

## SELENIUM-ENRICHED POLYSACCHARIDE FRACTION ISOLATED FROM MYCELIAL CULTURE OF *LENTINULA EDODES* (BERK.) – PRELIMINARY ANALYSIS OF THE STRUCTURE AND BIOLOGICAL ACTIVITY

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### ABSTRACT

We hypothesized that enriched in selenium polysaccharide fraction extracted from *Lentinula edodes* would possess higher biological activity than non-enriched, currently used to treat cancer. Se-enriched exopolysaccharide fraction was isolated from *L. edodes* mycelium, cultivated under submerged conditions in selenium-enriched medium. The monosaccharide composition of selenated polysaccharide, the amino acid content and the total concentration of Se was determined by reversed-phase high-performance liquid chromatography (RP HPLC) methods. X-ray absorption spectroscopy (XAFS) was used to probe the oxidation state and chemical speciation of selenium. The molar weight of Se-enriched polysaccharide fraction was determined by gel permeation chromatography (GPC). IR and NMR spectra were used to determine the type of glycosidic bonds. Se-enriched polysaccharide was tested for its antioxidant, cytotoxic and immunomodulating properties. All results regarding Se-enriched fraction were compared with that of reference polysaccharide fraction, extracted from mycelium not enriched in Se. Concentration of selenium in tested polysaccharide was 67 µg/g. Se-enriched fraction contained mainly glucose (89%) and mannose (8%). The molecular weight of the polysaccharide was 200 KDa; it contained 3% of protein. The type of glycosidic bounds was mainly β. XAFS analysis showed that the degree of Se oxidation in the polysaccharide was equal to –II and 0.

The comparison of cytotoxic profiles of tested fractions revealed that polysaccharide was not toxic toward HeLa (cervix carcinoma) and HUVEC (normal) cells. However, the Se-enriched mycelial polysaccharide fraction of high molecular weight significantly enhanced viability of cells; that may be an effect of the enhanced antioxidant activity. When assayed at concentrations 1-100 µg/ml, Se-enriched fraction caused no activation of lymphocytes T and B

**Keywords:** *Lentinula edodes*, selenium, exopolysaccharides, biological activity

## INTRODUCTION

*Lentinula edodes* (Berk.) Pegler, known commonly as shiitake mushroom, is one of the medicinal mushrooms postulated to exert highly potent enhancement of the immune system. The significant antitumor activity of *lentinan* and other pharmacologically-active polysaccharides and polysaccharide-protein complexes contained in shiitake extracts results from activation of the host immune system. The mechanism by which selenium – one of the trace elements of fundamental importance to human health - exerts anticancer and immunomodulating activity differs from that of *L. edodes* polysaccharide fractions, but a similar pharmacological effect suggests a possible synergism of these two agents. We hypothesized, therefore, that high concentrations of selenium in mycelial biomass would enhance the antioxidant and immunomodulating activity of mushroom mycelial extracts.

In a previous study, we demonstrated that *L. edodes* mycelium effectively accumulated Se from the cultivation medium [1, 2]. We found, that selenium is also incorporated into the mycelial polysaccharides [3, 6].

Thus, the present study deals with the isolation, structural analysis and examination of cytotoxic, antioxidant and immunomodulating activity polysaccharide-protein complexes isolated from the *L. edodes* Se-enriched and not enriched mycelial biomass.

## MATERIALS AND METHODS

**Microorganism and cultivation media.** The *Lentinula edodes* (Berk.) Pegler strain used in this study was ATCC 48085. The mycelial cultures were grown under the same conditions described in our previous reports [2, 4]. The fermentation medium was Se enriched to a concentration of 20 µg/ml by the addition of sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>, Sigma, Cell Culture Tested).

**Extraction and isolation of Se-enriched exopolysaccharide fraction.** Se-enriched polysaccharide fraction was isolated from the mycelial biomass by use of Chihara method [5]. Reference fraction was extracted from not enriched in Se mycelium of *L. edodes*.

### Structural analysis of polysaccharide fractions.

**RP-HPLC determination of monosaccharide composition.** A freeze-dried sample of polysaccharide fraction was hydrolyzed with 3M TFA at 120°C for 5 hrs. Samples were evaporated and neutralized. Monosaccharide composition was determined by use of RP HPLC method described in our previous paper [6].

