Selenized bioactive polysaccharide fraction from *Lentinula edodes*, pharmaceutical composition comprising selenized bioactive polysaccharide fraction, the selenized bioactive polysaccharide fraction for use as medicaments and a method of preparation thereof

Field of the invention

The present invention relates to a composition of selenized bioactive polysaccharide fraction from *Lentinula edodes*, a pharmaceutical composition comprising selenized bioactive polysaccharide fraction, the selenized bioactive polysaccharide fraction for use as medicaments and a method of preparation thereof.

Background of the invention

Many Basidiomycota mushrooms have the ability to synthesize bioactive compounds, including anticancer agents that can be isolated from fruit bodies, mycelial cultures, or culture media. *Lentinula edodes* (Berk.) Pegl. is one of the most widely used medicinal mushrooms. This mushroom is the source of two drugs approved in several countries. Both drugs are immunomodulators and are used in cancer therapy. In addition, *L. edodes* forms compounds with antibacterial, antiviral, cholesterol lowering, and anticoagulant activities. Water-soluble lignans derived from *L. edodes* culture medium are being tested as potential drugs for the treatment of hepatitis B and AIDS. *L. edodes* protein is made up of 18 amino acids, including all essential amino acids, in ratios appropriate for humans. The fruit bodies of the mushroom contain considerable amounts of vitamins C, B1, B2, PP, B12 and D. Immunomodulatory polysaccharides isolated from *L. edodes* are being used in chemoprevention and as adjuvants in cancer treatment because of their ability to alleviate undesirable side effects of such therapies. This valuable mushroom is also used to produce dietary supplements (Turło, 2013).

Mushroom-derived polysaccharides (MPS), in particular β-D-glucans, arouse great interest as they are immune modulating substances, recognized by the human immune system (Cheng et al., 2014; Liu et al. 2015; Rathore, Presad & Sharma, 2017). Most of biologically-active MPS are extracted from mushroom cell wall, which is composed of inner layers of chitin and β-D-glucans (Latge, 2010; Hardison & Brown, 2012)

As listed in several articles, the cell wall β-D-glucans are typically 1,3- and 1,6-linked chains with varying amounts of side chains in position *O*-6 or *O*-3 (Synytsya & Novak, 2013; Dalonso, Goldman&. Gern, 2015; Zhu, Du,. Bian & Xu, 2015)

Such a structure is described for lentinan, a highly purified polysaccharide fraction, extracted from *Lentinula edodes* (shiitake mushroom) fruiting bodies. This substance is a highly potent immune system enhancer (Wasser & Weis, 1999; Zheng, Jie, Hanchuan, & Moucheng, 2005), approved for use in cancer treatment as an adjunct to conventional therapy (Boon & Wong, 2004; Sullivan, Smith, & Rowan, 2006; Yeung & Gubili, 2008). However, β-1,3-β-1,6- structure of cell wall MPSs appears not to be universal amongst the Basidiomycota, as there are mushrooms such as *A. ovinus* that does not contain this type of β-D-glucan (Samuelsen et al. 2019). Furthermore, strain variations, developmental stage, culture method and conditions, medium composition, extraction method, and even drying method may result in a variability in monosaccharide composition and combinations among polysaccharides (Ma, Chen, Zhu &Wang, 2013; Diamantopoulou et al., 2014; Xu, Li & Hu, 2014; Chien, Yen, Tseng & Mau, 2015; Su, Lai, Lin &Ng, 2016).

Moreover, according to the Commission Decision of 2 February 2011, authorising the placing on the market of a mycelial extract from *Lentinula edodes* (Shiitake mushroom) as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council (notified under document C(2011) 442, the *Lentinula edodes* mycelial extract is defined as follows: the novel food ingredient is a sterile aqueous extract obtained from the mycelium of *Lentinula edodes* cultivated in a submerged fermentation. It is a light brown, slightly turbid liquid. Lentinan is a β-(1-3) β-(1-6)-D-glucan which has a molecular weight of approximately 5×10^5 Daltons, a degree of branching of 2/5 and a triple helical tertiary structure. Composition of the mycelial extract from *Lentinula edodes* comprises: moisture 98 %, dry matter 2 %, free glucose less than 20 mg/ml, total protein less than 0,1 mg/ml (Bradford method), N-containing constituents less than 10 mg/ml (Kjeldahl method), and lentinan 0,8 – 1,2 mg/ml.

In the prior art, the immunomodulatory effect of polysaccharides isolated from *L. edodes* is widely described.

Patent publication TW201923087A discloses [beta]-1,3-1,6-glucan powder having high solubility in water, and a glucan-containing composition in which the powder or the like is used. The β-1,3-1,6-glucan can be isolated and extracted by bacteria or from seaweed, mushrooms, for example, from Lentinula edodes.

Moreover, European patent application EP3277271A1 relates to compounds having unique antiviral properties found in mushroom mycelium and their analogues. The invention includes active principle ingredients found in the combination of products from the mycelia of multiple

mushroom species in a form to have the accumulated effect of restricting the growth, spread and survivability of viruses in animals, especially humans, birds and bees. The present invention also includes the combination of products from multiple mushroom species in a form useful for preventing, treating, alleviating, mitigating, ameliorating or reducing viruses, including oncoviruses, in animals including humans. Such forms may have the additional advantages of functioning as antibacterials, antiprotozoals, immunomodulators, nutraceuticals and/or prebiotics as well as enhancing innate immunity defence mechanisms and host immune response, resulting in healing.

American patent application US5756318A discloses novel polysaccharides originated from culture broth of liquid culture including a mixture of a culture medium containing plant tissues and the mycelia of microorganisms belonging to the class Basidiomycetes and isolated and purified therefrom. The polysaccharides have a molecular weight from 500 to 10,000 that are comprised by lineally (1→4) linked α-D-glucose units whose 2,3-hydroxyl groups are partially acetylated in a ratio of about 30%, and have properties of biological response modifiers (BRM) and is useful as an immuno-enhancing agent or immuno-activator.

WO2020000015A1 relates to immunomodulatory compositions comprising one or more extracts from medicinal fungi, and the use of such compositions in the treatment or prevention of conditions or diseases associated with immunological dysfunction, and/or to provide one or more health benefits to an animal subject. In certain embodiments, the invention relates to compositions comprising multiple extracts from a number of medicinal mushrooms, and the use of such compositions to provide one or more health benefits to a subject in need thereof.

International publication WO2019209706A2 provides a dried product comprising bioactive fungal compounds. In some embodiments, the dried product is characterized by the following properties - 60%-90% by weight carbohydrates, comprising polysaccharides including a(1 -4) glucans and b(1 -3) glucans as determined by 1H and 13C NMR spectrum, the polysaccharides having a median molecular weight of about 10,000 Da; 3%-5% by weight protein; 0,1 %— 3% by weight lipid; 2 or 3 retention time peaks by HPLC of de-lipidized sample on Agilent Hi-Plex Na column; and immunomodulatory bioactivity.

Furthermore, US20190249211A1 provides a method of preparing a high yield of mushroom β-glucan. The method includes: providing a liquid culture to culture the mushroom mycelium by fermentation, to increase the yields of the mushroom mycelium and polysaccharide, wherein the liquid culture comprises at least two ingredients selected from the groups consisting of glucose,

trehalose, a dietary fiber and mannose or derivatives thereof; and rupturing the mushroom mycelium with a continuous multiple-ultrasonic equipment; and removing insoluble matters from the liquid culture. A method of preparing highly pure mushroom β-glucan powder and solution and the products thereof are also provided. By the method of the disclosure, the yield of mushroom β-glucan is effectively increased, its activity loss is reduced, and the stability of product thereof is improved. One of the mushroom of the mushroom mycelium is Lentinula edodes.

Additionally, CN110437343A patent application refers to the extraction method of lentinan.

Polish patent document PL225547B1 discloses a biosynthesis method resulting in a formation of a pharmacologically active preparation from *Lentinula edodes* and the use of this preparation. The preparation obtained by that method combines the immunomodulatory effect of polysaccharides derived from *L. edodes* with a similar effect of selenium compounds, which act through a different mechanism. However, this study focuses on properties of non-separated fractions, comprising mixtures of different polysaccharides.

