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PHYSICOCHEMICAL AND CYTOTOXIC PROPERTIES OF *BACILLUS SUBTILIS* IMV B-7724 EXTRACELLULAR LECTIN

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Aim. *To study the chemical composition, sugar specificity and physicochemical properties of the extracellular lectin isolated from Bacillus subtilis IMV B-7724.* ***Methods.*** *Biochemical, spectrophotometric, immunological and cultural methods were used to assess the physicochemical and a number of biological properties of lectin isolated from the culture fluid of bacteria B. subtilis IMV B-7724. Molecular weight of the lectin was estimated in polyacrylamide gel electrophoresis. Analysis of the elemental composition was done using Perkin-Elmer 2400 CHNS analyzer. Temperature and pH stability of lectin were examined based on residual hemagglutination activity of the lectin. Cytotoxic activity was determined by the MTT-assay. The statistical analysis was made using Student's t-test.* ***Results.*** *B. subtilis IMV B-7724 lectin is a glycoprotein (protein – 86.0 %, carbohydrates – 7.0 %) with molecular weight of 18–20 kDa (major). Analysis of the elemental composition revealed that it contains 34.00 % of carbon, 7.04 % of hydrogen, 16.61 % of nitrogen, 42.35 % of oxygen. Amino acid composition analysis determined that it is rich in leucine, tyrosine and phenylalanine. The lectin exhibited high sugar-binding specificity toward N-acetylneuraminic and N-glycolylneuraminic acids (minimal inhibitory concentration – 0.3 mM for both sugars). The lectin is heat and acid stable, has long shelf life.* ***Conclusions.*** *These results provide the rationale to pursue further investigation for possible ways and modes of B. subtilis IMB B-7724 lectin application in clinical settings.*

Keywords: lectin, Bacillus subtilis IMV B-7724, physicochemical properties, sugar specificity, stability, cytotoxic activity.

Among the natural substances possessing anticancer activities the rapt attention is drawn to lectins. Lectins are proteins or glycopeptides possessing highly specific carbohydrate-binding activities. Specific binding with the carbohydrate receptors on the cell surface may activate intracellular reactions – apoptosis, autophagy or proliferation inhibition – leading to elimination of the cell [1–3]. This property of lectins can be applied in the development of new biotherapeutic drugs to improve the effectiveness of anticancer therapy. In recent decades, the number of investigations devoted to the application of lectins in cancer diagnosis and treatment is increasing. Yet, the majority of works deal with the antitumor properties of herbal lectins [4–7] at the same time bacterial lectins in regard of its anticancer activity and application in medical (oncological) practice are much more poorly investigated [8–10]. Although in terms of biotechnological production, the process of bacterial lectins extracting is much easier and can be standardized therefore is promising for large scale production.

Thus far, anticancer activity of the lectins produced by the members of the *Bacillus* genus: *B. polymyxa* 102, *B. subtilis* 316M, *B. subtilis* 7025 et al. have been investigated [11–13]. It was shown that the majority of these lectins are pH and thermostable glycoproteins not dependent on the metal ions with long shelf-life. Extracellular lectins of saprophytic bacilli strains possess rare carbohydrate specificity for sialic acids or their derivatives. Given that malignant cells can overexpress sialic acids on their cell surface, sialospecific lectins can be considered as possible antitumor agents.

It is important to note that the physicochemical and biological properties (including antitumor activity) of lectins are highly strain-dependent. Even one genus strains' lectins vary in quantitative and qualitative composition, differing significantly in terms of biological activity. Therefore, it is necessary to study in detail the properties of the extracted bacterial lectin.

In the previous experiments, from *B. subtilis* IMV B-7724 cultural fluid the substance possessing high hemagglutinating activity and therefore characterized as a lectin was extracted. The isolated lectin exhibited cytotoxic activity towards Ehrlich carcinoma cells *in vitro* [14]. The scope of this research was to explore chemical composition, carbohydrate specificity and physicochemical characteristics of the lectin isolated from *B. subtilis* IMV B-7724 cultural fluid.

Aim. To study the chemical composition, sugar specificity and physicochemical properties of the extracellular lectin isolated from *B. subtilis* IMV B-7724.