**RP-HPLC determination of Se.** RP HPLC method of selenium determination after precolumn derivatization, described in our previous reports was used [2, 4].

**RP-HPLC determination of amino acids.** Amino acids were determined in hydrolysates of the polysaccharide dry weight, by high performance liquid chromatography of o-phthalaldehyde derivatives (OPA method).

**IR and NMR spectral analysis.** The IR spectrum was recorded with a Nicolet Fourier transform infrared (FTIR) spectrometer (Shimadzu). Test specimens were prepared by the KBr-disk method.

**NMR Analysis.** <sup>1</sup>H-NMR spectra were determined at room temperature. Fractions were dissolved in 6% NaOD solution in D<sub>2</sub>O. <sup>13</sup>C-NMR spectra were determined in solid state by use of CP MAS technic. Bruker DMX400 WB instruments was used.

*X-ray absorption spectroscopy (XAFS).* XAFS analysis was performed according to the method described by Lee *et al* [7].

**Tests of biological activity of polysaccharide fractions.** Cells viability assay was performed by MTT test. The cytotoxicity of all compounds was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, after 24 h or 48 h of incubation with polysaccharides, the cells were treated with the MTT reagent and incubation was continued for 2 h. MTT-formazan crystals were dissolved in 20 % SDS and 50 % DMF at pH 4.7 and absorbance was read at 562 and 630 nm on an ELISA-PLATE READER (ELX800, Bio-Tek, USA). As a control (100 % viability), cultured cells treated with vehicle (saline or DMSO) were used. The DMSO concentration in cell cultures didn't exceed 2%.

*Antioxidant activity of polysaccharides in cell cultures.* The HeLa cells grown as above were pre-incubated with polysaccharides or crude extracts from *L. edodes* (at the final concentration of 50 or 200 µg/ml, respectively) for 30 minutes. Following this incubation, H<sub>2</sub>O<sub>2</sub> was added to the cells (final concentration 100 µM or 300 µM) for 24 hours. Cell viability was determined as above using MTT assay.

*Tests of immunomodulating activity of polysaccharides.*

Cells isolation and *L. edodes* fractions preparation. Mononuclear cells (MNC) and granulocytes were isolated from peripheral blood of healthy blood donors by centrifugation in ficoll gradient (1077 g/l for MNC and 1119 g/l for granulocytes) and then were counted in Turk medium in Bürker counting chamber. MNC were suspended in Parker medium with addition of L-glutamine (2mM), 3-β-mercaptoethanol, Hepes (0,23%), fetal bovine serum (FBS, 10%) and gentamycin (0,1 mg/ml), and granulocytes in PBS containing glucose (0,036%) and bovine serum albumin (BSA, 0,1%). Fractions of *L. edodes* monosaccharide were diluted in 0,9% NaCl to 0,1-0,001 mg/ml concentration.

Proliferation assay. Lymphocytes cultures were established in 96- well flat-bottom microplate (concentration 1×10<sup>6</sup> cells/well) and induced with specific mitogen: anti-CD3 mAb (OKT3, 1µg/ml), phytohemagglutinin (PHA, 20 µg/ml) and suspension of *Staphylococcus aureus* Cowan strain (0,004% w/v). *L. edodes* monosaccharide fractions were added in a proportion of 100 µl of prepared dilution per well. Control cultures contained an equivalent amount of NaCl. Lymphocytes were cultured for 3 days at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, then 17 hours more with tritiated thymidine and then harvested. Lymphocytes DNA, with incorporated thymidine, was transferred onto a paper filters and read off in scintillation counter.

