



# Chitosan of Peppery Milky Cap Fungi (*Lactarius Pergamenus* (Fr.) Fr): Isolation, Study of Physico-Chemical Properties and Biological Activity

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**Abstract:** Chitosan is widely used in biology and medicine. Usually, it is isolated from chitin of crustaceans, while chitosan of the basidiomycetes fungi is poorly studied. The purpose of this study was: 1) to develop a method for isolation of chitosan from dried pomace of the peppery milky cap (*Lactarius pergamenus*); 2) to study of physico-chemical and biological properties of the isolated chitosan. As traditionally, chitosan was obtained by the alkaline hydrolysis of chitin. The determination of its molecular mass was performed using the viscometric method and further analyzed by disk electrophoresis (pH 5.0). The anti-microbial and cytotoxic activities were measured spectrophotometrically as a conversion of MTT dye to formazan, while the antifungal activity was evaluated using by counting colony-forming units (CFU). Chitosan of *L. pergamenus* fungi was shown to have lower molecular mass and a lower degree of deacetylation compared to shrimp chitosan. A yield of chitosan from a pomace of *L. pergamenus* was 6.27%. A heterogeneous product was obtained with an average molecular mass of 72 kDa and a degree of deacetylation equal - 87.1%. In 10 mg/ml dose, it inhibited by 29% the growth of gram-positive of *Staphylococcus aureus* (ATCC25923) bacteria and inhibited growth of gram-negative *Echerichia coli* dH5a - by 86% and *Pseudomonas aeruginosa* (ATCC9027) bacteria by 55%. Approximately 50 and 90 % of *Candida albicans* (pat.) cells were killed at the action of chitosan in doses of 0.025 mg/mL and 0.1 mg/mL correspondingly. It should be noted that this chitosan preparation did not affect a growth of human embryonic kidney pseudonormal cells of HEK 293 line and human breast carcinoma cells of MCF7 line.

**Keywords.** *Lactarius pergamenus*, chitin, chitosan, purification, physico-chemical properties, antimicrobial activity.

## 1. Introduction

Chitin, 2-acetamino-2-deoxy- $\beta$ (1 $\rightarrow$ 4)-D-glucan, is one of the structural biopolymers in fungal cell walls. Chitosan is commercially produced by deacetylation of chitin from crustacean shells, but the technology requires strong alkali, high temperatures, and a long processing time. It is considered, that fungal chitin demand extraction process is simpler and requires less harsh solvents. Fungal chitin and chitosan potentially differ from those isolated from crustaceans in molecular weight, degree of acetylation (DA), and distribution of charged groups. These differences can alter their functional properties and bioactivity. Numerous studies were conducted in which the antifungal action of chitin and chitosan were described [1-3]. They demonstrated that chitosan shows a much stronger inhibitory effect towards growth of pathological fungi than the chitin [1,2].

Chitin and product of its alkaline hydrolysis - chitosan, were obtained from different sources and intensively studied regarding their toxicity, biocompatibility, biodegradability, and the expression of their hemostatic and antimicrobial properties [4-7]. Besides, chitosan is of interest in dentistry and orthopedics [8,9] where it is used for creation of composites for targeted delivery of drugs and prolongation of their action [10].

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Earlier, we conducted a series of studies devoted to investigation of chemical composition of fungi of the *Lactarius* genus [11-15]. It was found that *Lactarius pergamenus*, *L. quietus* and *L. volemus* contain substances capable of protecting the fruiting bodies from damaging and, furthermore, may be of interest for medicine. It was shown that some substances present in cell wall of the studied mushrooms also play a protective role, and chitin is of the most interesting among them. But the isolation and studied of chitin and chitosan from these fungi is not described in the literature.

In this work, a method for the isolation of chitin and chitosan from dried pomace of *L. pergamenus* is described. The method developed by us allows to use fungal material in a complex, to receive chitin and chitosan after extraction of other valuable biologically active substances. This method also was used for isolation of chitin and chitosan from other fungi. Besides, the results of studying of their physical, chemical and biological characteristics are presented.

## 2. Materials and methods

Fruit bodies of the *L. pergamenus* and other mushrooms used in this work (lurid bolete - *Boletus luridus* Fr; suede bolete - *Xerocomus subtomentosus* (Fr.) Quel; tree oyster mushroom - *Pleurotus ostreatus* (Fr.) Kumm; pheasant's back mushroom - *Polyporus squamosus* Huds.: Fr.) were harvested during their mass growing in a mixed forest in Skole district of Lviv region. The mushrooms were delivered in no more than after 6 h to the laboratory for further processing.