Further structural analysis of selenized polysaccharides – the lentinan analog – was presented in Malinowska et al. (Malinowska, E., Klimaszewska, M., Strączek, T., Schneider, K., Kapusta, C., Podsadni, P., Łapienis G., Kleps, J., Dawidowski, M., Górska, S., Pisklak, D.M., Turło J. (2018). Selenized polysaccharides–biosynthesis and structural analysis. *Carbohydrate Polymers* 198, 407-417). The authors of that publication expected that incorporation of selenium into the polysaccharide would enhance immunostimulatory activity of the isolated fraction versus lentinan. It has been also suggested that isolated Se-polysaccharide is a non-toxic immunosuppressant with a strong antioxidant activity, that even enhances cell viability (Kaleta, B., Gorski, A., Zagozdzon, R., Cieslak, M., Kazmierczak-Baranska, J., Nawrot, B., Turlo, J. (2019). Selenium-containing polysaccharides from Lentinula edodes - Biological activity. Carbohydrate Polymers, 223). The biological effect was completely different from lentinan. It has been also suggested that it depends in-part on the selenium incorporation into the polysaccharide molecule, but probably also on the dissimilar structure of the fractions isolated from mycelial cultures versus lentinan. Preliminary structural studies have shown that the isolated immuno-active fraction is a protein-containing mixture of high molar mass polysaccharides (M_w $3.9x10^6$ g/mol and $2.6x10^5$ g/mol), α- and β-glucans, composed of glucose or mannose. The X-ray absorption fine structure (XAFS) spectral analysis in the near edge region (XANES) confirmed that selenium in the Se-polysaccharides structure is present at the –II oxidation state and that Se is organically bound. The simulation analysis in the EXAFS region

suggested that selenium is most likely bound by a glycosidic-link in a β-1,3 or a-1,4-glycosidic bond or substituted for oxygen in a pyranosidic ring (Malinowska et al., 2018). However, as in the case of the above publications, the presented studies concerned structural studies related to non-separated fractions, which are a mixture of various polysaccharides, and it has not been explained, which of the components of the complex fraction was responsible for its immunosuppressive activity, non-typical for polysaccharides of fungal origin. Moreover, the complex composition of the preparation of the selenized polysaccharides synthesized by Lentinula edodes, would make this preparation difficult to use in pharmaceutical applications, because it is required that any medicinal product authorized for use has a fine control of purity and components.

Thus, the aim of the present invention is to provide a composition of selenized bioactive polysaccharide fractions from *Lentinula edodes* comprising a mixture of fraction A and fraction B-C, wherein:

fraction A consists of α-1,4-D-glucan polymer; and

fraction B-C consists of the following glucan polymers: 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan, and

wherein the content of fraction A and fraction B-C in the composition is equal to about 85% w/w and about 4% w/w, respectively.

In the present description term "about" should be understood as comprising similar values such as, equal ± 6% of such value, i.e., term about 85% w/w should be understood as comprising the range of 85% ± 6% of 85% value, i.e., the range from 79,90% to 90,10%, and wherein about 4% should be understood as comprising the range of 4% ± 6% of 4% value, i.e., the range from 3,76 % to 4,24 %. Preferably, the content of fraction A in the composition is in the range between 84%-86%, whereas the content of fraction B-C in the composition is in the range between 3,76 % to 4,24 %. Even more preferably, the content of fraction A is equal 85,35% and the content of and fraction B-C is 4,23%.

The present invention also provides a method of preparation of a composition of selenized bioactive polysaccharide fraction from *Lentinula edodes*, that comprises the following steps:

a) culturing *Lentinula edodes* in culture medium fortified with at least 30 μg/ml selenium;

b) harvesting mycelia;

c) extraction with boiling methanol 1:4 w/v;

d) extraction of the insoluble in methanol portion of c) with hot water;

e) precipitation of the fraction extracted in d) with 1 volume of ethanol;

f) solubilization of the precipitate of e) in water and precipitation with 0.2 M cetyltrimethylammonium hydroxide;

g) extraction of the precipitate of f) with 20% acetic acid;

h) extraction of the insoluble part from g) with 50% acetic acid;

i) solubilization of the insoluble part from h) with 6% NaOH;

j) precipitation with 3 volumes of ethanol;

k) deproteinization of the precipitate of j); and

l) purification of polysaccharide fraction from the deproteinized precipitate.

In a preferred embodiment of the present invention, step l) involves using ion exchange chromatography and gel permeation chromatography. More preferably, the first polysaccharide fraction (fraction A) eluted from the gel permeation column, the fraction containing at least 80% w/w of α-1,4-D-glucan polymer forming an α-helix structure, is retained; the second polysaccharide fraction (fraction A/B-C) eluted from the gel permeation column, the fraction containing mixture the following glucan polymers: α-1,4-D-glucan, 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan, is retained; or the third polysaccharide fraction (fraction B-C) eluted from the gel permeation column, the fraction containing mixture of three glucan polymers: 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan, is retained.

The present invention furthermore provides a composition of selenized bioactive polysaccharide fraction from *Lentinula edodes* obtained by the method according to present invention, wherein said composition comprises a mixture of fraction A of α-1,4-D-glucan polymer; and fraction B-C of 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan, and wherein the content of fraction A and fraction B-C in the composition is equal to about 85% w/w and about 4% w/w, respectively. Preferably, said composition is obtained by a method, wherein the second polysaccharide fraction eluted from the gel permeation column, the fraction containing mixture the following glucan polymers: α-1,4-D-glucan, 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan, is retained.

Moreover, the present invention provides a composition of selenized bioactive polysaccharide fraction for use as a medicament.

In a preferred embodiment of the present invention, the composition of selenized bioactive polysaccharide fraction is used as a medicament in selective inhibition of T-cell proliferation. More preferable, selective inhibition of T-cell proliferation is required in conditions selected from the group comprising allogeneic organ transplantation, autoimmune diseases, and T cell-dependent allergies. Most preferable, the autoimmune diseases are selected from rheumatoid arthritis, multiple sclerosis, celiac disease, glomerulonephritis, inflammatory bowel disease and psoriasis. In another most preferred embodiment, the T cell-dependent allergy is contact dermatitis.

In a preferred embodiment of the present invention, the said composition is administered orally, preferably in the form of tablets, capsules, granules for solution preparation or solution; intravenously; intraperitoneally, and externally, preferably in the form of ointments, creams, suppositories, pessaries, rectal enemas.

The present invention also provides a pharmaceutical composition comprising an active ingredient and a pharmaceutically acceptable excipient, which, as the active ingredient comprises a composition of the selenized polysaccharide fraction.

By the method of the present invention the composition of selenized bioactive polysaccharide fractions from *Lentinula edodes* might be obtained, which is, due to unexpected synergistic effect provided by fraction A and fraction B-C, more active and effective. What is more, the present invention overcome disadvantages of the known in the art methods as well obtained by such methods compositions, since it provides the compositions that is much purer and might be directly use as the active ingredient for pharmaceutical composition without any additional treatments, particularly additional purification. It is especially important, since such composition might be used for treatment rheumatoid arthritis, multiple sclerosis, celiac disease, glomerulonephritis, inflammatory bowel disease and psoriasis.

Brief description of figures

Preferred, non-limiting examples which embody certain aspects of the invention will now be described, with reference to the following figures.

Fig. 1. A. Chihara's method of isolation of lentinan; B Method modified for isolation Se-enriched lentinan analogue (fraction Se-Le-30).

Fig. 2. Selected parts of the 1H-13C HSQC-DEPT NMR spectra of fraction II (A, B) and fraction III. (C, D).

Fig. 3 The structure of polysaccharides presented in fraction II separated from Se-L-30.

Fig. 4. RI traces from SEC of the Se-Le-30, A (I), A/B-C (II), and B-C (III) fractions. The lines for the A, A/B-C, and B-C fractions were smoothed to remove noise.

Fig. 5. Deconvolution of the RI signal of product B-C.

Fig. 6. Structural models of the investigated polysaccharides; side view (left) and a view along the axis (right). A. Polysaccharide A (1,4-α-D-glucan). B. 1-3-β-D-glucan - component of B-C fraction. C. 1-6-β-D-glucan - component of B-C fraction. D. 1-3-β-branched 1-6-β-D-glucan - component of B-C fraction. E. Distances (Å) between oxygen atoms, suggesting an opportunity for stabilization of the helical structure of 1-3-β-branched 1-6-β-D-glucan by hydrogen bonds. Created using the Chem3D Pro module of ChemOffice Professional 2017 (PerkinElmer). Energy minimization was performed using the MM2 method.

Fig. 7. The effects of polysaccharides (Se-L, Se-Le-30, A, B-C, and A/B-C) on the proliferation of peripheral blood mononuclear cells (PBMCs). A. Non-stimulated (autostimulation). B. Stimulated with anti-CD3 monoclonal antibody (OKT3). C. Stimulated with phytohemagglutinin (PHA). D. Stimulated with suspension of Staphylococcus aureus Cowan strain (SAC). The results are presented as the percentage of control (without polysaccharides) proliferation. * $p<0.05$ versus control (K). The error bars correspond to the standard deviation. The results for the Se-L fraction were described by Kaleta et al. (2019).