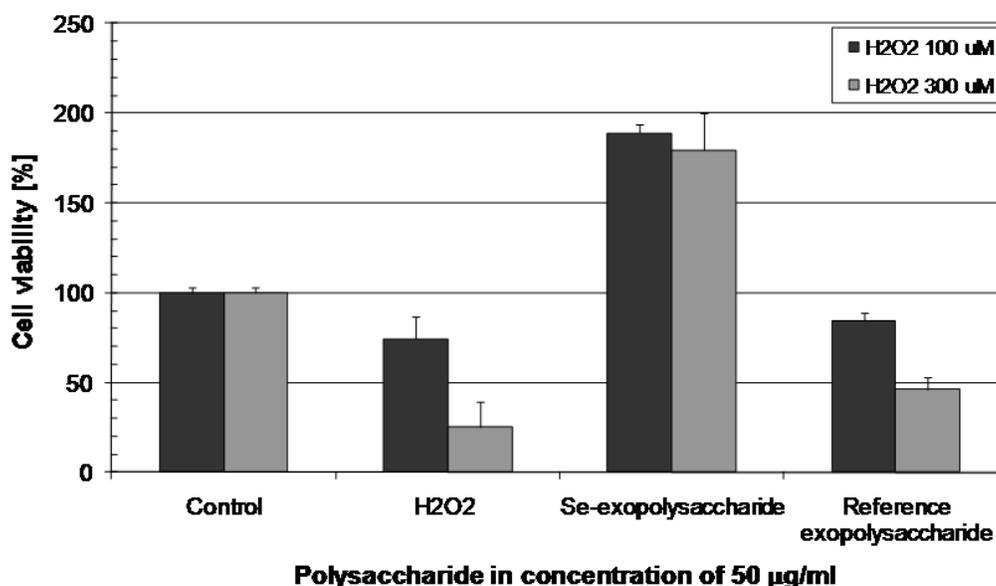
## RESULTS AND DISCUSSION

**Structure of the polysaccharides.** Total selenium amount in the *L. edodes* mycelium cultivated in medium enriched with 20 ppm Se medium was 748 µg/g. Approximately 13% of total mycelial selenium was combined to the polysaccharide fraction. Concentration of selenium in the isolated polysaccharide fraction corresponding to the Japanese drug *Lentinan* was 67 µg/g. Examined Se-enriched polysaccharide fraction contained mainly glucose (89%) and mannose (8%). Monosaccharide composition of reference fraction not enriched in selenium was similar. Isolated fraction contained 3% of protein. Main amino acids present in the protein component of the isolated fraction were lysine, arginine, serine, glutamic acid and glycine. The IR and NMR spectra confirm the type of glycosidic bounds was mainly β. The samples exhibited

characteristic absorption at 890 cm<sup>-1</sup> for the β-configuration. Assignment of the carbon-13 NMR spectra of polysaccharide fractions was made by comparison with previously published spectra [8]. The anomeric carbon signal apparent around 103 ppm was assignable as a β –configuration of glucan. XAFS spectra suggest the selenium in the isolated Se-exopolysaccharide is present at –II and 0 oxidative stage. Important problem to solve is the way the selenium is combined to the polysaccharide structure. The continuation of examination of the structure of isolated Se-polysaccharides is in progress.

**Biological activity of the polysaccharides.** In general isolated EPS fractions (selenated and not selenated) displayed no cytotoxic activity. Based on the dose-response curves, the IC<sub>50</sub> values were determined. The IC value for Se-exopolysaccharide fraction was >25 whereas it was >50 for not enriched in selenium exopolysaccharide. The comparison of cytotoxic profiles shows that the polysaccharides are not toxic toward HUVEC or HeLa cells. Cell viability in the presence of both, selenated and not selenated, fractions was higher than the control. It could be the result of the antioxidant activity of tested fractions, so the test of the protection of cells against oxidative stress by polysaccharide fractions was carried out.

Antioxidant activity of polysaccharides in cell cultures was expressed by the higher cells viability after H<sub>2</sub>O<sub>2</sub> exposure. Both tested exopolysaccharide fractions displayed antioxidant activity. Cell viability in the presence of the selenated polysaccharide was approximately two times higher than with not selenated polysaccharides. This is particularly evident for cells exposed to 300μM H<sub>2</sub>O<sub>2</sub> (Fig1).



**Fig.1** :Anti-oxidative properties of selenated and not selenated exopolysaccharides in HeLa cell cultures exposed to H<sub>2</sub>O<sub>2</sub> to induce the oxidative stress (24h)

These results strongly suggest the presence of selenium in polysaccharide enhance its antioxidant properties.

When assayed *in vitro*, *L. edodes* fractions in concentrations 1-100 ug/ml caused no stimulation of human T and B lymphocyte activation, even immunosuppressive activity was observed.

Selenium-enriched polysaccharide fraction display higher immunomodulating activity than not enriched fraction.

## CONCLUSIONS

Selenium- enriched exopolysaccharide fractions isolated from *L. edodes* mycelium cultivated in selenium enriched medium show interesting biological activities, not characteristic for mushroom polysaccharides. The continuation of the structural and biological activity tests is necessary.

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