A part of the collected fruit bodies of the fungi was dried in a drying cabinet at  $52 \pm 3^\circ\text{C}$  and used for isolation of chitin and chitosan, while the another part, in particular *L. pergamenus*, was crushed on a meat grinder and pressed for juice preparation. The juice obtained was used for preparing lectin [16], and after the pomace was dried and extracted with the methanol. The methanol extract was fractionated with the organic solvents and subjected to chromatography on a silica gel column. As a result of such treatment different fractions containing sesquiterpenes, phthalates, and stearic acid were obtained [11-15]. After treatment with methanol, the extracts were re-dried and used for preparation of chitin and chitosan.

All reagents and standards used were purchased from Sigma-Aldrich (Sigma-Aldrich GMBH, Munich, Germany). All reagents used were of analytical quality.

*Purification of the "raw chitin"*. The purification of chitin from the fruit bodies of fungi and pomace of *L. pergamenus* was carried out according to a procedure described earlier for isolation of chitin from tree oyster mushroom [17]. The obtained product was called as "raw chitin" or "alkali-insoluble chitin material" (AICM) due to a presence of big quantity of impurities.

Dried fruit bodies of *L. pergamenus* fungus were powdered in the electromixer and sieved (pore size of 0.5 mm). The obtained material was placed in a glass bowl filled with 0.1 N NaOH solution in 1:10 ratio of raw material (extracting solution). A suspension was stirred for 4 h, by mechanical stirrer at room temperature, the extract was removed, and the precipitate was washed with a distilled water on a Buchner funnel until the neutral reaction ( $\text{pH } 7.0 - 7.6$ ) of the washing water. Then the enriched chitin material was treated with a 0.6 M solution of the hydrochloric acid with a continuous stirring for 4 h at room temperature for elimination of mineral substances. An insoluble material was washed with a distilled water in a Buchner funnel for performing neutralization of the reaction mixture ( $\text{pH } 7.0 - 7.6$ ). The obtained material was washed with 95% ethanol and the AICM was dried in a drying cabinet at  $60^\circ\text{C}$ .

In order to obtain chitosan, we used AICM obtained from mushrooms, as well as shrimp chitin purchased from "Sigma" Chem. Co., USA. These were put in 50% NaOH solution at 1:10 ratio and placed for 60 min on a boiling water bath. After that, the chitin powder from shrimp and AICM was washed with a distilled water until the washing water was discolored. The obtained chitosan preparation was extracted for 30 min with a 2% solution of the acetic acid using an intense mixing. Treatment of this material with 2% acetic acid solution was repeated, and the supernatants of both extractions were combined. The obtained chitosan was precipitated by 0.1 N NaOH solution and adjusting  $\text{pH}$  to 9.5 -

10.0. Chitosan precipitate was washed with a distilled water, 96 % ethanol, acetone, diethyl ether, and then dried at room temperature.

Chitosan of “Tyanshi” company (China) was bought by at a local pharmacy and purified from the filling substances by re-precipitation.

To measure of chitin content in the AICM, an exact mass of a well-shredded AICM isolated from the fungal fruit bodies (*Lactarius pergamenus*, *Boletus luridus* and *Pleurotus ostreatus*) and from shrimp chitin (“Sigma” Chem. Co., USA) obtained as described below were used for comparison after dissolving in 50.0 mL of 36% HCl with stirring 1 h in a wet ice bath. The supernatant was separated from the precipitate by centrifugation (3,000g, 10 min). Chitin was pelleted through adjusting a solution in wet ice bath to pH 10.0 without allowing a solution to warm above 5°C. The pellet was washed several times with a distilled water, 95% with ethanol, acetone, diethyl ether, and then dried in a drying cabinet at 60°C.

The molecular mass of the resulting products was determined by the viscometric method, as described [18]. The viscosity was measured at 25°C with a viscosimeter Ubbelohd of VPZh-4 type (VPZh-4, Soyuznauchpribor, USSR) with 0,82 mm diameter of the capillary. Measurement of the viscosity of chitosan solutions was carried out in a mixture of 0.17 M acetic acid: 0.2 M NaCl (1: 2).

A degree of deacetylation of chitosan samples was determined by a titration using the following procedure: 50.0 mg of chitosan obtained from various fungal sources (*Lactarius pergamenus*, *Polyporus squamosus*, *Pleurotus ostreatus*), “Tianshi” chitosan and shrimp chitosan obtained from chitin of “Sigma”, were dissolved in 0.02 N HCl. Samples were gently dissolved with a constant stirring of solution for 2-4 h. The resulting solutions were titrated with 0.1 N NaOH using phenolphthalein as a pH indicator.