Fig. 8. RALS traces for Se-Le-30 and fractions A, A/B-C, and B-C.

Fig. 9. SEC traces. a) Se-Le-30, b) A/B-C fraction, c) A fraction, d) B-C fraction

Fig. 10. Deconvolution of the Se-Le-30 RI signal. Curves corresponding to fractions A and B-C are marked by arrows.

Fig. 11. Deconvolution of the RI signal of product A/B-C. Curves corresponding to fractions A and B-C are marked by arrows.

Fig. 12. Deconvolution of the RI signal of product A. The curve corresponding to fraction B-C is marked by an arrow.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related.

For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide definitions commonly accepted in the field for many of the terms used in this disclosure.

In this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. The terms "a" (or "an"), as well as the terms "one or more," and "at least one" can be used interchangeably herein.

As used herein the terms "treat," "treatment," or "treatment of" refers to (i) reducing the potential for a disease or disorder (e.g., a disease associated with increased activity or proliferation of T lymphocytes), (ii) reducing the occurrence of a disease or disorder, (iii) reducing the severity of a disease or disorder, preferably, to an extent that the subject suffers less or no longer suffers discomfort and/or altered function due to it, (iv) reducing an indication or marker of a disease or disorder such as reducing the blood or serum levels of T-cell markers, or (v) a combination thereof.

The terms "subject", "individual" or "patient" as used herein refer to any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy of a disease or disorder is desired. As used herein, the terms "subject," "individual" or "patient" include any human or nonhuman animal. The term "nonhuman animal" includes all vertebrates, e.g., mammals and non-mammals, such as mice, nonhuman primates, sheep, dogs, cats, horses, cows, bears, chickens, amphibians, reptiles, etc. In preferred embodiments, a subject is a human.

The term "administration" or "administering" of a drug or a medication, as used herein, includes delivering, applying, or giving the therapy or drug to a subject including self-administering by the subject.

The term "therapeutically effective amount" as used herein refers to an amount of a compound or a mixture (e.g., polysaccharide fraction) effective to "treat" a disease or disorder in a subject and/or to prevent or reduce the risk, potential, possibility or occurrence of a disease or disorder.

The therapeutic effective amount for the treatment may in practice depend on factors including the blood or serum levels of proinflammatory factors, severity of the disease or disorder in the subject before the treatment, the presence or absence of various conditions (e.g., presence or absence or severity of cardiovascular disorders and/or allergic diseases or reactions), age and gender of the subject and can be adjusted by a person of ordinary skill in the art (e.g., a doctor).

Said treatments may include the treatment of diseases requiring modulation of T cell activity, including, but not limited to, allogeneic organ transplantation, as well as a range of autoimmune diseases, especially the ones with known aberrant activity of T cells, such as rheumatoid arthritis, multiple sclerosis, celiac disease, glomerulonephritis, inflammatory bowel disease, psoriasis etc., and also in T cell-dependent allergy, such as contact dermatitis etc.

"Deproteinization" is intended to mean any method as known in the art of decreasing protein content or removing proteins from a mixture, i.e., Savage method (Ruthes, A.C.; Smiderle, F.R.; Iacomini, M. D -Glucans from Edible Mushrooms: A Review on the Extraction, Purification and Chemical Characterization Approaches. Carbohydr. Polym. 2015, 117, 753–761, doi:10.1016/j.carbpol.2014.10.051).

A glucan is a polysaccharide derived from D-glucose, linked by glycosidic bonds. A glycosidic bond or glycosidic linkage is a type of covalent bond that joins a carbohydrate (sugar) molecule to another group, which may or may not be another carbohydrate. α- and β-glycosidic bonds differ by the relative stereochemistry of the anomeric position and the stereocenter furthest from C1 in the saccharide. An α-glycosidic bond is formed when both carbons have the same stereochemistry, whereas a β-glycosidic bond occurs when the two carbons have different stereochemistry.

By the term "analog" as used herein it should be understood a chemical compound with a similar structure, but which may exhibit a different biological activity.

The term "Se-L" as used herein refers to an refers to the lentinan analog isolated by Yap and Ng method.

The term "Se-Le-30 fraction" as used herein refers to an refers to a lentinan analog isolated by a modified Chihara method.

The term "fraction A" or "fraction I", as used herein refers to an α-1,4-D-glucan characterized by the elution time expressed in milliliters at a flow rate of 0.5 ml/min: ion exchange chromatography: 6 – 24 ml and gel permeation chromatography: 62 – 74 ml.

The term "fraction A/B-C" or "fraction II", as used herein refers to an α-1,4-D-glucan, 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan characterized by the elution time expressed in milliliters at a flow rate of 0.5 ml/min: ion exchange chromatography: 79,5 – 97,5 ml and gel permeation chromatography: 62 – 76 ml.

The term "fraction B-C" or "fraction III", as used herein refers to an 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan characterized by the elution time expressed in milliliters at a flow rate of 0.5 ml/min: ion exchange chromatography: 87 – 109,5 ml ml and gel permeation chromatography: 72 – 90 ml. (Calculated from the fractions: LC-1KP after DEAE A4B1 on HW C7D1, LC-1KP after DEAE D8E5 on HW C7D2, LC-1EM after DEAE D13E13 on HW C11D9).

Hot water is intended to mean water having a temperature within the range of 90-100ºC.

The polysaccharide fractions obtained by the method of the present invention may be administered using various routes, e.g. orally, intravenously, topically or other. The polysaccharide fractions can be administered alone or in a pharmaceutical composition comprising pharmaceutically acceptable excipients.

The polysaccharide fractions of the present invention or pharmaceutical compositions thereof can be presented in various dosage forms. They may be administered in liquid form (e.g. as a solution, dispersion, slurry, sterile solution for injection or infusion etc.) or a solid form (e.g. powder, such as freeze-dried). If the polysaccharide fractions of the present invention or pharmaceutical compositions thereof are to be administered orally, they may be presented in a form of e.g. tablets, capsules, lozenges or other.

When the pharmaceutically effective amount of the polysaccharide fraction of the invention is administered orally, e.g., comprised in a pharmaceutically acceptable oral dosage form, such oral dosage forms can also comprise a suitable amount of one or more pharmaceutically acceptable excipients, including a diluent, suspending agent, solubilizer, binder, disintegrant, preservative, coloring agent, lubricant, and the like. The pharmaceutical excipients can be a liquid, such as water or oil, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. The pharmaceutical excipient can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea, and the like. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents can be used. In some embodiments, the pharmaceutically acceptable excipient is sterile when administered to a human subject. Suitable pharmaceutical excipients also include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. The pharmaceutical compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. Examples of pharmaceutically acceptable carriers and excipients that can be

used to formulate various dosage forms are known in the art, e.g., described in the Handbook of Pharmaceutical Excipients, American Pharmaceutical Association (1986).

Detailed description of the invention

The aim of the present invention is to provide the Se-polysaccharides fraction, which exhibits improved immunosuppressive activity compared to isolated Se-polysaccharide-protein fraction *Se-L*.

Lentinan, the Japanese immunostimulatory drug for which the inventors intended to obtain a selenized analogue, was isolated for the first time by G. Chihara from the fruiting bodies of *L. edodes* in a multi-step fractionation procedure using strong bases and organic acids (Chihara et al., 1970). It was disclosed that, during the isolation of Se-polysaccharides from *L. edodes* mycelium using this method, a significant portion of selenium may be lost. The selenium-containing polysaccharides (selenoglycosides) may be unstable in a strongly basic and acidic environment. Therefore, the Yap and Ng (2001) method of isolating lentinan was chosen. The advantage of this method is the mild conditions of the isolation process, resulting in higher polysaccharide yield and less loss of selenium. The disadvantage, however, is lower homogeneity and reduced purity of the obtained fraction *versus* the Chihara method (87% *versus* 96%).

