelial culture of *Sarcodon imbricatus* (L.) P. Karst., and included isolation of polysaccharides fractions and investigation of antibacterial and antiviral activity. *Sarcodon imbricatus* is popular edible species, common in the south-west Europe. This species is under strict protection in Poland.

In the past, some studies of chemical composition of fruit bodies of this species were conducted in our laboratory (9) but there are no reports about *in vitro* culture conditions and secondary metabolites, expecially polysaccharides, obtained in this system. *In vitro* cultures of a related species *Sarcodon aspratus* (Berk.) S. Ito**.** have been successfully maintained in Korean and Japanese research centers (10).

EXPERIMENTAL

In vitro cultures of *Sarcodon imbricatus*

In vitro culture derived from fruit bodies collected from natural environment in south Poland and was established on solid medium (11). Experimental cultures were maintained as agitating cultures on optimized medium composed of: 5% glucose, 1% yeast extract, 1% casein hydrolyzate, 0,3% KH_2PO_4 and distilled water to 100%, pH 5.5. The culture was maintained at 23 ± 1°C under alternate light (24 hours period) and subcultured every 3 weeks.

Isolation of polysaccharides

Polysaccharide fractions were isolated using Mizuno method with our modifications (12). Lyophilized mycelium was preextracted with 80% methanol at room temperature. The post-extraction filtrate was discarded, while the residue was dried at room temperature and extracted twice with distilled water at 100°C for 6 h. Aqueous extracts were combined and concentrated. In order to precipitate polysaccharides, the concentrated extract was added in portions to absolute ethanol. The precipitate was centrifuged, frozen and lyophilized. We obtained 2.786 g of crude polysaccharide fraction.

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Purification and fractionation of polysaccharides by ion-exchange column chromatography

Polysaccharides were isolated and fractionated using ion-exchange column chromatography. Unpurified crude polysaccharide fraction (1 g) was dissolved in 100 mL of distilled water and loaded on DEAE-Sephacel column. The column was first washed with water, then with phosphate buffer pH 6.0 with increasing ionic strength and finally with aqueous NaOH solution (0.2 mol/L). Two-milliliter fractions were collected. In order to detect polysaccharides, a 0.2 mL sample was collected from each eluted fraction that was mixed with sulfuric acid and phenol to yield color reaction (13). Polysaccharide-containing fractions were combined, concentrated under vacuum, dialyzed and lyophilized.

Polysaccharides were divided into 2 fractions: acid (A) and neutral (B). The isolated fractions were tested for sugar content according to Dubois et al. (14) and for monosaccharide composition by HPLC Merck using an Supelcosil LC-NH_2 column, mobile phase – acetonitryl : H_2O (8:2, v/v) and a refractive index detector.

Assessment of antibacterial activity of purified polysaccharide fractions using disc diffusion method (Kirby-Bauer method)

Antibacterial activity of polysaccharide fractions A and B was tested on five laboratory bacterial strains: Gram-positive: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and Gram-negative- *Escherichia coli* ATCC 25922 *Klebsiella pneumoniae* ESßL+ ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853. The strains were cultured on appropriate media at 37°C under aerobic conditions for 24 h. The obtained bacterial cultures were used to prepare suspensions in physiological saline matching a 0.5 standard on the McFarland scale. The suspension was inoculated on Mueller-Hinton Agar 2 medium. Polysaccharide fractions A and B were dissolved in dimethylsulfoxide (DMSO) at a final concentration of 0.5% and sterilized using membrane filters with 0.22-µm pore size. Subsequently, 10 µL samples of 0.5 % solution of a polysaccharide fraction were loaded on a paper filter 6 mm in diameter (Oxoid, Argenta Sp. z.o.o) and the discs were placed on the medium with bacteria. Plates were incubated at 37°C for 24 h and growth inhibition zones of test bacterial strains were measured.

The result was considered to be positive and indicative of anti-inflammatory activity of a substance contained fungal polysaccharide fractions if bacterial growth inhibition zone was ≥ 9 mm (14).

