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DEVELOPMENT OF NEW METHODS FOR ISOLATION OF MUSHROOM BETA-GLUCANS FOR THE USE IN THE FOOD INDUSTRY AND THEIR COMPARATIVE EVALUATION

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Abstract

Mushroom beta-glucans are well-known for their immune-modulating activity. Oyster mushroom - *Pleurotus ostreatus* is one of the most largely produced industrial species, at the same time one of the most well studied for its medicinal properties. Beta-glucans from oyster mushroom are widely used in various food supplements. Traditional methods of extraction of these compounds include a routine multi-stage extraction using sequential ethanol and aqueous extraction. Therefore, a novel process for extraction of beta-1, 3/1, 6- D-glucan from *Pleurotus ostreatus* mushroom fruit bodies, has been investigated using microwave technology.

Microwave extraction method (with a frequency of 2450 MHz) and its influence on the yield of beta-glucans and their immune-modulating activity were studied. Concentration of beta-glucans in obtained extracts was determined using enzymatic method. Immune-modulating activity was determined by detection of the production of active forms of oxygen by human blood neutrophil cells using luminal-dependent chemiluminescence method.

The yield of beta-glucans obtained by microwave extraction was much higher comparing with traditional. Immune-modulating activity of microwave extracts was also higher. In addition microwave extraction allows to decrease the number of required stages comparing to the traditional scheme and significantly to reduce the time and energy consumption.

Therefore, it is likely that the microwave method could be used to extract beta-glucan for food, and pharmaceutical industries.

Key words: *Beta-glucans, Extraction, Microwave, Immune-modulation*.

1. Introduction

Beta-glucans are a family of polysaccharides of D-glucose monomers, connected by beta-glycoside bonds and differing in molecular weight, density, and three-dimensional structure. The biological activity of glucans is multi-vector and depends on many factors, primarily on the type and configuration of bonds between the constituent sugar residues, the degree of branching of the side chains of biopolymers, the molecular weight of polysaccharides, and solubility in water. The most biologically active form of beta-glucans is beta-1,3 / 1,6-glucan, in the molecule of which glucose is attached to positions 1 and 3, and the molecule also has branches in positions 1 and 6.

Beta glucans are large molecules that do not undergo enzymatic fragmentation in the gastrointestinal tract. They are captured by intestinal mucosa cells and actively transferred to the submucosal layer where macrophages are activated, and through them the lymphocytes responsible for protecting the endothelium, that is, for local immunity (Seijelid *et al.,* [1], Young *et al.,* [2]). Thanks to the repopulation mechanism, activated lymphocytes from the intestinal mucosa are disseminated into the mucous membranes of various organs, thus ensuring their protection against infections.

The mechanism of action of beta-1,3 / 1,6-glucan in general can be explained by its pronounced selectivity for specific receptors (dectin-1, complement 3,

lactosylceramide, etc.) existing on the surface of macrophages binding only to the unbranched portion of the beta-glucan, and resulting in the activation of macrophages, which leads to the implementation of the trigger mechanisms of a number of processes aimed at the immune defense of the body (Thornton *et al.,* [3], Brown and Gordon, [4], Brown *et al.,* [5]). On the one hand, the phagocytic function of macrophages is activated, on the other hand, such substances as cytokines (interleukins, interferon), which are a signal for other cells of the immune system, such as T-lymphocytes, growth factor of epidermal cells, angiogenesis factor (Okazaki *et al.,* [6], Williams, [7]).

Part of beta-glucans with blood flow through the portal vein enters the liver, where they are captured by Kupffer cells, which in response to interaction with polysaccharides secrete cytokines that activate systemic immunity. In particular, beta-1,3 / 1,6-glucan stimulates the production of tumor necrosis factor, which, in turn, activates the monocytic system of immunity (Sandula *et al.,* [8]).

Thus, beta-glucans activate both local immunity, providing protection of the body against invasions of antigens, and systemic immunity, which leads to the destruction of foreign genetic material that has already penetrated the body and restore immune homeostasis. It should be emphasized a distinctive feature of the immunomodulatory effects of beta-1,3/1,6-glucan, which consists in an adequate increase in the activity of the immune system without its excessive stimulation, which often causes autoimmune diseases.