As the inventors wanted to exclude the immunosuppressive effect of the previously examined *Se-L* fraction being caused by components other than polysaccharides (proteins or other undefined impurities), it was decided to obtain pure lentinan analogue via Chihara's isolation method. To compensate for the loss of selenium during isolation, the mycelium culture was cultivated in medium containing a higher concentration of selenium (30 ppm). By raising the concentration of selenium in the culture medium to 30 ppm, a very significant increase in the concentration of Se in the *L. edodes* mycelium was achieved: 1753 μg/g *versus* 1040 μg/g for the culture enriched in 20 ppm. A significant increase in the concentration of selenium in the *Se-L* fraction isolated according Ng and Yap method was also observed (305 μg/g *versus* 190 μg/g). However, the lentinan analogue *Se-Le-30* isolated by Chihara's method contained a much lower concentration of selenium (48 μg/g). Thus, a change in the isolation method resulted in a significant loss of selenium, but also had an expected positive effect, a significant increase in the purity of the fraction in terms of protein and sugars other than glucose content.

Previously defined *Se-L* fraction (Kaleta et al., 2019) had an immunosuppressive effect, which is opposite to the Japanese drug lentinan, which is isolated by the same method from the fruiting bodies of the same species of mushroom. Structural studies of the *Se-L* fraction have indicated

that it contains a mixture of 1,4-α-, 1,6-β- and 1,3-β-glucans and mannans (Malinowska et al., 2018), whereas lentinan is a (1,3;1,6)-β-glucan (Zhang et al., 2011). The proportion of 1,6- and 1,3-β-glycosidic linkages in the mycelial fraction was also different from lentinan (Malinowska et al., 2018).

Se-Le-30 fraction

Similar to *Se-L* fraction, data on the type of glycosidic bonds were recorded for the *Se-Le-30* fraction. The NMR and TRISEC studies showed that the *Se-Le-30* fraction was not homogenous. The product A was the main component of the *Se-Le-30* fraction, and product B-C with a much lower molar mass was present only in small quantities. The approximate mass ratio of the *A* and *B-C* polysaccharides in the *Se-Le-30* fraction was 26:1. In addition, the *Se-Le-30* fraction contained approximately 6% mannose and 4% protein by weight (perhaps in the form typical for mushroom cell wall mannoproteins). The selenium content in the *Se-Le-30* fraction was nearly 4-fold lower than in the previously isolated *Se-L* fraction (Malinowska et al., 2018).

Fraction A (I)

The sugar analysis and absolute configuration determination showed that both isolated polysaccharides (*A* and *B-C*) are composed of D-glucose.

Methylation analysis indicated that polysaccharide *A* is linear. Analysis of the sequence of the monosaccharide residues within the repeating unit of this polysaccharide obtained by assigning the inter-residue interactions observed in the 2D NOESY and HMBC spectra revealed connectivities between C-1 and C-4. Complete assignment of the ^{1}H and ^{13}C resonances and information on the sequence and linkage obtained from a series of 2D NMR experiments suggested that residue *A* was α-linked. This indicated that *A* is a →4)-α-D-Glc*p*-(1→ moiety. The data obtained on the structure of fraction *A* indicated that this natural polysaccharide of fungal origin, most likely a component of the *L. edodes* cell wall, has a structure similar to amylose, a polysaccharide of plant origin. Its molar mass determined by TRISEC was significantly higher than the average masses typical for amylose. According to Joye (2019), the molar mass range of amylose is quite broad and varies between 8×10^4 and 10^6 g/mol depending on the plant species, varieties, and maturity of the starch under study.

The >80% content of *A*, an amylose-like α-1,4-D-glucan in the *Se-Le-30* polysaccharide fraction isolated from *L. edodes* mycelium, was surprising. The amylose-like polysaccharide has not yet been described as a main hot water-soluble component of the cell wall of *L. edodes*. However, McCleary and Draga (2016) determined the α and β-glucans in commercial preparations of

fungal origin, including *L. edodes* extracts; they contain up to 80% α-glucans, instead of the β-glucans declared by the manufacturer. The authors explained the presence of α-glucans as an artefact caused by the cultivation and isolation method. They stated that commercial mushroom mycelia are cultivated over a sterilized cereal grain base. In the latter process, the mycelium-infested grain is harvested and the polysaccharide fractions isolated. Based on the analytical data, they suggested that the starch from the grain is the major α-glucan present in the final product (McClery & Draga, 2016).

This was possible with mycelium grown on a solid medium (grain). However, the present invention discloses mycelium that was grown in a liquid medium that did not contain starch or solid components. When harvested, it was thoroughly filtered and rinsed, meaning that contamination with culture medium was not possible. Polysaccharide *A*, a water-soluble 1,4-α-D-glucan, was a component of *L. edodes* mycelium.

The secondary structure of polysaccharide *A* predicted on the basis of molecular modeling is an α-helix (Fig. 6A), analogous to amylose, which also tends to adopt a natural helical structure (Joye, 2019). Natural polysaccharides have polydisperse molar mass, but using fractionation techniques, such as size exclusion chromatography, a low polydispersity index can be obtained. This is the case with fraction *A* having a low polydispersity index ($Đ$ =1.2), which indicates its homogeneity. The deconvolution of the RI signal of product *A* allowed for determination of the residual content of the *B-C* fraction equal to 2.73%. The purification by GPC in general did not ensure the isolation of completely pure polysaccharides. In particular, the *B-C* fraction was very difficult to separate from fraction *A*, despite the very significant difference in molar mass, without being bound by any theory, probably due to intermolecular interactions. A similar problem occurs when separating the amylose from amylopectin using gel-permeation chromatography. Perhaps amylose is cross-linked to amylopectin and, thus, eluted with amylopectin (Ai & Jane, 2018), or is interspersed with amylopectin molecules. Similar interactions may occur between polysaccharides *A* and *B-C*.

Fraction B-C (III)

The same analytical methods used to investigate the structure of fraction *A* (sugar analysis and absolute configuration determination, methylation analysis, ^{1}H, ^{13}C, and 2D NMR) showed that the *B-C* fraction is a mixture of three glucans: 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan. The molar mass determined by TRISEC was significantly lower than

that of fraction *A*. The homogeneity of fraction *B-C* was also lower, which indicates a higher polydispersity index (*Đ* =2.18).

1,6-β-Glucan has been shown to be an important component of *S. cerevisiae* and *C. albicans* cell walls (Aimanianda et al., 2009; Klis et al., 2006; Lesage & Bussey, 2006). However, a number of publications reported the presence of 1-6-β-glucans in the cell wall of higher fungi (Malinowska et al., 2018; Li et al., 2019; Samuelsen et al., 2019). The structural analysis of water and alkali extracts from *A. ovinus* fruit bodies suggested the presence of two different β-D-glucan backbone structures: a 1-6-linked β-D-glucan with single β-D-Glc*p* residues at *O*-3, and a (1→3)-linked β-D-glucan with branches (Samuelsen et al., 2019). The *B-C* polysaccharide of similar structure was present in low proportions in the tested *Se-Le-30* fraction isolated from *L. edodes*, which is probably due to its low solubility in hot water. A similar compound was much more efficiently isolated by Samuelsen et al. (2019) and by Li et al. (2019) with hot alkali solution.

In contrast to lentinan, the polysaccharide isolated from the fruit bodies of *L. edodes* by G. Chihara - a β-D-glucan with 1-3-β-linked backbone and 1-6-β-linked side chains, the polysaccharide fraction isolated by the same procedure from the submerged cultured *L. edodes* mycelium proved to be a mixture of linear 1-4-α-D-glucans, 1-3-β-D-glucans, 1-6-β-D-glucans and branched 1-6-β-D-glucan with 1-3-β-linked backbones.

EXAMPLES

MATERIALS AND METHODS

Biosynthesis and isolation of Se-enriched polysaccharide fractions

Mushroom strain and cultivation conditions

The *Lentinula edodes* (Berk.) Pegler strain used in our study was ATCC 48085. The seed culture was grown under the conditions described in our previous reports (Turło et al., 2010; 2011). Mycelia used as the raw material for extraction of polysaccharides were cultivated under submerged conditions in a 10-L fermenter (BioTec FL 110, Stockholm, Sweden). The culture media was fortified with 30 μg/ml selenium by the addition of sodium selenite (Na_2SeO_3, Sigma, Cell Culture Tested). The initial pH of the medium was 6.5. The medium was inoculated with 5% (v/v) seed culture and cultivated at 26°C. Fermentation was performed for 10 days under the following conditions: aeration rate, 0.5 volume per volume per minute (vvm); agitation speed, 200 rev/min; and working volume, 8 L. Mycelia were harvested by filtration, washed three times with distilled water, and freeze dried.

Extraction of Se-enriched polysaccharide fraction Se-Le-30 by Chihara method

The Se-enriched polysaccharide fraction, the analog of the Japanese anticancer drug lentinan, was isolated from the Se-enriched *L. edodes* mycelium using the modified Chihara method (Chihara et al., 1970). The original method was completed by the preliminary extraction of lipids, small carbohydrate molecules, and other non-polysaccharide compounds in boiling methanol (1:4 w/v) for 4 hours (Fig. 1).