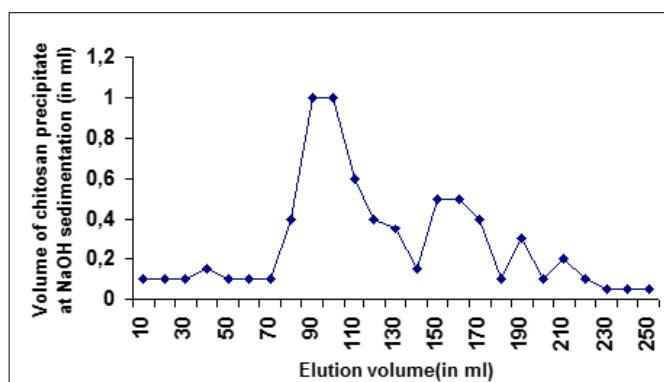
A degree of deacetylation (CA) was calculated using formula:

$$CA = \frac{2032 \times 100}{42.0 + \frac{1000 \times M}{C_{NaOH} \times V_{NaOH}}}$$

where:

M - mass loss in grams,  $C_{NaOH}$  - molar concentration of alkali (in M),  $V_{NaOH}$  - volume of alkali used for titration (in ml).

Separation of the sample of *L. pergamenus* chitosan was conducted by gel chromatography on a column of Acrylex P-60. Chitosan sample (120 mg) was loaded on a column of Acrylex P-60 (h = 56 cm, d = 4.0 cm), pre-washed with 0.1 M acetate buffer, pH 6.0. After entering of chitosan solution into the gel, the column was washed with 0.1 M acetate buffer, pH 6.0 and 10.5 mL fractions were collected in the centrifuge tubes. To detect chitosan in the selected fractions, 0.5 mL of 20% NaOH was added to each fraction. The tubes were centrifuged, and volume of the formed precipitate was determined.



**Figure 1.** The graph of elution of *Lactarius pergamenus* chitosan on a column filled with Acrylex P-60

Fraction number 1 (0 - 70 mL); No. 2 (71-130 mL); No. 3 (131-170 mL); No. 4 (171-210 mL); No. 5 (211-260 mL)



A plot of the dependence of volume of sediment formed upon the volume of elution was done, and the united fractions (No. 1 - 5) were used for further analysis by disk-electrophoresis.

Electrophoretic study of chitosan was carried out in a plate of the polyacrylamide gel in the acidic buffer system ( $\beta$ -alanine-acetic acid, pH 5.0), as described by Audy and Asselin [19] with a modification of Lootsik et al. [20]. A polyacrylamide gel with a gradual gradient concentration - 5-20% acrylamide was used, that improves a resolution of chitosan samples. Gel staining was carried out with 0.1% solution of the Coomassie G-250 dye (SERVA Electrophoresis GmbH, Heidelberg Germany).

Chitosan samples isolated by gel chromatography on a P-60 Acrylex column, were subjected to disk electrophoresis in glass tubes in 10% of PAAG.

### Study of cytotoxicity, antimicrobial and antifungal activity of *L. pergamenus* chitosan

The antimicrobial activity was determined using *Staphylococcus aureus* ATCC25923, *Escherichia coli* dh5a and *Pseudomonas aeruginosa* ATCC9027 as the microbial targets (see Grela et. al. [21]). This method of evaluation of the antimicrobial activity can be used for both water-soluble and insoluble substances. At studying the chitin and chitosan samples, 10 mg of chitosan powder were placed in a test tube, and 0.1 mL of a bacterial suspension were added. Preliminary, the bacteria suspension was sown for 24 h in a test tube with peptone-glucose medium, in order to achieve an absorbance value in the range of 0.3-0.6 at 590 nm. The incubation of tested bacteria with chitin and chitosan samples was carried out for 4 h, followed by adding the MTT [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide] dye in 10  $\mu$ L (5 mg / mL) per sample, and incubation for 1 h at 37°C. After incubation, samples were centrifuged at 3,000 rpm for 10 min. A supernatant of each sample was collected and added to 1 mL of the dimethylsulfoxide (DMSO, REALAB, Ukraine), after which it was incubated for 1 h at 37°C for complete dissolution of the formazan. Then the absorption of the formazan solution was determined quantitatively on a spectrophotometer UV102 at a wavelength of 570 nm. A viability of the microorganisms was evaluated according to their ability of transforming the MTT dye into the formazan of the purple color.