Rational drug correction of the functional activity of the immune system is a necessary measure for many diseases and pathological conditions of the body. The most appropriate and pathogenetically justified is the use of agents that activate the primary component of the immune system - macrophages. Macrophage activators can be substances of various chemical structures and origins, such as endotoxins, viruses, and bacteria. However, their use is not always highly effective and safe regarding the complications of therapy, and compounds of beta-1,3/1,6-glucan and beta-1,3 (D) -glucan classes are, on the contrary, safe, including toxicological (class generally recognized as safe (GRAS) according to the classification Food and Drug Administration (FDA), USA) and they can be used both enterally and parenterally. This pharmacokinetic feature of beta-glucans and determines their widespread use in medical practice.

Thus, the study of Dőll *et al.,* [9], demonstrated good tolerability of beta-glucan preparations for oral administration, which was accompanied by a pronounced increase in the concentration of immunoglobulin A.

A pronounced immunomodulating activity of beta-1,3/1,6-glucan was shown in a clinical study on the possibility of preventing infectious diseases, sepsis, and pneumonia in patients with severe multiple injuries. (Lehne *et al.,* [10]).

In addition to pronounced immunomodulatory effects in terms of both specific and nonspecific immunity, beta-glucans have antioxidant properties, which was confirmed in experimental conditions regarding the reduction of ischemic and, most importantly, reperfusion injury. In addition, it should be noted that the class of polysaccharides has: anticancer (prevention of the onset and dissemination of tumors), anti-inflammatory and antiallergic, hypocholesterolic and hypolipidemic activities (de Felippe *et al.,* [11], Babineau *et al.,* [12], Meira et *al.,* [13], Shamtsyan, [14]). Of the local effects of beta-glucans, their ability to stimulate regeneration processes by activating keratinocytes and fibroblasts deserves special attention (Sugiyama *et al.,* [15], Woo et *al.,* [16]).

Analysis and generalization of the pharmacodynamic effects of beta-glucans identified in experimental and clinical conditions, allow us to recommend them for use as a means of preventing and treating recurrent bacterial, viral, fungal, parasitic infections in conditions of primary and secondary immunodeficiency of various etiologies, as well as in allergic diseases (allergic rhinitis, bronchial asthma, atopic eczema).

Beta-glucans have great potential for use as food additives and for the development of functional foods (Shamtsyan, [17], Antontseva *et al.,* [18], Frioui *et al.,* [19]). Thus, it is currently shown that beta-1,3/1,6-glucans are multi-vector modulators of biological reactivity of the body with significant immunomodulatory potential, allowing the use of drugs based on this polysaccharide for the treatment and prevention of many diseases and pathological conditions.

Usually, the isolation of beta-1,3/1,6-glucans is a multistage process, which includes many hours of treatment by boiling in 80% ethanol solution and subsequent extraction with water (Mizuno, [20]). We studied the possibility of simplifying and increasing the efficiency of the procedure for isolating beta glucans.

2. Materials and Methods

Oyster mushroom fruit bodies were purchased from the local market in St. Petersburg. After cutting into pieces, the fruit bodies were dried in a stream of air at 70 ^{0}C and powdered. Dry powder of mushroom fruit bodies was used for extraction of beta-glucans.

Traditionally to extract water-soluble beta-glucans from fruit bodies, fruit body power is initially boiled in 80% solution of ethanol (3 times for 3 hrs. each time) and then beta glucans are extracted from the residue by boiling it in the water (3 times for 3 hrs. each time).

We studied the yield and activities of the obtained extracts after triple, twice and single ethanol treatment and water extraction procedures.

Also, the influence of microwave extraction process on the yield and activity of beta-glucans was studied. Microwave oven LG Model MB4049F/01 at maximum power 800W with a frequency of 2450 MHz was used for extraction. Mushroom powder was pretreated by boiling in 80% solution of ethanol for 3 hrs. and then water-soluble fraction was extracted by its treatment in microwave oven for 30 minutes. Water soluble fraction was extracted by microwave treatment for 30 minutes from mushroom fruit body powder without any pretreatment with ethanol solution.

Obtained water extracts were separated from residue by filtration, then concentrated, and high molecular compounds, including beta-glucans, were precipitated by addition of 5 volumes of 96% ethanol and incubation of the mixture at the chilling temperature (4 ^{0}C).