It is also possible to use a simplified method, that allows for a lower loss of selenium, in which after the precipitation step with alcohol, the obtained sediment is subjected to lyophilization, then redissolved in hot water, again precipitated with alcohol, lyophilized and subjected to chromatographic separation.

Separation of Se-enriched polysaccharides fraction Se-Le-30

The polysaccharides were purified according to Górska et al. (2016). Briefly, the freeze-dried Se-Le-30 crude extract were purified by ion exchange chromatography on a DEAE-Sephadex A-25 column (1.6x20 cm; Pharmacia Biotech, USA) and further by gel permeation chromatography on a Toyopearl HW-55S column (1.6×100 cm; Tosoh Bioscience LLC, Germany) fitted to an FPLC system (Amersham Pharmacia Biotech, Sweden). The fractions were eluted with a NaCl gradient (0-2 M in 20 mM Tris buffer, pH 8.2) and 0.1 M ammonium acetate buffer, respectively. The column effluents were monitored at 220, 260 and 280 nm with an UV-VIS absorbance detector and Knauer differential refractometer and for the carbohydrate content (Dubois., Giller, Rebers & Smith, 1956). The fractions containing carbohydrates were collected.

Structural analysis

The molar mass of the analyzed fractions was estimated by size exclusion chromatography (SEC) with triple detection (TRISEC) as described in Malinowska et al. Namely, three TSK-GEL columns (G5000 PW_{XL} + 3000 PW_{XL} + 2500 PW_{XL}; 7.8×300 mm; Tosoh) with a Knauer K-501 pump, a refractive index (RI) detector (LDC), and a double TDA 270 detector (laser light scattering [RALS + LALS] and viscosity detector; Viscotek, USA) were used. The analysis was performed at 26°C with a mobile phase of 0.1% NaN_3 and flow rate of 1.0 ml/min. The injection volume was 100 µl. Before injection, samples were filtered through 0.2-µm pore size membrane filters. OmniSEC software (Viscotek, USA) was used to calculate the number average molar mass (M_n) for $dn/dc = 0.145$ (β-glucans in NaN_3 solution). The procedure for calculating molar mass using OmniSEC software was reported previously (Huang et al., 2004).

RP-HPLC determination of monosaccharide composition

The monosaccharide composition of the polysaccharides was determined by a reversed phase high performance liquid chromatography (RP HPLC) method described in Malinowska et al. Prior to the analysis, the polysaccharides were hydrolyzed for 5 hours in 3M TFA at 120°C.

RP HPLC determination of Se content

The RP HPLC procedure to determine Se content was a modified fluorometric method for Se determination after derivatization with 2,3-diaminonaphtalene (3,5-benzopiazselenol formation) with fluorescence detection as described in Turło et al. and Malinowska et al.

Determination of protein content in the lentinan analogue (Se-Le-30)

The protein content was determined using two independent methods as described in Malinowska et al. The results of the well-known spectrophotometric Bradford method (Bradford, 1976) were verified by measuring the nitrogen content in the analyzed fractions using a CHNS elemental analyzer (Vario EL III, Elementar, Germany). The protein content was calculated using a conversion factor of 6.25 for total nitrogen to protein.

Sugar and methylation analysis and determination of the absolute configuration

Polysaccharide samples (0.5 mg) were hydrolyzed with 10 M HCl at 80°C for 25 min and evaporated under a stream of N_2. The resulting monosaccharides were converted into alditol acetates according to Sawardeker, Sloneker, and Jeanes (1956). For methylation analysis, the polysaccharide samples were permethylated according to the method described by Ciukanu and Kerek (1984). The product was purified by water-chloroform extraction. The methylated polysaccharides were hydrolyzed in 2 M TFA at 120°C for 2 h and evaporated under N_2. Finally, methylated monosaccharides were reduced with $NaBD_4$ and acetylated for gas-liquid chromatography-mass spectrometry (GLC-MS) analysis using the same conditions as for the sugar analysis and butyl glycosides as described below. The absolute configurations of the sugars were determined by GLC-MS of their acetylated glycosides using (S)-/+2-butanol essentially as described by Gerwig, Kamerling, and Vliegenthart (1979). Alditol acetates and acetylated 2-butyl glycosides were analyzed by GLC-MS using an ITQ 700 Thermo Scientific system equipped with a ZB-5HT (Phenomenex) capillary column with a temperature gradient from 150 to 270°C at 8°C/min.

Determination of protein content in the lentinan analogue,Se-Le-30

The protein content was determined using two independent methods as described in Malinowska et al. The results of the well-known spectrophotometric Bradford method (Bradford, 1976) were verified by measuring the nitrogen content in the analyzed fractions using a CHNS elemental analyzer (Vario EL III, Elementar, Germany). The protein content was calculated using a conversion factor of 6.25 for total nitrogen to protein.

Sugar and methylation analysis and determination of the absolute configuration

Polysaccharide samples (0.5 mg) were hydrolyzed with 10 M HCl at 80°C for 25 min and evaporated under a stream of N_2. The resulting monosaccharides were converted into alditol acetates according to Sawardeker, Sloneker, and Jeanes (1956). For methylation analysis, the polysaccharide samples were permethylated according to the method described by Ciukanu and Kerek (1984). The product was purified by water-chloroform extraction. The methylated polysaccharides were hydrolyzed in 2 M TFA at 120°C for 2 h and evaporated under N_2. Finally, methylated monosaccharides were reduced with $NaBD_4$ and acetylated for gas-liquid chromatography-mass spectrometry (GLC-MS) analysis using the same conditions as for the sugar analysis and butyl glycosides as described below. The absolute configurations of the sugars were determined by GLC-MS of their acetylated glycosides using (S)-/+2-butanol essentially as described by Gerwig, Kamerling, and Vliegenthart (1979). Alditol acetates and acetylated 2-butyl glycosides were analyzed by GLC-MS using an ITQ 700 Thermo Scientific system equipped with a ZB-5HT (Phenomenex) capillary column with a temperature gradient from 150 to 270°C at 8°C/min.

NMR spectral analysis

NMR spectra were obtained on a Bruker 600 MHz Avance III spectrometer using a 5-mm QCI $^{1}H/^{13}C/^{15}N/^{31}P$ probe equipped with a z-gradient. The NMR spectra were obtained for a $^{2}H_2O$ solution of the polysaccharide at 25°C using acetone (δ_H 2.225, δ_C 31.05 ppm) as an internal reference. The polysaccharide (10 mg of fraction I and II and 2 mg of fraction III) was repeatedly exchanged with $^{2}H_2O$ with intermediate lyophilization. The data were acquired and processed using Bruker Topspin software (version 3.1) and SPARKY (Goddard & Kneller, 2001). The signals were assigned using one-dimensional (^{1}H, ^{13}C, ^{31}P) and two-dimensional (2D) experiments: correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), ^{1}H-detected heteronuclear single quantum coherence spectroscopy (HSQC) with and without carbon decoupling, HSQC-TOCSY, and ^{1}H-^{13}C

heteronuclear multiple-bond correlation spectroscopy (HMBC). The TOCSY experiments were carried out with mixing times of 30, 60, and 100 ms, NOESY with mixing times of 100 ms and 300 ms, and HMBC with a mixing time of 60 ms.

Molecular modeling

The structural models of the investigated polysaccharides were built with Chem3D Pro module of ChemOffice Professional 2017 (PerkinElmer). The energy minimization was performed using MM2 method implemented in the software.

The effects of polysaccharides on human peripheral blood mononuclear cells (PBMCs) proliferation

To investigate the effect of polysaccharide fractions and isolated polysaccharides on human lymphocyte proliferation, a method described in Kaleta et al. was used. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood of healthy donors by density-gradient centrifugation on a Histopaque-1077 (Sigma). PBMCs were cultured in Parker medium (Biomed) supplemented with 2 mM L-glutamine (Sigma), 0.1 mg/ml gentamycin (KRKA), β-mercaptoethanol (Sigma), 0.23% Hepes (Sigma), and 10% heat inactivated fetal bovine serum (FBS, Gibco).