A cytotoxic activity of chitosan towards the mammalian cells was determined by using the MTT test. Human embryonic kidney cells of HEK 293 line and human breast carcinoma cells of MCF7 line were obtained from Cell culture collection of R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine. Cells were cultured in the Dulbecco's modified Eagle's medium (DMEM, Sigma Chem. Co, St. Louis, USA) supplemented with 10% fetal bovine serum (Sigma), 50  $\mu$ g/mL streptomycin ("Sigma") and phenol red used as pH indicator.

Substances under study was added in 100  $\mu$ L of cell suspension. After 24 h incubation, 10  $\mu$ L of the MTT reagent was introduced into the well and kept in the CO<sub>2</sub>-incubator for 2 h. Then 100  $\mu$ L of the DMSO was added to each well for dissolving the formazan crystals, and colored solution was measured at a wavelength of 490 nm on a universal automatic microphotometer BioTek ELx800 (BioTek, Instruments, USA).

Antifungal activity was determined using a method of the colony-forming units (ECO). Tested substances were incubated for 4 h in a suspension of yeast (approximately 1 million cells), that's as diluted 10,000 times, yeast were sown in 200  $\mu$ L per Petri dish, and the number of colonies was counted after 24 h incubation.

In order to calculate the inhibition degree, the control was taken for 100 %, from it the calculated of number living cells after the action of the substance was deduced. The result obtained from division of this numbers was taken as a percentage of the inhibition (Figures 4, 5, 6).

### Statistical analysis

Each experiment was performed in triplicate and average values were recorded. The data were evaluated statistically using Student's t-test, and a value of  $p \leq 0.05$  was considered to be statistically reliable.



### 3. Results and discussions

#### 3.1. Purification of chitin and chitosan

A juice of fruiting body of the peppery milky cap fungi was used to obtain the lectin [16], then it was dried at 55°C and extracted with the methanol.  $9.7 \pm 1.2$  g of extracted dry material was obtained from 100.0 g of fresh fruit bodies. The methanol extraction of dry raw material gave  $22.3 \pm 1.5\%$  of the extracted material that was used for preparation of substance effective in treatment of foot mycoses [22]. Thus, from 100.0 g of fresh fruit bodies, 7.5 g of dry pomace that were devoid of water- and methanol-soluble substances were obtained. In mushrooms, unlike insects and arthropods, chitin is only a part of material present in cell walls. Different methods used for detection of chitin, gave the non-uniform results - from 3 to 20% [23]. Besides, various species of mushrooms differ in the amount of chitin present in their fruit bodies. For example, a mycelium of the basidiomycota fungi contains 8.5% (*Agaricus bisporus*) of chitin, while *Auricularia auricula-judae* - 19.6% [4]. The main difficulty at measuring the amount of chitin in mushrooms is caused by its insolubility in water and the organic solvents.

It is known [4] that chitin of crabs and shrimp is dissolved in concentrated mineral acids, however, strong acids hydrolyze the chitin. Chitin present in acid solution can be sedimented by its alkalization. We used a concentrated hydrochloric acid, sulfuric acid and nitric acid for dissolving of chitin. It was found that 36% hydrochloric acid was the best, however, the process should be carried out in the wet ice bath.

Optimal result of dissolving chitin of the mushroom material with 36 % HCl was obtained by using a continuous mixing of the AICM fungi ( $d < 1$  mm) in 1:40 ratio for 2 h with a subsequent precipitation of acid-soluble material with alkali. Neutralization of acid-soluble material should be carried out gently and slowly with adding ice to a solution. Chitin precipitate was released from present pigments by washing twice with water, then with alcohol, acetone and the diethyl ether. The results of chitin isolation from different sources are presented in Table 1. Thus, chitin content in the fruiting bodies of the studied mushrooms ranges from 3.48 to 8.16%.

A dissolution of shrimp chitin from "Sigma" in concentrated hydrochloric acid was carried out in parallel with a dissolution of chitin of the studied fungi in order to establish a degree of its hydrolysis with strong acid. After precipitation of 500 mg of shrimp chitin with alkaline solution of the hydrochloric acid, 471.5 mg of chitin was obtained, that is 94.3%. Under these conditions, a degree of chitin hydrolysis did not exceed 6%.