Materials and methods. Strain *B. subtilis* IMV B-7724 [15], spore forming, aerobic, gram-positive bacteria, was used as a source of the lectin. The lectin was isolated from *B. subtilis* IMV B-7724 cultural fluid on the 4th day of culturing as described in [16]. Isolated lectin was freeze dried at temperatures between −32 °C and +24 °C; the lectin was stored as a powder at −20 °C.

Molecular weight of the lectin was estimated in polyacrylamide gel electrophoresis [17]. Polyacrylamide gel electrophoresis in the presence of SDS was performed on 12 % gels in Tris-glycine buffer, pH 8.9. The proteins were stained with Coomassie Brilliant Blue R (Ferak, Germany). The molecular weight of the lectin was determined by comparing its electrophoresis mobility with the standard molecular weight marker proteins (Sigma, USA).

Absorbance of proteins and sugars was performed with NanoDrop™ 1000 Spectrophotometer (Thermo-Scientific, USA) at 280 nm [18] and 620 nm [19] respectively.

Determination of the carbon, hydrogen, nitrogen and oxygen content was done using Perkin-Elmer 2400 CHNS (Perkin-Elmer Inc., USA) analyzer according to the standard protocols.

Absorbance at UV spectrum was performed with NanoDrop™ 1000 Spectrophotometer (Thermo-Scientific, USA) at 280 nm. The measurements were carried out using a standard protocol Protein A280 according to the user manual. The spectra and absorption values obtained by measuring micro-volume are normalized to a 10.0 mm pathlength equivalent.

IR spectrum of the lectin were recorded in the range of 4000–400 cm^{-1} on a Perkin-Elmer FTIR System Spectrum BX spectrometer (Perkin-Elmer Inc., USA) using the pellets with KBr [20]. Data treatment was performed according to the databases [21].

The lectin was tested for the presence of organic contaminants by the use of qualitative tests for proteins (amido black 10B, coomassie brilliant blue G-250 dyes, Sigma-Aldrich, USA), mucopolysaccharides and nucleoproteins (toluidine blue), lipoproteins (Sudan black B), DNA (diphenylamine), RNA (methylene blue, Sigma-Aldrich, USA) [22].

Amino acid analysis of the lectin was performed on Amino Acid Analyzer T339 (Czech Republic) using method of hydrolyzation [23]. Samples were loaded into the Analyzer with standards (Sigma, USA). Optical density of the resulting products (which is directly proportional to concentration of the substance in the solution) was measured at 520 nm. Amino acid content per 100 mg of the lectin was calculated.

Sugar specificity of the lectin was studied in the sugar inhibition test. Briefly, serial dilutions of the sugar were mixed with the lectin prior to addition of erythrocytes [24]. The lowest concentration of the sugar giving full inhibition of agglutination was determined. The following sugars were used: D-glucose, D-glucosamine, D-galactosamine, lactose, D-glucuronic acid, fructose-1 6-diphosphate, N-acetylneuraminic and N-glycolylneuraminic acids. The assay was performed in U-bottomed microtiter plates using 2 % solution of rabbit erythrocytes.

Hemagglutination studies of the lectin were carried out using trypsinized and aldehyde-fixed 2 % solution of rabbit erythrocytes [25]. A serial two-fold dilution of the lectin solution in PBS (pH 7.2) was performed at room temperature in 96-well U-shaped microtiter plates. The hemagglutination activity of the lectin was assessed as the reciprocal of the highest dilution exhibiting visible agglutination of erythrocytes and was expressed as $titer^{-1}$ hemagglutinating activity. Identical 2 % solution of rabbit erythrocytes without addition of the lectin was used as a control of the auto-agglutination.