The polysaccharide fractions from *L. edodes* (*Se-L*, *Se-Le-30*, I (*A*), II (*A/B-D*) and III (*B-D*)) were diluted in 0.9% NaCl (Fresenius Kabi) to achieve concentrations of 0.1 – 0.001 mg/ml. PBMCs were seeded into flat-bottom 96-well microplates at a density of 1×10^6 cells/well and stimulated with specific T- and B-cell mitogens: anti-CD3 mAb (OKT3, 1 µg/ml, BD Pharmingen, T-cell mitogen), phytohemagglutinin (PHA, 20 µg/ml, Sigma, T-cell mitogen), and a suspension of *Staphylococcus aureus* Cowan strain (SAC, 0.004% w/v, Calbiochem, B-cell mitogen). Polysaccharides were added to cell cultures in 100 µl aliquots of the prepared dilution per well. Control cultures contained an equivalent amount of 0.9% NaCl. As an internal control, an analogous test was carried out without any mitogens (autostimulation). PBMCs were cultured for 72 h at 37°C in a humidified atmosphere with 5% CO_2, pulsed with 1 μCi/well of [^{3}H]-thymidine (113 Ci/nmol, NEN) for 18 h, and harvested using a Skatron cell harvester. Thymidine incorporation into the DNA of proliferating cells was analyzed in a liquid-scintillation counter (Wallac Microbeta). Results were expressed as counts per minute (cpm). All experiments were performed in triplicate.

All participants provided written informed consent to participate in the study. The study was approved by the local ethics committee. The procedures were carried out in accordance with the Helsinki Declaration of 1975 as revised in 2000.

Statistical analysis

The Mann-Whitney U-test and Spearman correlation were applied using Statistica 9.0 (StatSoft Inc). Differences from control cultures were considered significant at $p<0.05$.

RESULTS

The composition of the polysaccharide fraction Se-Le-30

Selenium content

The selenium concentration in Se-enriched mycelium cultivated in medium enriched with 30 µg Se/ml was 1753 µg/g. The concentration of selenium in the *Se-Le-30* isolated using the Chihara method was 48 µg/g.

Monosaccharide composition

The monosaccharide composition of the Se-enriched fraction, *Se-Le-30,* was determined after complete hydrolysis using trifluoroacetic acid. As with the previously described *Se-L* fraction (Malinowska et al., 2018), mannose and glucose constituted 99.5% of total monosaccharides by weight. However, the mannose content in the *Se-Le-30* fraction was lower than for the previously described *Se-L* fraction (6% versus 14%).

Protein content

The total protein content in the *Se-Le-30* fraction was 3.3% when determined by the Bradford method, nearly 2-fold lower than in the *Se-L* fraction isolated by Ng and Yap method (8,4%) (Malinowska et al. 2018). The protein content in the *Se-Le-30* fraction, calculated on the basis of the elemental analysis and conversion of total nitrogen to true protein, was similar to the Bradford method (4.8% for *Se-Le-30* and 10.2% for *Se-L).* The use of conversion factors for total nitrogen to true protein is controversial, and considerable variation has been found in the conversion factors for different foods (Mariotti, Tomé, & Mirand, 2008). It was assumed that the isolated polysaccharide fraction was free of impurities containing nitrogen and applied a conversion factor of 6.25.

Structural analysis on separated polysaccharides of Se-L-30 crude extract.

The Se-containing polysaccharide crude extract (Se-L-30) was purified by ion-exchange chromatography on DEAE-Sephadex A-25 and finally by gel filtration according to Górska et al. (2016). Three fractions were obtained (I, II, III) and analyzed by chemical analysis (sugar, methylation, and determination of absolute configuration analysis) and NMR spectroscopy. The sugar and absolute configuration determination analysis showed that all fractions are composed of D-glucose residues with pyranose ring (D-Glcp). Methylation analysis of fraction I revealed the presence of 4-substituted glucopyranose (1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol-1-d). In fraction III were identified the 6-substituted glucopyranose (1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol-1-d), 3,6-disubstituted glucopyranose (1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-D-glucitol-1-d), 3-substituted glucopyranose (1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol-1-d) and terminal glucopyranose (1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol-1-d) in the molar ratios 2:0.4:1.8:1.8. In fraction II, which is a mixture of both fraction I and III, all of above mentioned monosaccharide derivatives were identified.

A complete structural analysis was performed by 2D NMR spectroscopy on all fractions (I-III). The 1H-1H COSY spectra allowed for identification of H-2 signals of the residues and subsequently 1H-1H TOCSY spectra with different mixing times and 1H-13C HSQC-DEPT spectrum allowed for the assignment of the H-3 to H-6, 6' signals for each residues of three fractions. The structural analysis of fraction II was described in details further in the description as it is a mixture of polysaccharides presented in both I and III fractions.

The HSQC-DEPT spectrum of fraction II contained signals for four anomeric protons and carbons, respectively (Fig.2A,B and Table 1).

Residue **A** (δ_H/δ_C 5.33/99.7 ppm, $^1J_{C\text{-}1,H\text{-}1}$ ~172 Hz) was recognized as the 4-substituted α-D-Glc*p* based on the characteristic proton spin system. The downfield shift value of C-4 (δ_C 76.7 ppm) indicated that this is a 4-substituted residue (Górska *et al.*, 2016; Mandal *et al.* 2012).

Residue **B** (δ_H/δ_C 4.48/102.6 ppm, $^1J_{C\text{-}1,H\text{-}1}$ ~165 Hz) was recognized as the 3-substituted β-D-Glc*p* based on basis of the large vicinal couplings between all protons in the sugar ring and the characteristic high chemical shifts of the C-3 at δ_C 84.5 ppm signals.

Residue **C** (δ_H/δ_C 4.46/103.0 ppm, $^1J_{C\text{-}1,H\text{-}1}$ ~163 Hz) and residue **C'** (δ_H/δ_C 4.63/103.0 ppm, $^1J_{C\text{-}1,H\text{-}1}$ ~163 Hz) were recognized as the →6-substituted β-D-Glc*p* based on the large vicinal couplings between all protons in the sugar ring and characteristic for substitution chemical shifts of C-6 signals at δ_H/δ_C 3.80, 4.15/68.8, and at δ_H/δ_C 3.77, 4.13/68.9 ppm, for **C** and **C'**, respectively. The significant difference in chemical shift values was observed only for anomeric

protons of **C** and **C'** residues at δ_H 4.46 and 4.63 ppm, respectively. The presence of two →6-β-D-Glc*p* residues in fraction II can be explained by fraction heterogeneity, the presence of mixture of three polysaccharide structures.

The monosaccharide sequences in fraction II was established using a ^{1}H-^{1}H NOESY and ^{1}H-^{13}C HMBC experiments. NOESY spectra showed strong inter-residue cross-peaks between the following transglycosidic protons: H-1 of **A**/H-4 of **A**, H1 of **B**/H3 of **B**, H-1 of **C**/H-6 of **C**, and H-1 of **C'**/H-3 of **B**. The HMBC experiment of fraction II confirmed substitution positions of the all monosaccharide residues with exception the glycosidic bound between **B** residues in β(1→3) glucan.

Table 1. ^{1}H and ^{13}C NMR chemical shifts of fraction II separated from Se-L-30 crude extract.

Sugar residue	Chemical shifts (ppm)					
	H1/ C1	H2/ C2	H3/ C3	H4/ C4	H5/ C5	H6, H6' C6
A	5.33	3.54	3.90	3.57	3.77	3.77, 3.80
→4)-α-D-Glc*p*-(1→	99.7	71.6	73.4	76.7	71.2	60.5
B	4.48	3.45	3.69	3.48	3.46	3.67, 3.85
→3)-β-D-Glc*p*-(1→	102.6	72.8	84.5	69.2	75.5	60.7
C	4.46	3.26	3.39	3.40	3.56	3.80, 4.15
→6)-β-D-Glc*p*-(1→	103.0	73.1	75.8	69.4	74.8	68.8
C'	4.63	3.31	3.44	3.40	3.56	3.77, 4.13
→6)-β-D-Glc*p*-(1→	103.0	73.3	75.4	69.5	74.8	68.9

Spectra were obtained for ^2H_2O solutions at 25°C, and acetone (δ_H 2.225, δ_C 31.05 ppm) was used as an internal reference.

Each of →6-β-D-Glc*p* (**C** and **C'**) residues belongs to different polysaccharide structures presented in fraction II. While the **C** residue builds a main β(1→6)-glucan, the **C'** residue substitutes **B** residue in position 3 in other polysaccharide – β(1→3) glucan, presented in fraction II. The amount of **C'** residue in comparison with **B** is significantly lower.