**Table 1.** The yield of chitin isolation (in % to mass of AICM)

Number	Chitin source	Mass used to dissolve in 36% HCl (mg)	Chitin after alkaline precipitation (mg)	% chitin in AICM mass
1	Dried fruiting bodies of <i>Lactarius pergamenus</i>	42,009 mg	1930 mg	4.6
2	Dry pomace of fruiting bodies of <i>Lactarius pergamenus</i>	2,504 mg	224 mg	8.96
3	Dried fruiting bodies of <i>Boletus luridus</i>	27,070 mg	947 mg	3.48
4	Dried fruiting bodies of <i>Xerocomus subtomentosus</i>	2,512 mg	205 mg	8.16
5	Dried fruiting bodies of <i>Pleurotus ostreatus</i>	20,000 mg	876 mg	4.38

A procedure described above can be also used for isolation of chitosan from chitin, however, due to a big quantity of highly concentrated of strong acid and alkali it is not appropriate to use it. Thus, it is easier and cheaper to obtain chitosan by a direct alkaline hydrolysis of the AICM of fungi fruiting bodies, as described in the "Materials and methods" section. The results of such procedure are presented in the Table 2.

**Table 2.** The yield of chitosan isolation (% of mass of the AICM)

Number	Chitosan source	Mass of AICM (g)	Mass of the obtained chitosan (g)	% of chitosan in the mass of AICM
1	Dried fruit bodies of <i>Lactarius pergamenus</i>	42.01	1.51	3.59
2	Dry pomace of fruit bodies of <i>Lactarius pergamenus</i>	10.0	0.627	6.27
3	Dried fruit bodies <i>Boletus luridus</i>	34.80	0.825	2.37
4	Dried fruit bodies <i>Pleurotus ostreatus</i>	20.03	0.661	3.30
5	Dried fruit bodies <i>Polyporus squamosus</i>	34.8	0.821	2.36

In the reaction of chitin hydrolysis, theoretically, it is possible to obtain  $\approx 81\%$  chitosan, however, we received a smaller amount that is 68,1% for *B. luridus* and 78,0% for *L. pergamenus* (Table 1 and 2).

### 3.2. Physico-chemical properties

Measurement of the viscosity of chitosan solutions was carried out in a mixture of 0.17 M acetic acid: 0.2 M NaCl (1: 2) in studies used for determining its average molecular mass. In control, solutions of the polyethylene glycol of different concentration with a molecular mass of 40 kDa, were used taking into account an assumption that constants of Schwadinger equation are similar for both substances – chitosan and polyethylene glycol (Table 3).

**Table 3.** The molecular mass of chitosan of different fungi species determined by means of the viscometry method

Number	Chitosan source	Mol. mass (kDa)
1.	Chitosan of <i>Lactarius pergamenus</i> fruit bodies	72 $\pm$ 3
2	Chitosan of <i>Polyporus squamosus</i> fruit bodies	36 $\pm$ 3
3	Chitosan of <i>Pleurotus ostreatus</i> fruit bodies	97 $\pm$ 3
4	Chitosan purchased from “Tyanshi” company	249 $\pm$ 5
5	Chitosan obtained from shrimp chitin	625 $\pm$ 10

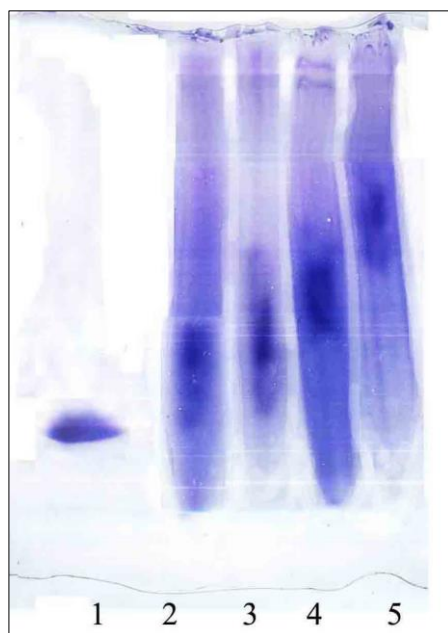
Thus, chitosans of the basidiomycota fungi species differ significantly in their molecular mass. It was noted that the chitosans of studied fungi have a significantly lower molecular mass comparing to the chitosan isolated from shrimp.

The next step in our studies was a determination of a degree of deacetylation of chitosan obtained from fruit bodies of *Lactarius pergamenus* and other fungi species (Table 4).