Precipitates were filtered and dried at 70 ^{0}C in air flow and powdered. The weights of obtained powdered preparations was evaluated gravimetrically. In the obtained powder beta-glucan content was determined by enzymatic method (Megazyme International, 2013) [21], and immune-modulating activity was detected studying the influence of the preparations on the production of oxygen active forms by phagocyting cells.

The formation of active oxygen by whole blood cells was detected with luminol dependent chemiluminescence method (LDCL) 8. The detection of LDCL was carried out in 96-hole white non-transparent microplates (Costar) with the use of the device Victor-2 (Finland) connected with PC which provided sequent registration of chemiluminescence. The reaction result was termed as the light sum (sum of impulse accumulated during the certain time). The base luminol solution (10^{-2} M) was prepared in dimethyl sulfoxide DMSO and stored at 4 ^{0}C. The working solution (10^{-4} M) was prepared fresh diluting the base solution with Hanks solution (pH 7.2).

The experiment was carried out by the following scheme: 120 mL of Hanks solution, 20 mL of fresh donor blood, 40 mL of the working luminol solution, and 20 mL of the studied preparation in the appropriate dilution were placed in each hole of the 96 well microplates. Each test was carried out in 3 parallels. The reaction was fixed in 1 h. Results were expressed as index of stimulation compared to the level of chemiluminescence of the fresh blood (without addition of mushroom preparation).

3. Results and Discussion

Influence of different number of repetitions of ethanol pretreatment and aqueous extraction procedures on the yield of water-soluble fraction, its content of beta glucans and immune-modulating activity was studied. Results are represented in the Table 1.

Obtained results are demonstrating, that decrease of the number of ethanol pretreatment procedures from 3 to 1 is not significantly decreasing the yield of the water-soluble extract and has no influence on concentration of beta glucans and their activity in the extracts.

Number of aqueous extractions has influence on the yield of the extracts. While there is not much difference between the yields of the extracts after triple or twice water extractions, in case of only one extraction procedure the yield of the extract was significantly lower.

Water extraction without ethanol pretreatment resulted in the lowest yield of the extract and its lowest activity. This is probably, because extraction of beta-glucans from mushroom cell walls, without their pretreatment with ethanol is more complicated. Ethanol is dissolving lipids and low-molecular compounds, making cell-walls more permeable.

Table 1. Influence of ethanol and water extraction regimes on the yield and activity of beta-glucans

Preparation procedure	Yield from 100 g of mushroom fruit body powder	Concentration of beta-glucans in the preparation, %	Stimulation Index
Ethanol treatment (3 times) + water extraction (3 times)	6.8	23.5	1.38
Ethanol treatment (2 times) + water extraction (3 times)	6.7	24.1	1.40
Ethanol treatment (1 time) + water extraction (3 times)	6.5	22.3	1.34
Ethanol treatment (1 time) + water extraction (2 times)	6.2	23.8	1.33
Ethanol treatment (1 time) + water extraction (1 time)	5.1	21.2	1.35
Water extraction (1 time)	3.8	7.9	1.0

Table 2. Yield of beta-glucans from oyster mushroom powderized fruit bodies using microvae treatment

Preparation procedure	Yield from 100 g of mushroom fruit body powder	Concentration of beta-glucans in the preparation, %	Stimulation Index
Microwave treatment	10.5	31.2	1.59
Ethanol treatment (once for 3 hrs.) + Microwave treatment	8.2	40.5	1.96

We also studied the possibilities of intensifying the process of isolation of beta glucans from mushroom fruit bodies using microwave methods. Results are demonstrated in the Table 2.

It appears, that microwave treatment provides higher efficiency in extraction of beta-glucans from mushroom powder. This could be due to partial destruction of cell-walls by microwaves. The yield of the extract powder obtained by Ethanol + microwave treatment was lower, than when only microwave treatment was used. Probably, some low molecular compounds were removed with ethanol, and thus concentration of beta-glucans in the final extracts and stimulation index were higher. In case of using of microwave technologies the concentration of beta glucans in the obtained preparation, and thus, activity of such type of preparations was significantly higher.

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4. Conclusions

- Using of microwave technology to extract beta-glucans from mushrooms has significant advantages.

- Developed technology could be less complicated and more efficient, as in relation to the amount of extracted beta-glucans, as regarding the activity of the preparations.

5. References

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