Structure and Functions of Biopolymers

CHARACTERIZATION OF DEFENSIN-LIKE PROTEINS FROM SCOTS PINE SEEDLINGS

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Two defensin-like proteins with molecular weight 11.3 and 9.8 kDa were isolated from 7-day-old Scots pine seedlings (Pinus sylvestris L.) using ion exchange chromatography. The quality and the activity of purified preparations were analysed by gel electrophoresis and growth-inhibitory assays. We found that both defensin-like proteins inhibited the growth of mycelium of the phytopathogenic fungus Fusarium oxysporum at concentration <1 mkg/ml, and caused morphological changes of hyphae.

Keywords: *Scots pine, defensins, inhibition of growth*

Introduction. All living organisms, from bacteria to plants and then to mammals, have developed defense mechanisms from unfavorable environmental abiotic and biotic factors in the process of evolutionary development. Among the most dangerous biotic factors, the first place is given to microorganisms, which surround the plants throughout the whole process of ontogenetic development and often are causative agents of infectious diseases. The synthesis of antimicrobial proteins (AMP) in response to pathogenic action of microorganisms is a survival mechanism developed during the evolution by many classes of living organisms [1]. The family of plant AMP is very diverse and includes в-gluconase, chitinases, ribosome inactivating proteins, inhibitors of serine and cysteine proteases, lipid-transporting proteins, etc [2]. Plant defensins which obtained its name due to their structural and functional

similarities to defensins from mammals and insects, are members of AMP family [3].

Plant defensins are low-molecular weight and cysteine-rich proteins. In addition, most defensins are positively charged due to high percentage of arginines and lysines in their amino acid sequences. The tertiary structure of these proteins is formed by б-helixes and three anti-parallel в-layers, which are connected by disulfide bridges. Anti-fungal and anti-bacterial activities are inherent to defensins [4, 5]. The expression of these compounds is constitutive, but could be strongly induced in response to pathogens or stress factors [6].

Previous studies on the anti-fungal activity of defensins revealed significant changes in membrane potential of fungal cell membranes and the amplification of Ca^{2+} consumption and K^{+} output [7]. It was also shown that plant defensins interact with specific lipid components of fungal plasma membrane. Furthermore, defensin RsAFP2 from *Raphanus sativus* was fond in complex with glycosylceramides, while defensin DmAMP1 from *Dihlia merskii* accumulated in membrane microdomains – rafts. Internalization of defensins and their regulatory interaction with intracellular components has been recently proposed as a potential mechanism for anti-fungal activity of these proteins [8].

First plant defensins were isolated from seeds, where they comprise up to 0.5% of total protein content. Nowadays, defensins from vegetative and generative organs of metasperms have been purified and characterized. It has been found that in these organs defensins are localised on the extracellular surface of cells, suggesting their involvement in the first line of defense in the most vulnerable to pathogen tissues [6]. Recently, genes encoding defensine-like proteins, were identified in genomes of gymnosperms of *Picea* and *Ginkgo* species and their expression in the roots of spruce seedlings was shown [9]. Using affinity purification approach, we have recently isolate and identified by mass spectrometry defensin from the extracts of *Pinus sylvestris* seedlings [10]. The main goal of current study was the isolation and functional characterization of the pool of defensin-like proteins from *Pinus sylvestris* seedlings. Using ion exchange chromatography, two defensin-like proteins were isolated nearly to homogeneity and their anti-fungal activities characterized.

Materials and methods. The extraction of heat-stable proteins from *Pinus sylvestris* seedlings. In this study we used viable seeds of *P.sylvestris*, obtained from Bug State Forestry, L'viv region. The seeds were incubated in the thermostat at 26 C, on filter paper soaked with distilled water in Petri dishes. Seven days later, seedlings were isolated, frozen in liquid nitrogen and stored at -70 C until their usage.