**Table 4.** A degree of deacetylation of different chitosan samples

Number	Chitosan source	Degree of deacetylation (in %)
1	Chitosan of <i>Lactarius pergamenus</i> fruit bodies	87.1
2	Chitosan of <i>Polyporus squamosus</i> fruit bodies	74.7
3	Chitosan of <i>Pleurotus ostreatus</i> fruit bodies	78.5
4	Chitosan purchased from the “Tyanshi” company	92.1

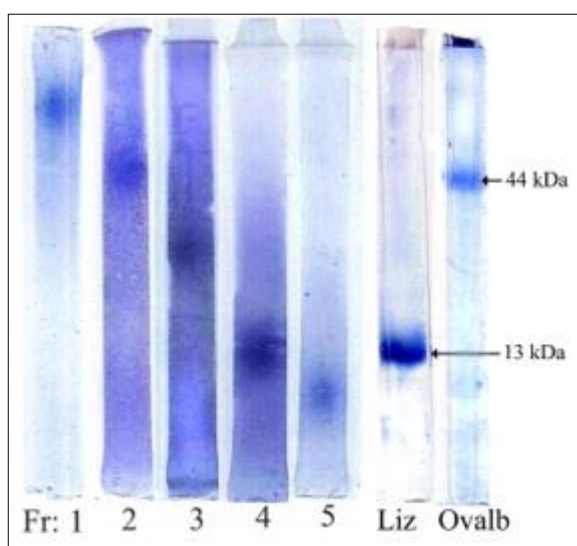
Figure 2 presents the results of disk-electrophoresis of chitosan samples performed on a plate of the polyacrylamide gel with 5-20% gradient of PAA concentration in the acidic buffer system at the absence of sodium dodecyl sulfate.



**Figure 2.** The results of disk electrophoresis of chitosans isolated from various sources in 5-20% gradient PAAG in the acidic buffer system, pH 5.0. 1 - Lysozyme from chicken egg white (Merck KGaA, Darmstadt, Germany); 2 - chitosan of *Lactarius pergamenus* fruit bodies; 3 - chitosan of *Polyporus squamosus*; 4 - chitosan isolated from shrimp; 5 - Chitosan purchased from “Tyanshi”

The results of the performed electrophoretic study suggest a significant heterogeneity of the obtained chitosan samples. They migrate very stretched that suggests a heterogeneity of their molecular mass and a charge of the molecule.

We used gel chromatography for isolation of chitosan fractions of the *Lactarius pergamenus* fungi. A series of sorbents (Sephadex G-75, Sephacryl S-200, Acrylexes P-200 and P-60) were tested and Acrylex P-60 was found to be the best for at purpose, although full separation of chitosan fractions could not be achieved. This was evidenced by the results of disc-electrophoresis in 10% PAAG in the acid buffer system (Figure 3). The presented results of disk-electrophoresis demonstrate a satisfactory separation of fractions. They also show that these fractions demonstrate molecules of similar type whose charge/molecular mass ratio is the same. Thus, at disk-electrophoresis, it is possible to obtain a distribution of molecules in fractions depending on their molecular mass.

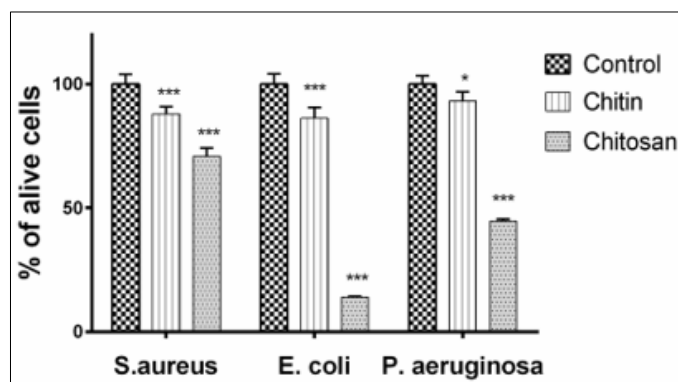


**Figure 3.** Disk electrophoresis of chitosan fractions of *Lactarius pergamenus* separated in tubes of 10% PAAG using the acidic buffer system (pH 5.0). Liz – egg lysozyme (mol. mass – 13.6 kDa) Ovalb - egg albumin (mol. mass – 44 kDa) The numbers indicate the combined fractions.