Cytotoxic activity of the lectin was tested in MTT-assay [26]. Ehrlich carcinoma cells were used as target cells. In brief, target cells (1×10^5 per well), in RPMI medium supplemented with 10 % fetal bovine serum (all reagents from Sigma, USA) and antibiotics, were placed in a flat-bottom 96-well plate and incubated for 24 h in 100 % humidity atmosphere with 5 % CO_2 at 37 °C. On the next day the lectin in concentration 0.1; 0.25; 0.5; 1.0; 1.5; 2.0 mg/ml was added to each except for the control wells and the plate was incubated for 1h. After that, 0.01 ml of MTT solution/well (5 mg/ml, Sigma, USA) was added, and incubation continued for 2 h. Then the plates were centrifuged (1500 g for 15 min) and medium was replaced with 0.12 ml of KOH (2 mole/liter) and 0.14 ml of DMSO (50 % solution). Optical density was measured at λ = 545 nm *vs* λ = 630 nm with a MicroELISA reader (StatFax-2100, USA). Each sample was measured in triplicate.

Cytotoxic Activity Index (CTAI, %) was calculated as follows:

$$CTAI = [1 - (OD_{tc+l} - OD_{tc})/OD_{tc}] \times 100\%,$$

where OD_{tc} – optical density of wells in which only tumor cells were incubated; OD_{tc+l} – optical density of wells in which target cells were incubated with the lectin.

To assess the stability during long-term storage, the cytotoxic activity of the lectin samples towards cancer cells was determined and the IC_{50} value was calculated (with the help of on-line program available on http://www.antimalarial-icestimator.net). The following concentrations of lectin were used in this series of studies: 0.1; 0.25; 0.5; 1.0; 1.5; 2.0 mg/ml. Measurements were performed every third month of the storage during the year.

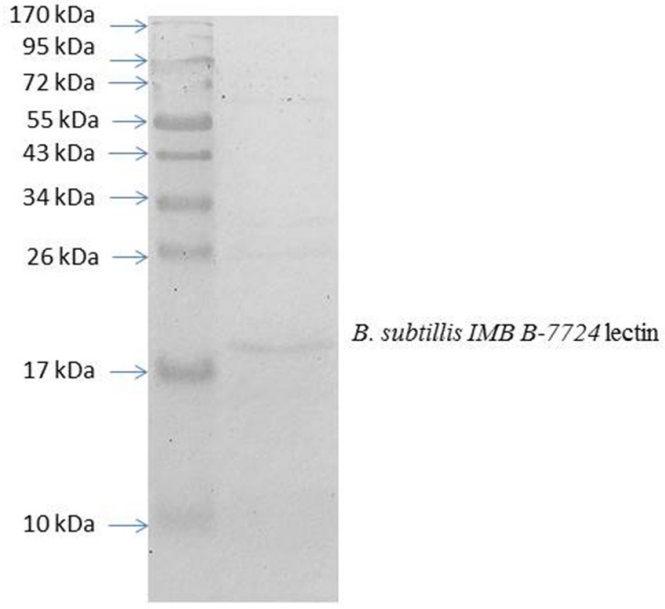
The pH stability was studied based on lectin's (in concentration 1 mg/mL) hemagglutinating and cytotoxic activity against Ehrlich carcinoma cells after it was dialyzed in buffers at different pH values (pH 6–9) for 24 h at room temperature [27, 28]. The following buffers were used: 20 mM acetate buffer (pH 6.0); 20 mM phosphate buffer (pH 6.5; 7.0); 20 mM M Tris-HCl buffer (pH 7.5; 8.0; 8.5; 9.0). The agglutination titer of lectin in PBS (pH 7.2) was used as control.

To investigate the thermal stability, the lectin (in concentration 1 mg/mL) was incubated at different temperatures of 20, 30, 40, 50, 60, 70, 80, and 90 °C for 60 min. After the samples were cooled to room temperature, their hemagglutinating and cytotoxic activity against Ehrlich carcinoma cells was determined [27, 28].

The statistical analysis was made using Student's t-test [29]. The difference was considered as significant when $p<0.05$.

Results. The lectin isolated from cultural fluid of *B. subtilis* IMV B-7724 can be stored as a brown colored powder. It is easy soluble in water, buffers (PBS, Tris-HCl), bases (20 % NaOH) and some organic solvents (65 % ethanol, 50 % acetone).

Non-reducing SDS electrophoretic analysis of the lectin (Fig. 1) showed three bands with approximate molecular weight of 18–20 (major band) kDa, 26 and 70–72 kDa (minor).