The frozen seedlings (20g) were powdered in liquid nitrogen. The proteins were extracted using 50mM sulphate acid (3ml/g of raw mass) for 1 hour at 4 C. The extract was gauze filtered and centrifuged at 14000g, for 20min at 4 C in K-24 centrifuge (Janetzki, Germany). The supernatant was removed and its pH equilibrated to 7.8 by 10M NaOH. After incubation at at 4 C for 30min the supernatant was centrifuged again (14000g, 20min, 4 C). Then, ammonium sulphate precipitation was applied to precipitate proteins at 35%, and then at 80% saturation. At each precipitation stage, the sediment was formed for 18 hours, 4 C, followed by centrifugation at 14000, 30min, 4 C. The sediments were dissolved in 20mM Tris-HCl buffer, pH 7.4, 10mM NaCl. The fraction of heat-stable proteins was obtained by heating re-suspended sediments at 85 C, 10min, followed by centrifugation at 20000g, 20min, 4 C. Precipitated proteins were removed and the supernatant used for ion exchange chromatography.

Ion exchange chromatography of thermostable proteins on P-cellulose**.** The fraction of heat-stable proteins was dialyzed against buffer, containing 20mM, Tris-HCl, pH 7.4, 100mM NaCl and then applied onto P-cellulose P11 column (volume 4ml) (Whathman, Great Britain) equilibrated by dialysis buffer. The elution of bound proteins was conducted by linear gradient of sodium chloride (0.1-1M NaCl). The concentration of proteins in fractions was determined at 280nm on BIOTECH photometer (WPA, Great Britain).

The fractions of eluted proteins were analyzed by SDS-PAGE electrophoresis in Laemmli system [11]. To do so, TSA was added to fractions in small aliquots to the final concentration of 10%. Precipitated proteins were harvested by centrifugation and washed with cold acetone. The pellet was dissolved in sample buffer (62.5mM tris-HCl, pH 6.8, 1% SDS, 2% 2-mercaptoethanol, 10% glycerol) and boiled for 2 min. Proteins were separated by electrophoresis in 5-22% polyacrylamide gradient gel in the presence of 0.1% SDS. Then, the gel was silver stained [12].

The fractions containing the proteins of interest were pooled together, dialyzed against double distilled water and stored at -70 C.

The investigation of anti-fungal activity of isolated proteins. The culture of phytopathogenic fungus *Fusarium oxysposum* was kindly provided by the Institute of Forest Biology (Poland).

Anti-fungal activity of proteins was investigated as previously described [13]. The solutions of tested proteins (0.3ml in volume) were sterilized by filtration