### 3.3. Biological activity of *L. pergamenus* chitin and chitosan

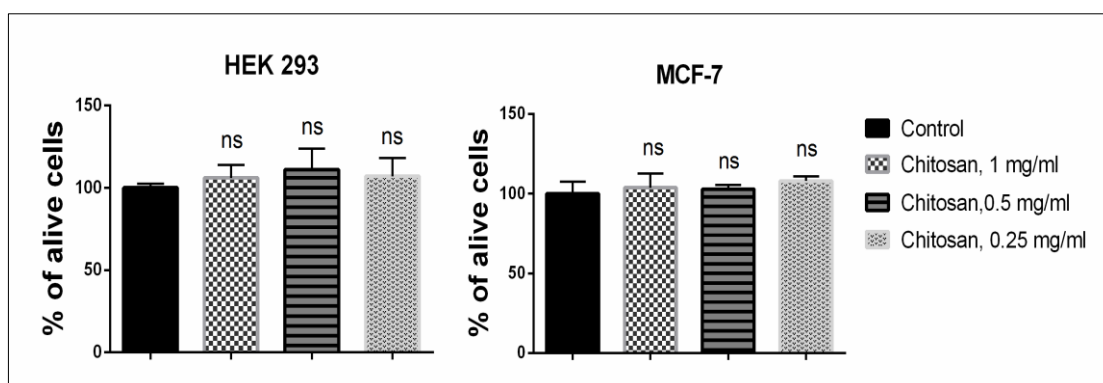
It is known that chitin and chitosan of the arthropods (shrimps and crabs) [24] possess several properties of the biological value, for example, the antibacterial one. The antimicrobial activity of chitosan originating from true mushrooms is poorly studied. It was found that, chitosan isolated from

shiitake mushrooms possessed an antimicrobial activity comparable with that of crab's chitosan [25]. Properties of chitins and chitosans isolated from different sources might depend upon their molecular mass, a degree of deacetylation, and presence of the biologically active impurities [20, 26]. We have studied the antibacterial action of chitin and chitosan isolated from the fruit bodies of *L. pergamenus* towards *Staphylococcus aureus* ATCC25923, *Echerichia coli* dH5a and *Pseudomonas aeruginosa* ATCC9027 bacteria (Figure 4). It was found that chitosan in 10 mg/mL dose suppressed by 29% a growth of gram-positive *S. aureus* (ATCC25923) bacteria, by 86% - of gram-negative *E. coli* dH5a, and by 55% - of *P. aeruginosa* (ATCC9027) bacteria. At the same time, chitin did not exhibit an antimicrobial activity (Figure 4). That is why we excluded chitin from the next studies.



**Figure 4.** The level of antibacterial activity of chitin and chitosan, isolated from *Lactarius pergamenus* towards *S. aureus*, *E. coli* and *P. aeruginosa* bacteria. \*  $p < 0.05$ ; \*\*\*  $p < 0.0005$

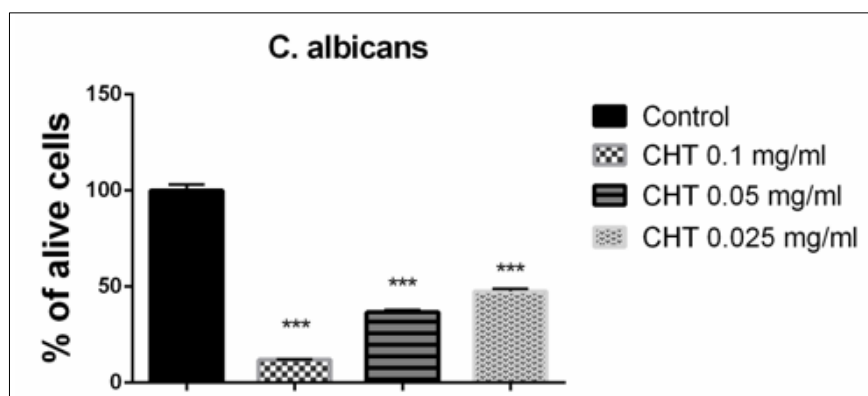
Chitosan of *L. pergamenus* did not show a cytotoxic effect toward pseudonormal human embryonic kidney cells of HEK 293 line and human breast carcinoma cells of MCF7 line (Figure 5).



**Figure 5.** The level of cytotoxic activity (MTT-test) of chitosan isolated from *Lactarius pergamenus* towards human embryonic kidney cells of HEK 293 line and human breast carcinoma cells of MCF7 line. ns – not significant.

Chitosan of *L. pergamenus* fungi also possessed a pronounced antifungal activity. In concentration of 0.025 mg/mL it killed 50% of *Candida albicans* (pat.) yeast cells, and in concentration of 0.1 mg/mL it killed  $\approx 90\%$  of these cells (Figure 6).