F i g. 1. SDS-polyacrylamide gel electrophoresis of the lectin isolated from cultural fluid of *B. subtilis* IMV B-7724

Study of the lectin chemical composition revealed that it is a glycoprotein with 86.0 % of protein and 7.0 % of sugars. The lectin contains 34.00 % of carbon, 7.04 % of hydrogen, 16.61% of nitrogen and 42.35 % of oxygen. Our results are analogous with data on the chemical composition of extracellular lectins of other saprophytic bacteria strains of the *Bacillus* genus. Thus, it was shown that the lectins of 5 different strains of *B. subtilis* contain 53.7–76.0 % of protein and 5.0–11.5 % of carbohydrates, in some samples small amount (<1.0 %) of nucleic acids was revealed [11]. As compared to the above mentioned data, isolated by us lectin has slightly higher protein content (86.0 %) and a standard amount of carbohydrates (7.0 %). Comparatively high carbohydrates content is characteristic of saprophytic bacteria lectins, contrary to pathogenic ones.

Based on the results of the qualitative tests, the isolated lectin was proved to be free of nonprotein impurities. Positive tests with amido black 10B and coomassie brilliant blue confirm the presence of protein constituent. Negative tests with Sudan black B, toluidine blue, diphenylamine and methylene blue indicate that such substances as mucopolysaccharides, nucleoproteins, lipoproteins, DNA and RNA are not detected in the solution of the isolated lectin.

Spectrometric methods, which are based on the relations between the atomic-molecular structure of the substances and the absorption of electromagnetic waves by them, provide an effective assessment and quality control of various biological objects [30, 31]. For proteins, carbohydrates and lipids absorbance at IR spectrum differs due to different vibrations of C–H, C=O, N–H, O–H, S–H chemical groups and their combinations [32]. It was shown (Fig. 2, A) that *B. subtilis* IMV B-7724 lectin's maximum IR absorbance was caused by vibration of atoms in bonds and functional groups characteristic of proteins and peptides: C–H (616 cm^{-1}); C–O (1067 cm^{-1}); C=C (1386 cm^{-1}); C–C (1530 cm^{-1}); C=O and –NH (1650 cm^{-1}); N–H (2957 cm^{-1}), carboxyl group –COOH (3409 $см^{-1}$). This finding proved ones more that the lectin solution contains no nonprotein impurities.