Antiviral activity testing

Only fraction B was tested for antiviral activity (because of poor aqueous solubility of fraction A). Herpes simplex virus type 1 (HSV-1), laboratory strain McIntyre, grown in cell culture at $TCID_{50} = 10^{-5.83}$/mL was used for the study. Cytopathic effect on the rabbit kidney epithelial (RK-13) cell culture infected with different amounts of virus (10, 100, 1000 $TCID_{50}$) was examined. Cultures were maintained on 96-well flat-bottomed plates. There were a minimum of 6 cultures per one virus dilution.

Examination of inhibitory effect on HSV-1 replication

The highest concentration of the isolated polysaccharides, nontoxic to cell culture, i.e., 500 mg/mL was used. Virus concentration was 10 and 100 $TCID_{50}$. RK-13 cells infected and cultured for 48 h were overlaid with Eagle's medium supplemented with appropriate compounds and incubated for 96 h. The cultures were examined every day for cytopathic effect.

RESULTS

Two polysaccharide fractions A and B were isolated from *Sarcodon imbricatus* mycelium cultured *in vitro*. Sugar content in fraction A was 30%.

Table 1. Biological activity of 0.5% DMSO solution of polysaccharide fractions A and B against selected bacterial strains, evaluated using disc diffusion method, growth inhibition zone [mm]; (–) lack of inhibition.

Bacterial strains	Fraction A	Fraction B
Staphylococcus aureus ATCC 25923*	27	13.5
Enterococcus faecalis ATCC 29212*	22	–
Escherichia coli ATCC 25922**	9.5	–
Klebsiella pneumoniae ESßL+ ATCC 700603**	–	–
Pseudomonas aeruginosa ATCC 27853**	–	–

*G (+) bacteria, ** G (-) bacteria. Values are the mean of triple determinations.

HPLC analysis of acid hydrolysis products showed that it is a heteropolysaccharide composed of galactose and fucose. Sugar content in fraction B was estimated at 23% and based on HPLC analysis it was identified to be a sugar built of glucose and fucose. Besides, the fractions A contained uronic acids.

Disk diffusion method (Kirby-Bauer method) allowed for preliminary evaluation of antibacterial activity of both polysaccharide fractions. The results of antibacterial tests for fraction A and B are presented in Table 1.

Polysaccharide fraction A was demonstrated to possess antibacterial activity against Gram-positive bacterial strains *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. Moreover, fraction A exhibited weak growth-inhibiting effect on Gram-negative bacterial strain *Escherichia coli* ATCC 25922. No action on other Gram-negative tested bacterial strains (*Klebsiella pneumoniae* ESßL+ ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853) was detected. Polysaccharide fraction B produced antibacterial activity only against a Gram-positive bacteria *Staphylococcus aureus* ATCC 25923.

Only fraction B was tested for antiviral activity. First, we investigated toxicity of this polysaccharide fraction to RK-13 cell line. No cytopathic effect suggesting cell damage was observed after a 5-day incubation of these cells with fraction B at 50–500 mg/L.

Polysaccharide fraction B inhibited replication of HSV-1 at doses of 10 and 100 $TCID_{50}$. In the subsequent experiments, concentrations of preparations under study were gradually decreased, and antiviral activity was still detected at 5 mg/mL. When virus dose was 100 $TCID_{50}$, the concentration inhibiting virus replication by 100% was 15 mg/mL.

DISCUSSION AND CONCLUSION

Polysaccharide fractions isolated in our study from mycelium of *Sarcodon imbricatus* cultured *in vitro* contained fucose, galactose (fraction A) and fucose and glucose (fraction B), respectively. Polysaccharide isolated from fruit bodies of *Sarcodon aspratus* contains also fucose and galactose (5). Polysaccharides fraction isolated by us, mainly fraction A, showed pronounced antibacterial activity. Only some of mushroom polysaccharides posses this type of activity (3). Very interesting were also the antiviral effects of fraction B isolated by us from mycelial culture. There are only a few studies which describe this type of activity of mushroom polysaccharide e.g., PSK (krestin) from *Trametes versicolor* mycelial culture which inhibited HIV and cytomegalovirus replications (15). This results underlined the usefulness of *in vitro* cultures of Basidiomycota to obtain biologically active metabolites.

Recently, Barros (16) described the first study on the antioxidant activity of *Sarcodon imbricatus* and its correlation with phenol contents. This interesting report additionally justifies undertaking of research of the fruit bodies and mycelium cultured *in vitro* of this species.

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