**Figure 6.** The level of the antifungal action of chitosan of *L. pergamenus* towards *C. albicans* yeast (CHT – chitosan of *L. pergamenus*)

\*  $p < 0.05$ ; \*\*\*  $p < 0.0005$

Thus, chitosan isolated from *L. pergamenus* fungi possesses the anti-fungal and antibacterial activity toward gram-negative bacteria, however, being non-toxic towards the mammalian (human) pseudonormal and tumor cells. These results suggest a perspectiveness of using chitosan of *L. pergamenus* fungi as an antimicrobial or antifungal agent. The biocompatibility and biodegradability of chitosan also suggests a possibility of its use as a carrier of the delivery of biologically active compounds in the organism.

The production of chitin and chitosan from fungal sources has gained increased attention in recent years due to potential advantages over the traditional source. For example, fungal mycelia have lower levels of inorganic materials compared to crustacean shells, and no demineralization treatment is required during processing [27]. Fungal mycelia and/or carpophores can be cultivated on a large scale and consequently chitin can be more easily isolated, avoiding aggressive treatment. By-products from *Agaricus bisporus*, *Ganoderma lucidum*, *Pleurotus ustreatus*, *Flammulina velutipes* can be proposed as an alternative source for chitin isolation [28,29]. However, the yield of crude chitin from mushrooms is lower. Thus, when purifying chitin from the mycelium of 7 species of basidiomycetes, the yield of raw chitin ranged between 8.5 and 19.6% of dry weight, and the yield of chitosan was approximately 1% [30].

Among various potential applications of chitin and chitosan in the food, agricultural, and pharmaceutical industries, the uses of these biopolymers as food antimicrobials and biopesticides are especially attractive. Chitosan showed strong antimicrobial activity on both Gram-negative and Gram-positive bacteria,

At the same time, we did not detect antibacterial or antifungal activity of *L. pergamenus* chitin. It is possible that a discovery of such activity in chitin by some researchers [31,32] is due to a presence of free amino groups, that most likely appears during of chitin isolation from raw material. The protocol used for chitin purification involves a processing of raw material with strong mineral acids and alkalis for removing the impurities. Such treatment might be accompanied by a certain degree of deacetylation. Chitin provided by some companies (for example, “Sigma” Chem. Co.) contains a small amount of amino groups and behaves like a weak cationite. As a proof for this is a non-specific sorbtion on columns where chitin is used as a sorbent at purifying chitinases and chitin-binding lectins. Therefore, at washing chitin (as sorbent) from ballast substances, a buffer solutions with an increased amount of salt is recommended [33,34]. A presense of amino groups is important for manifestation of the antimicrobial and antifungal properties of tested substances. However, there is no evidence that chitin present in the wall of fungi cells, as well as in the crustacean shells, has a certain a degree of their deacetylation. At the same time, chitosan has no toxic effects on mammals. Moreover, chitosan-fed rats significantly reduced the adverse effects of various chemical poisons [35].



## 4. Conclusions

1. A method for isolation of chitosan from the pomace of fruit bodies of *Lactarius pergamenus* by the alkaline hydrolysis has been developed. It allows obtaining chitosan with a yield of 6.27% of mass of dry pomace.

2. The molecular mass of *Lactarius pergamenus* chitosan was determined ( $72 \pm 3$  kDa) using a viscometric method. A degree of its deacetylation was shown to be 87.1%. Chitosans isolated from other species of the basidiomycetes fungi (*Lactarius pergamenus*, *Polyporus squamosus*, *Pleurotus ostreatus*) possessed a molecular mass in a range of 36-100 kDa, whereas chitosan produced by “Tyanshi” company -  $249 \pm 5$  kDa and shrimp chitosan -  $625 \pm 10$  kDa.

3. *Lactarius pergamenus* chitosan in 10 mg/ml concentration suppresses by 29% growth of gram-positive *S. aureus* bacteria, by 86% gram-negative - *E. coli*, and by 55% - *P. aeruginosa* bacteria.

4. Chitosan of *L. pergamenus* in concentration of 0.025 mg/mL kills  $\approx 50\%$  of *Candida albicans* cells, and in concentration of 0.1 mg/mL -  $\approx 90\%$  of these yeast cells.

5. Chitosan of *Lactarius pergamenus* fruit bodies does not demonstrate a toxicity towards pseudonormal and tumor human cells. Thus, perspectives of its use as an antimicrobial or antifungal agent, as well as a carrier of the biologically active compounds might be considered.

6. Chitin of *Lactarius pergamenus* fruit bodies does not possess any antimicrobial and antifungal activity.

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