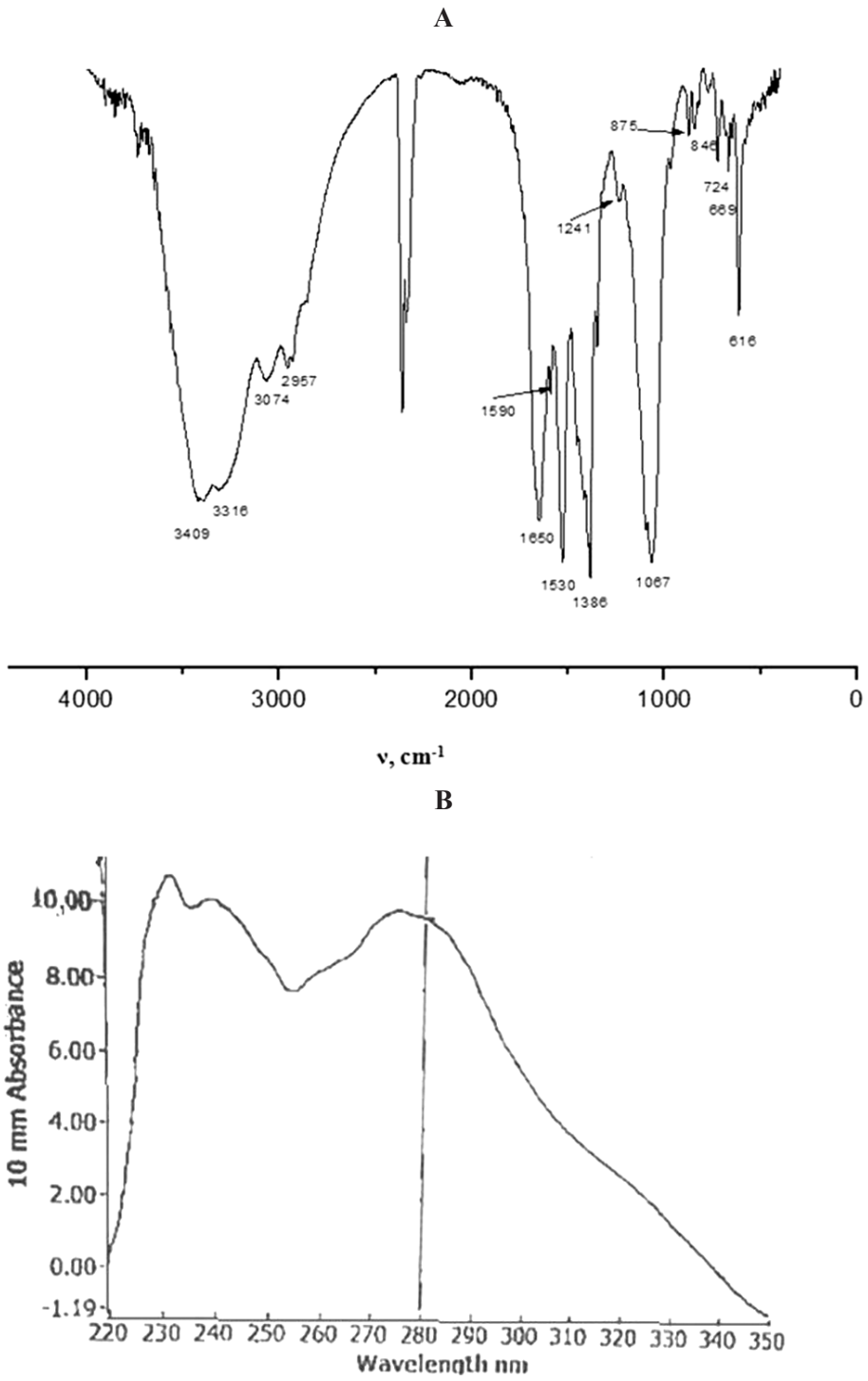
The lectin's UV absorption peaks were recorded at value of 230 and 280 nm (Fig. 2, B). UV absorption in the range of 180–230 nm is characteristic mainly for peptide bounds. Absorption in the range of 230–300 nm is dominated by the aromatic side-chains of tryptophan, tyrosine and phenylalanine residues. The wavelengths of maximum absorbance for these amino acids are 265–295, 265–285 and 245–270 nm respectively [33]. So, the solution of the isolated lectin contains molecules of a protein which is composed largely of aromatic amino acids.

The analysis of amino acid contents confirms findings revealed with the spectrophotometry. The lectin is composed of 15 amino acids among which leucine (15 %) and aromatic amino acids tyrosine (12 %) and phenylalanine (11 %) constitute the majority; isoleucine, alanine and valine made up 8–9 % (Fig. 3). The proportion of the last 9 amino acids varies between of 2–7 %. Of all the amino acid residues, neutral amino acids constituted about 60.6 %, aromatic – 22.5 %, basic – 11.0 % and acidic – 5.9 %.

One of any lectin main characteristics is its carbohydrate (or sugar) binding specificity. This property makes each lectin unique and is utilized in lectin classification. The higher sugar-binding specificity *B. subtilis* IMV B-7724 lectin exhibited toward 2 types of sialic acids (N-acetylneuraminic and N-glycolylneuraminic) and towards D-glucuronic acid and fructose-1 6-diphosphate (Table 1). Other carbohydrates (D-galactosamine, lactose, D-glucose and D-glucosamine) did not inhibit the agglutination reaction with rabbits' erythrocytes. This means that the isolated lectin, likewise the majority of lectins produced by different *B. subtilis* strains, is specific for sialic acid [11]. This finding substantiates the further investigation into anticancer activity of the isolated lectin, as long as sialic acids in large quantities are expressed on surface of cancer cells.

Resistance to the effects of different environmental factors and length of time that a biological agent may be stored without losing its activities are very important characteristic of any substance to be applied as a therapeutic agent. Thus, the study of thermal and pH stability of the isolated lectin was carried out.