The fractions obtained after separation of crude extract of Se-Le-30 were successively: (I) fr□ction of homopolys□cch□ride α(1→4) gluc□n; (II) fr□ction with a mixture of shorter chains of α(1→4) glucan, β(1→6) glucan, β(1→3) glucan and also β(1→6)-β(1→3)-glucan; (III) fraction comprising a mixture of shorter chains of β(1→6) glucan, β(1→3) glucan and β(1→6)-β(1→3)-

glucan, respectively. Simultaneously, signals of 3,6-disubstituted β-D-Glcp (residue **E**) and β-D-Glcp (residue **D**) have been identified (Figure 2 C, D), however the sequence of this branched molecules was not determined. It has to be pointed out that in fraction III, the β(1→6) and β(1→3) glucan dominated. Residues **E** and **D** have been observed in fractions II, III in methylation analysis.

Homogeneity and molar mass determination of the lentinan analogue (Se-Le-30) and isolated polysaccharides

RI traces from SEC of fractions Se-Le-30, A (I), A/B-C (II), and B-C (III) are shown in Fig. 4. The respective RALS traces of these products are shown on Fig. 8.

Additional SEC traces from the RI, light scattering (LS), and viscosity detectors are provided in the Fig. 9. Bimodal signals were observed from the LS detectors for all analyzed samples. A bimodal signal for RI was observed only for the *Se-Le-30* fraction (Fig. 9A). For all analyzed samples, both LS signals were shifted to the lower values of retention volume (higher molar masses) in relation to the RI and viscometer signals. The signals measured from the four detectors for each product do not fully overlap, resulting in a broader distribution of the molar masses (Table 2).

Table 2.

SEC analysis (RI, RALS, LALS, and visc) of analyzed products

Sample	Ret. vol. [ml]	M_n	M_w	$Đ$	Remarks
Se-Le-30	18.58	1.69×10^6	3.62×10^6	2.15	Bimodal signal for all detectors
A/B-C	18.48	1.72×10^6	2.18×10^6	1.27	Bimodal signal for LS and visc detectors
A	18.49	1.82×10^6	2.25×10^6	1.24	Bimodal signal for LS detectors
B-C	20.05	5.06×10^4	1.10×10^5	2.18	Bimodal signal for LS detectors

The OmniSEC calculation was based on glucan standard (glu-245). $Đ$ (dispersity) represents M_w/M_n

The molar masses for the analyzed products were determined by OmniSEC for the polysaccharide fractions as follows: *Se-Le-30*: $M_n = 1.69 \times 10^6$ g/mol, $M_w = 3.62 \times 10^6$ g/mol, $Đ = 2.15$ (dispersity); *A/B-C*: $M_n = 1.72 \times 10^6$ g/mol, $M_w = 2.18 \times 10^6$ g/mol, $Đ = 1.27$; *A*: $M_n = 1.82 \times 10^6$ g/mol, $M_w = 2.25 \times 10^6$ g/mol, $Đ = 1.24$; and *B-C*: $M_n = 5.06 \times 10^4$ g/mol, $M_w = 1.10 \times 10^5$ g/mol, $Đ = 2.18$. Fraction *B-C* had a much lower molar mass. Broad traces were observed for RI and viscosity, as well as bimodal traces for LS detectors. The character of the SEC curves suggests that the *B-C* fraction does not have a uniform structure. It was assumed that it is a mixture of linear and branched polysaccharides. The behavior of the *B-C* fraction is in agreement with its molecular modeling.

The *A*, *A/B-C*, and *B-C* fractions were isolated and purified on chromatographic columns. This procedure however did not ensure the isolation of completely pure products without any impurities. A more detailed analysis of the RI traces for the *Se-Le-30, A,* and *A/B-C* fractions (after the deconvolution procedure; Figs. 10-12) allowed to determine the content of the remaining fraction, *B-C*. Product *A* is the main component of the *Se-Le-30* fraction, and product *B-C* (with much lower molar mass) is present in only small quantities. Thus, in the *Se-Le-30* fraction, the *A* and *B-C* content was equal to 77.86% and 2.86%, respectively (Fig. 10). In fraction *A/B-C,* a higher amount of *A* (85.35%) and *B-C* (4.23%) was observed (Fig. 11). Finally, deconvolution of the RI signal of product *A* allowed for determination of the residual content of the *B-C* fraction to be 2.73% (Fig. 12). The overall purity of fraction *A* was estimated and equal to 86.48%. These calculations should be considered with caution because of the great difference in the content of both products.

Deconvolution of the RI signal of product *B-C* allowed the overall purity of fraction *B-C* to be determined to be 85.03%. We were also able to estimate the content of three other products (peaks 1-3) with slightly higher molar masses: 2.03% (18.5 ml), 3.32% (18.8 ml), and 9.63% (19.2 ml), respectively (Fig. 5).

In addition, based on linear calibration to β-glucans, the following molar masses were calculated for the respective peaks: 96 500 g/mol (peak 1), 74 000 g/mol (peak 2), 56 700 g/mol (peak 3), 31 300 g/mol (fraction B-C).

Molecular modeling

The MM2 calculations showed that all components of polysaccharide fractions, *A* and *B-C*, tend to adopt wide helical conformations (Fig. 6). Polysaccharide *A* is a linear 1,4-α-D-glucan and expected to adopt an amylose-like structure (Fig. 6A). It was previously described deformation

of the helical structure by replacement of the oxygen atom in the glycosidic bond with Se (Malinowska et al., 2018). However, Se incorporation cannot affect the overall structure of the polysaccharide *A* chain because of the very low selenium content in the samples tested; the Se incorporation effect is only local. Being an amylose-like polysaccharide, *A* is expected to form left-handed helices with six glucose units per turn and stabilized by hydrogen bonds.

According to the MM2 calculations, the α-helix was also the most probable conformation in the 1-3-β-D-glucan, 1-6-β-D-glucan and 1-3-β-branched 1-6-β-D-glucan, components of the polysaccharide fraction *B-C* (Fig.6B-D). However, in contrast to 1-3-β-D-glucan, the helical structure of 1-6-β-D-glucan and 1-3-β-branched 1-6-β-D-glucan may be flexible.

One reason is an additional degree of freedom arising from the link ω (C_6-C_5 in 1-6-glucans) compared to 1-3-, 1-4-, and1-2-glucans, in which only φ (C_1-O) and ψ (O-C_3, O-C_4, O-C_2) linkages are present (Fig.6D). The second reason is a limited possibility of creating intramolecular hydrogen bonds (Fig. 6D).

To investigate whether the presence of 1-3-β branches affect the adopted conformation of polysaccharide *B-C*, the MM2 experiments for its 1-3-β unbranched counterpart were performed (Fig.6C-D). The results show similar conformations for both branched and unbranched polysaccharides.

Characteristics of polysaccharides fractions

The individual fractions were characterized as follows. Fraction A refers to an α-1,4-D-glucan characterized by the elution time expressed in milliliters at a flow rate of 0.5 ml/min: ion exchange chromatography: 6 – 24 ml and gel permeation chromatography: 62 – 74 ml. Fraction B-C refers to an 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan characterized by the elution time expressed in milliliters at a flow rate of 0.5 ml/min: ion exchange chromatography: 87 – 109,5 ml ml and gel permeation chromatography: 72 – 90 ml. Fraction A/B-C refers to an α-1,4-D-glucan, 1,3-β-D-glucan, 1,6-β-D-glucan and β-1,3-branched 1,6-β-D-glucan characterized by the elution time expressed in milliliters at a flow rate of 0.5 ml/min: ion exchange chromatography: 79,5 – 97,5 ml and gel permeation chromatography: 62 – 76 ml. (Calculated from the fractions: LC-1KP after DEAE A4B1 on HW C7D1, LC-1KP after DEAE D8E5 on HW C7D2, LC-1EM after DEAE D13E13 on HW C11D9).

Effects of polysaccharides on the proliferation of human peripheral blood mononuclear cells

The effect of the Se-Le-30 fraction (lentinan analogue) and fractions A and B-C on non-stimulated, OKT3-, PHA-, and SAC-stimulated PBMC proliferation is presented in Fig. 7. The results are compared to those previously obtained for the Se-L fraction (Malinowska et al., 2018).

As reported in Kaleta et al. for the *Se-L* fraction, autostimulation showed some tendency to decrease the proliferation of PMBCs. The opposite tendency was noted for the *Se-Le-30* fraction, but these effects were not significant (Fig. 7A). The *Se-L* fraction demonstrated some tendency to decrease the proliferation of PMBCs stimulated with SAC (Kaleta et al., 2019), but this effect was not observed for the *Se-Le-30* fraction isolated by the Chihara method and the purified polysaccharides *A* and *B-C* (Fig. 7D).