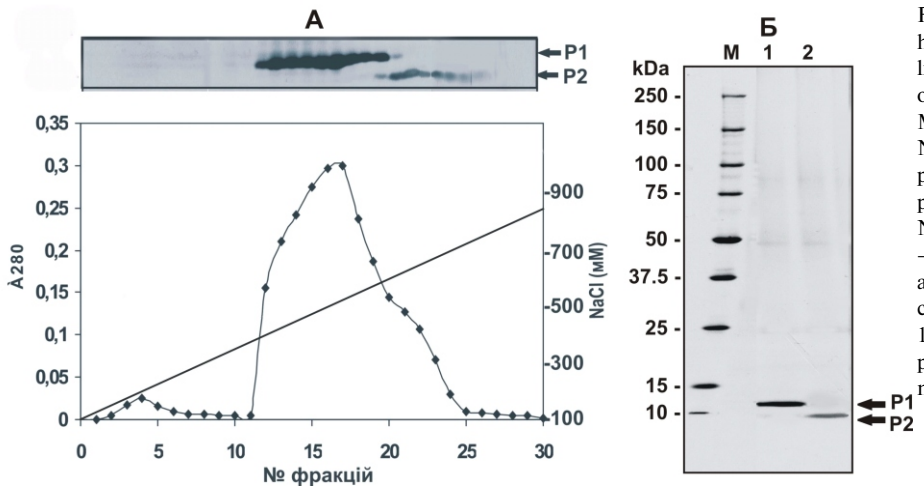

Fig.1 Ion exchange chromatography of heat-stable proteins from *P.sylvestris* seedlings on phosphocellulose: *a* – fractionation of proteins by NaCl linear gradient (0.1 – 1 M); fraction volume 0.6 ml; diagonal line – NaCl concentration gradient; additional panel shows the electrophoretic mobility of proteins eluted from the phosphocellulose by NaCl gradient; silver stained gel); *b* –SDS-PAGE density gradient (5 – 22 %) analysis of proteins separated by ion exchange chromatography (silver stained gel): 1– proteins, eluted by 0.35-0.6 M NaCl; 2 – proteins, eluted by 0.65 – 0.8 M NaCl; *M* - molecular weight markers).

through 0.22 m filter (Millipore, France) before the addition to tubes containing 3ml of liquid 1.8% potato-dextrose agar at 45 C. After mixing, the agar was poured onto Petri dishes (50x15mm). When the agar solidified, *F.oxysporum* mycelium pieces were inoculated in the center of every dish. The distilled water was used as a control. The dishes were incubated for 72 hours at 23 C. The area of micelle colony was then measured and the ratio of fungal colony inhibition growth was calculated as previously described [13].

The investigation of relationship of purified on P-cellulose proteins out of *P.sylvestris* seedlings to phosphotyrosine-sepharose. In this assay, 5 g of purified proteins was incubated on the wheel with 40 l of phosphotyrosine-sepharose in buffer contained 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1% Triton X-100, 5mM EDTA, 20mM NaF, for 2 hours at 4 C. The binding to tyrosine-sepharose was carried out under similar conditions. Affinity matrixes with tyrosine or phosphotyrosine sorbents were obtained according to [14]. Bound proteins were washed 3 times in the incubation buffer. Specifically associated proteins were eluted from beads by boiling in Laemmli buffer (100mM Tris HCl, pH 6.8, 2% SDS, 4% 2-mercaptoethanol, 20% glycerol) and separated by electrophoresis in 5-22% polyacrylamide gradient gel in the presence of 0.1% SDS. Separated proteins were then visualized by silver staining.

Results and discussion. It is well known that plant defensins are low-molecular weight and cysteine-rich proteins with high positive charge. Furthermore, they are highly resistant to extreme pH values and temperatures. The stability of defensins is determined by their unique 3D structure, known as Cys-stabilized бв fold, which is comprised of б-helix and three anti-parallel в-strands, linked by four disulfide bridges [15].

To purify defensine-like proteins from *P. sylvestris* seedlings, we employed following stages: sulfate acid extraction [16]; ammonium sulphate cut; and heat treatment. Then, the obtained fraction of heat-stable proteins was separated by chromatography on P-cellulose. Bound proteins were eluted from the sorbent by linear gradient of sodium chloride. The analysis of eluted fractions by gel electrophoresis indicated the presence of only two proteins with the molecular weight of 11.3 kDa (**P1**) and 9.8 kDa (**P2**) (Fig.1, B). **P1** peak was eluted in fractions between 0.35-0.65M NaCl, and **P2** peak - at salt concentration of 0.6-0.8M (Fig.1, A). The differences in the elution profile allowed us to obtain homogenous preparations of each protein.

Then, purified preparation of **P1** and **P2** were assayed for anti-fungal activity towards *F. oxysporum*. As shown in Fig. 2, **P1** protein inhibited colonies growth by 95% at 25 g/ml in the agar, while P2 protein had the same effect at lower concentration (10 g/ml). Further analysis indicated that the inhibition of hyphae elongation to 70% occurs at 1 g/ml (**P2**) and 3 g/ml (**P1**).

Plant defensins have a very diverse amino acid content and low sequence homology, which determines their biological activities. Based on structural and functional studies defensins could be classified into four groups [6]. The representatives of the 1st group inhibit the growth of a wide range of phytopathogenic fungi, prevent elongation of hyphae and amplify their branching. Therefore, this group is called morphogenic. The defensins of the 2nd group (non-morphogenic) inhibit the growth of fungi hyphae, but do not cause morphological changes. Plant defensins of the 3rd group are specific for their anti-bacterial activity.