Isolated lectin turned out to be temperature stable as long as heating (1 h, 20–90 °C) did not influenced nether hemagglutinating no cytotoxic activity of the lectin (Table 2). Neither did different pH values (pH 6.0–8.0) significantly affect the bioactivity of the lectin (Table 3). The maximum hemagglutinating activity (2048 $titer^{-1}$) was seen at pH 7.5–8.0; cytotoxic activity of the lectin was not affected significantly. So, these results revealed the high thermal and pH stability of *B. subtilis* IMV B-7724 lectin.

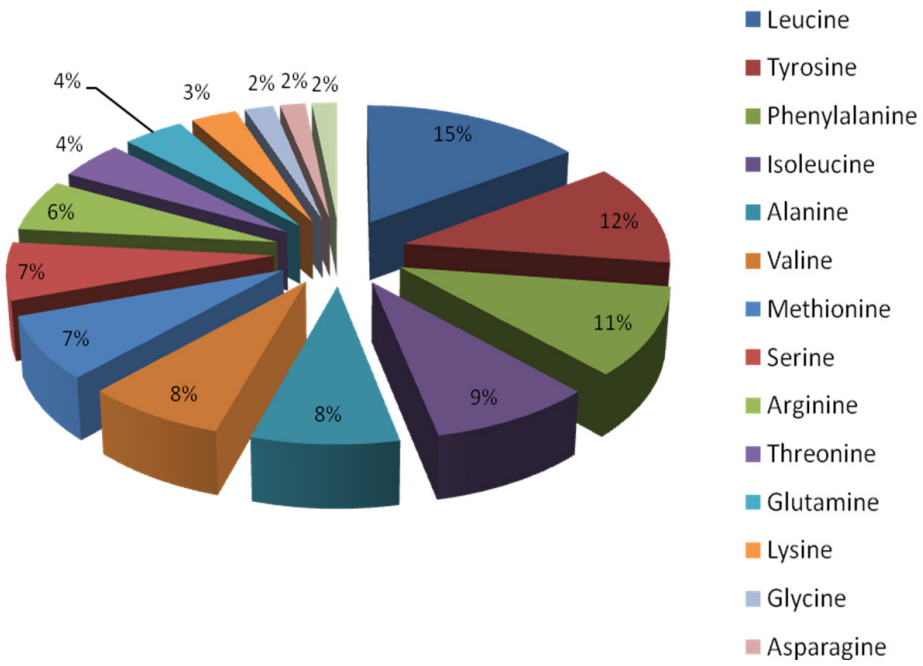


F i g. 2. Absorbance of *B. subtilis* IMV B-7724 lectin in infrared (A) and ultraviolet (B) spectrum

Table 1

Carbohydrate binding specificity of *B. subtilis* IMV B-7724 lectin

Carbohydrate	Minimal inhibitory concentration, mM
N-acetylneuraminic acid	0.3
N-glycolylneuraminic acid	0.3
D-glucuronic acid	0.9
Fructose-1 6-diphosphate	18.7



F i g. 3. Amino acid composition of *B. subtilis* IMV B-7724 lectin. Content of free amino acids (%) calculated per 100 mg of the lectin

Table 2

The effect of temperature on the hemagglutinating and cytotoxic activity of *B. subtilis* IMV B-7724 lectin

Temperature, ^{0}C	Bioactivity	
	Hemagglutinating, titer^{-1}	Cytotoxic, %
20	1024	97.4 ± 0.3
30	1024	97.6 ± 0.4
40	1024	97.5 ± 0.1
50	1024	97.5 ± 0.1
60	1024	96.9 ± 0.1
70	1024	96.8 ± 0.3
80	1024	95.8 ± 0.3
90	1024	95.9 ± 0.1