The OKT3-stimulated T-cell proliferation was significantly inhibited by all tested fractions (*Se-Le-30*, *A*, *B-C,* and *A/B-C*) at 100 µg/ml, but at a concentration of 10 µg/ml, this effect was shown only in the *Se-Le-30*, *B-C,* and, surprisingly, in *A/B-C* fractions. For the *Se-Le-30* fraction, the degree of inhibition at 100 and 10 µg/ml was approximately 82% and 48%, respectively. For polysaccharides *A* and *B-C,* the degree of inhibition at 100 µg/ml was approximately 82% and 83%, respectively.

However, the difference between the fractions was significant at 10 µg/ml; fraction *A* was practically inactive, whereas the degree of inhibition in fraction *B-C* was 47%. The relatively high standard dev iations in the *Se-L* and *Se-Le-30* fractions (Fig. 7) were due to the diverse response of the lymphocyte cultures from different donors to the mitogen. No significant inhibitory effect of polysaccharides *A* and *B-C* and mixture *A/B-C* on PHA-stimulated PBMCs was observed (Fig. 7C).

Unexpectedly, for fraction *A/B-C*, the impact on OKT3-stimulated PBMC proliferation was significantly stronger than for polysaccharides *A* and *B-C* separately (Fig. 7). Without being bound by theory, the stronger inhibition of proliferation by the *A/B-C* fraction as opposed to the *A* or *B-C* fractions alone may be due to synergism and/or the formation of active intermolecular structures. Therefore, an expert would not feel motivated to combine the non-inhibiting *A* fraction with the *B-C* fraction, obtaining a selective and most potent *A/B-C* fraction.

The cell viability assessment by trypan blue exclusion confirmed that the inhibitory effect on OKT3- and PHA-stimulated PBMC proliferation was not due to the toxicity of the examined polysaccharides.

Biological activity

The effects of the isolated Se-polysaccharide-protein fraction (*Se-L)* on the proliferation of human PBMCs were examined in publication Kaleta et al. Lymphocyte proliferation induced by OKT3 and PHA as a method to evaluate the T–cell proliferative response was used. The two mitogens use different mechanisms to promote T-cell effector functions; PHA stimulates T-cell proliferation via interactions with the *N*-acetylgalactosamine glycoprotein present on these cells (Lindahl-Kiessling & Peterson, 1969), whereas OKT3 stimulates T cells via CD3-mediated signaling (Van Wauwe, De Mey, & Goossens, 1980). The lymphocyte proliferation induced by SAC to evaluate B-cell responsiveness was also used.

Here, the same experiments for the *Se-Le-30*, *A (I)*, *B-C (III),* and *A/B-C (II)* fractions were conducted. The results clearly demonstrate that, for the immunosuppressive activity of the previously described *Se-L* fraction (Kaleta et al., 2019), polysaccharides *A* and *B-C* were primarily responsible. The selenium content also has a significant impact on the activity.

The suppression of T-lymphocyte proliferation by currently isolated fractions was weaker, but more selective than with the *Se-L* fraction (Fig. 7). In higher concentrations (100 µg/ml), there was no significant difference between the activity of the *Se-Le-30, A, B-C,* and *A/B-C* fractions. At lower concentrations (10 µg/ml), however, the differences become significant. Polysaccharide *B-C* is almost twice as active as polysaccharide *A*. Unexpectedly, the mixture of polysaccharides *A* and *B-C* is more active than the individual fractions, suggesting possible synergism or the formation of active intermolecular structures.

The effect of selenium on the polysaccharide activity is clearly visible, particularly at lower concentrations (10 µg/ml). A 4-fold decrease in the concentration of selenium in the *Se-Le-30* fraction *versus Se-L* correlated with an approximately 4-fold decrease in the inhibition of T-cell proliferation.

Selective immunosuppressive activity is not typical for polysaccharides of fungal origin. The probable mechanisms for this are not known and have not been described. For the previously isolated *Se-L* fraction (Malinowska et al., 2018), significant inhibition of the proliferation of the T lymphocytes was observed, most likely via CD3 receptor, but the suppression was also seen in PHA-stimulated PBMCs, though the effect was significantly weaker (Kaleta et al., 2019). In the autostimulation and SAC-stimulation settings, the *S-Le* fraction exhibited some tendency to decrease the proliferation of PMBCs, though it was not significant.

The suppression of T-cell proliferation for currently isolated fractions (*Se-Le-30, A, B-C*) in the OKT3 test, without an effect in the PHA test, suggests a mechanism *via* the CD3 receptors without interactions with the *N*-acetylgalactosamine glycoprotein. Therefore, it was concluded that impurities in the *Se-L* fraction that are absent from currently examined polysaccharides were responsible for the interaction with B lymphocytes and the *N*-acetylgalactosamine glycoprotein.

It was hypothesized, without being bound to any theory, that the completely different biological effect from lentinan depends at least in part, on the selenium incorporation into the polysaccharide molecule, but also on the dissimilar structure of the fractions isolated from mycelial cultures versus lentinan. Lentinan is 1-6-β-branched 1-3-β-D-glucan with a M_w of 500 KDa. Its helical structure is stabilized by strong internal hydrogen bonding, reducing polysaccharide hydration. The structure is inflexible and rigid (Burkus & Tamelli, 2005; Zhang et al., 2011). In contrast, the main component of the Se-enriched lentinan analogue amylose-like polysaccharide *A* is a linear 1-4-α-D-glucan. It also has a helical structure, but much more flexible and hydrophilic than lentinan (1,3-β-D-glucan) (Almond, 2005). This explains the good solubility and extractability of compound *A* (the polysaccharide with a $M_w > 2$ million Da) in hot water.

Polysaccharide fraction *B-C*, more active component of the *Se-Le-30* fraction (lentinan analogue), is a mixture of unbranched 1-6-β-D-glucan, unbranched 1-3-β-D-glucan, and 1-3-β-branched 1-6-β-D-glucan – all with probable helical conformation (Fig. 6). It was found that numerous branches formed by single β-D-glucose molecules in 1-3-β-branched 1-6-β-D-glucan do not affect the adopted conformation (Fig. 6C). However, the helical structure of two components of polysaccharide fraction *B-C* (unbranched 1-6-β-D-glucan and 1-3-β-branched 1-6-β-D-glucan) should be flexible and labile because of an extra degree of freedom in the link ω (C_6-C_5 in 1-6-glucans; Fig. 6D) compared to 1-3-, 1-4-, 1-2- glucans. The distances between some oxygen atoms (2.8-2.9 Å) suggest an opportunity for stabilization of the helical structure by hydrogen bonds (Fig.6D). Low polysaccharide *B-C* content in the *Se-Le-30* fraction suggests low content in the *L. edodes* cell wall and/or low extractability with hot water. The latter is very possible, as two papers have described the isolation of analogous 1-3-β-branched 1-6-β-D-glucan from mushroom cell walls using hot basic solution (Samuelsen et al., 2019; Li et al., 2019).

Elements of the polysaccharide structure determine their biological activity, and the activity-structure relationship is well known for 1,3-β-D-glucans. For other groups of fungal polysaccharides, this knowledge is poor (Zhang, Cui, Cheung, & Wang, 2007).

Notably, the results do not indicate a lack of 1-3-β-D-glucans in the cell wall of Se-enriched mycelium from *L. edodes*. These compounds are likely characterized by worse hot water solubility than 1-3-β-D glucans isolated by Chihara (1970).

The mushroom cell wall is a dynamic structure that is continuously evolving in response to environmental conditions (Latge, 2010). The culture method, conditions, and medium composition may affect the structure of cell wall polysaccharides. The results of the current research show that even a well-known and widely described species of fungus in submerged culture in a specific liquid medium may biosynthesize a polysaccharide with a completely different structure and biological activity then described thus far.

The modulation of T cell activity may be useful in allogeneic organ transplantation, as well as in a range of autoimmune diseases, especially the ones with known aberrant activity of T cells, such as rheumatoid arthritis, multiple sclerosis, celiac disease, glomerulonephritis, inflammatory bowel disease, psoriasis etc., and also in T cell-dependent allergy, such as contact dermatitis etc.

Moreover, the results of the present study show that no uniformity or predictability is found in the structural features or functional characteristics of bioactive polysaccharides; the biosynthetic pathways, the structure of the polysaccharides obtained using the same mushroom species, and the bioactivity mechanisms are highly variable and confusing to researchers. In addition, biotechnological methods of cultivating higher fungi offer an advantage, in many aspects, over the methods of cultivating crops. The mycelial cultures in bioreactors are carried out under repeatable conditions, resulting in a stable composition of the grown biomass. This facilitates the standardization of the preparations derived from fungi, such as for pharmaceutical use.

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