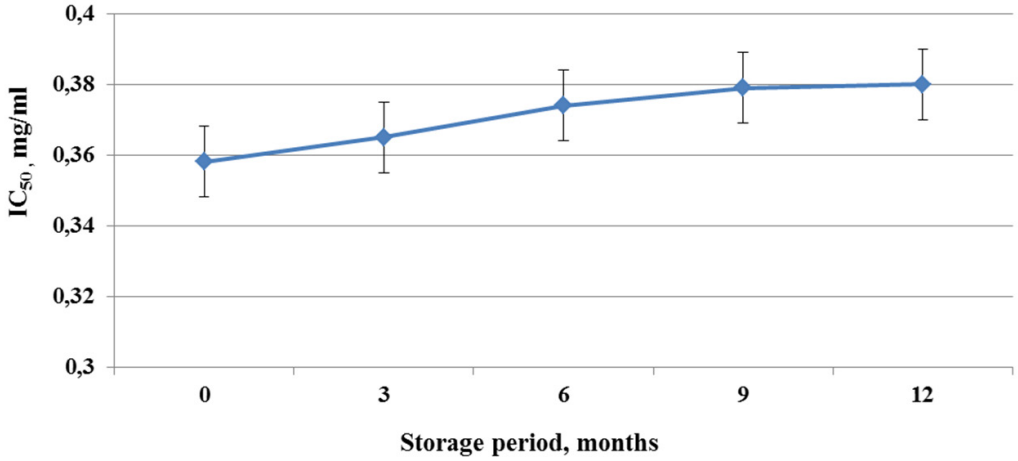
Table 3

The effect of pH on hemagglutinating and cytotoxic activity of *B. subtilis* IMV B-7724 lectin

pH	Bioactivity	
	Hemagglutinating, titer^{-1}	Cytotoxic, %
6.0	1024	96.2 ± 0.6
6.5	1024	96.4 ± 0.5
7.0	1024	96.5 ± 1.4
7.5	2048	97.0 ± 0.5
8.0	2048	96.8 ± 0.4
8.5	1024	95.9 ± 1.1
9.0	1024	95.0 ± 1.0

An important characteristic of any biological substance is an ability to maintain its bioactivity for a long time. To examined storage stability of the lectin (stored as lyophilized powder at −20 °C) it was tested for cytotoxic activity towards cancer cells every third month of the storage. Then the IC_{50} value (the amount a particular inhibitory substance needed to inhibit, *in vitro*, a tested biological process or biological component by 50 % [34, 35]) for each storage period was calculated and compared

to each other. Obtained results (Fig. 4) demonstrated that *B. subtilis* IMV B-7724 lectin can be stored at least for 12 months without losing its cytotoxic activity.



F i g. 4. Cytotoxic activity of *B. subtilis* IMV B-7724 lectin depending on storage period

Discussion. Summing up, it can be concluded that by its physicochemical characteristics the lectin isolated from *B. subtilis* IMV B-7724 culture fluid is similar to other lectins isolated from different strains of *B. subtilis* [11]. The isolated lectin is a glycoprotein. The electrophoresis of lectin revealed three bands corresponding to peptides with molecular weights of 18–20 kDa (major), 26 kDa and 70–72 kDa. As it was shown [13, 36], the presence of polypeptides with different molecular weights in the lectin solution is a characteristic of most bacterial lectins. In particular, similar results were obtained in the research devoted to lectin produced by *B. subtilis* IMV B-7025 – the most closely related strain. Its native molecule had a molecular weight of 26.5 kDa and consisted of 3 polypeptide chains (8.3 kDa). In the solution, native lectin molecules were present not only in the monomer form, but as a trimer with molecular weight of 72 kDa [13]. Similarly, lectins isolated from cultural fluids of *Lactobacillus* and *Bifidobacterium* formed major and several minor bands when separated in polyacrylamide denaturing gel electrophoresis [36]. Possibly this may be due to the simultaneous presence in the test samples of individual polypeptide chains, native molecules and their conglomerates.

The amino acid composition of *B. subtilis* IMV B-7724 lectin does not differ significantly from the currently known bacterial sialic lectins and is composed mainly of aromatic amino acids (tyrosine and phenylalanine), as well as leucine.

Carbohydrate specificity – the ability to selectively bind certain sugars – is one of the most important characteristics of any lectin. The isolated lectin of *B. subtilis* IMV B-7724 showed the highest affinity towards N-acetylneuraminic and N-glycolylneuraminic acids with minimal inhibitory concentration of 0.3 mM in both cases. This fact allows classifying the lectin as sialo-specific. Lectins of this group attract the attention of researchers elaborating new methods of cancer immunotherapy. Sialic acids are expressed on human cells and tissues and take part in physiological biological processes [37]. But in certain diseases qualitative and quantitative changes in these molecules appear. For instance, overexpression of sialic acids is characteristic of malignant cells. Lectins recognizing and binding carbohydrates expressed on cell surface leading so far to death of transformed cell can be considered as new promising means of anticancer therapy.

Summing up, the lectin isolated from cultural fluid of *B. subtilis* IMV B-7724 is a glycoprotein with 86.0 % of protein and 7.0 % of sugars and a native molecular weight of 18–20 kDa. The extraction technique allows isolating pure lectin free of nonprotein impurities. The lectin contains 34.00 % of carbon, 7.04 % of hydrogen, 16.61 % of nitrogen and 42.35 % of oxygen. The amino acid composition was characterized by a high content of leucine (15.0 %), tyrosine (12.0 %) and phenylalanine (11 %). The lectin exhibited the highest sugar-binding specificity towards N-acetylneuraminic and N-glycolylneuraminic acids with minimal inhibitory concentration of 0.3 mM in both cases. This suggests that the lectin can be classified as a specific for sialic acid. The isolated lectin is temperature and pH stable and has

a shelf-life not shorter than 12 months.

The physicochemical properties of the lectin isolated from *B. subtilis* IMV B-7724 cultural fluid highly resemble, thus, one's characteristics to other lectins produced by different strains of *B. subtilis.* On the other hands, easy was the lectin isolation and purification in addition to its sialic acid specificity and cytotoxic activity towards cancer cells, its temperature and pH stability and long shelf-life make the lectin very promising object to be applied in biomedical researches especially in oncology.

Conclusions. The further investigation into lectin's cytotoxic activity toward different types of transformed and normal cells as long as into the mechanisms underlining this cytotoxic activity is highly warranted.

ФІЗИКО-ХІМІЧНІ ТА ЦИТОТОКСИЧНІ ВЛАСТИВОСТІ ПОЗАКЛІТИННОГО ЛЕКТИНУ *BACILLUS SUBTILIS* IMB B-7724

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Р е з ю м е

Мета. Дослідити хімічний склад, вуглеводну специфічність та фізико-хімічні властивості позаклітинного лектину *Bacillus subtilis* IMB B-7724. **Методи.** Для оцінки фізико-хімічних та ряду біологічних властивостей лектину, виділеного з культуральної рідини бактерій штаму *B. subtilis* IMB B-7724, використані біохімічні, спектрофотометричні, імунологічні, культуральні методи. Молекулярну масу лектину визначали методом електрофорезу в поліакриламідному гелі. Аналіз елементного складу проводили з використанням аналізатору Perkin-Elmer 2400 CHNS. Термостабільність лектину та стійкість до зміни pH досліджували за результатами гемаглютинуючої активності. Цитотоксичну активність зразків лектину оцінювали в МТТ-тесті. Статистичну обробку результатів проводили з використанням t-критерію Ст'юдента. **Результати.** Отриманий бактеріальний лектин є глікопротеїном (білок – 86,0 %, вуглеводи – 7,0 %) з молекулярною масою 18–20 кДа. Елементний склад: вуглець – 34,00 %, водень – 7,04 %, азот – 16,61 %, кисень – 42,35 %. В амінокислотному складі превалюють лейцин та ароматичні амінокислоти (тирозин і фенілаланін). Найвищу спорідненість лектин *B. subtillis* IMB B-7724 виявляв до N-ацетилнейрамінової та N-гліколілнейрамінової кислот (мінімальні інгібуючі концентрації вуглеводів – 0,3 мМ). Лектин має високу термостабільність, стійкий до зміни pH середовища, стабільний при зберіганні протягом тривалого часу. **Висновки.** Одержані результати можуть скласти підґрунтя для подальшого дослідження напрямків і методів використання лектину *B. subtilis* IMB B-7724 в клінічних умовах.

Ключові слова: лектин, *Bacillus subtilis* IMB B-7724, фізико-хімічні властивості, вуглеводна специфічність, стабільність, цитотоксична активність.

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