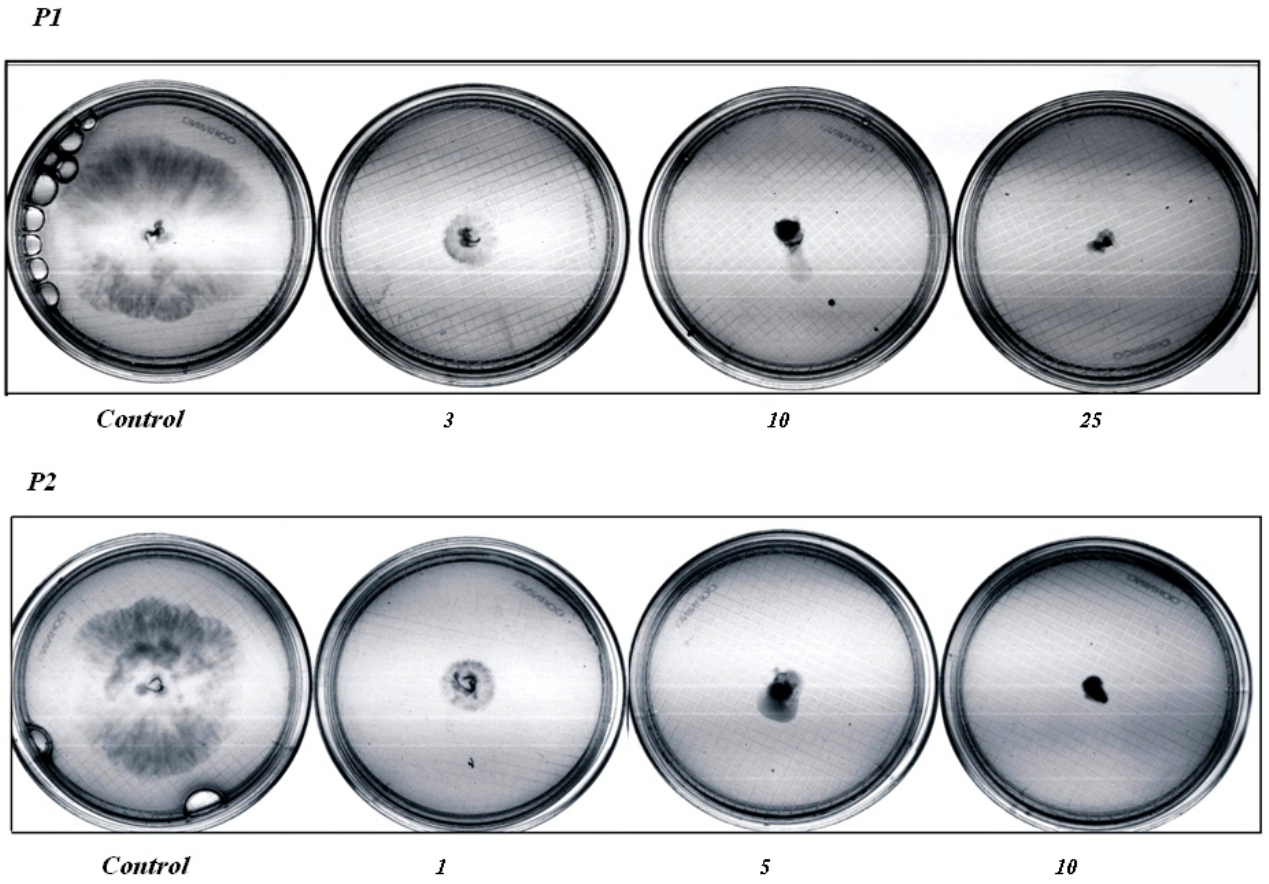

Fig.2 Concentration-dependent effect of P1 and P2 preparations from Scotch pine seedlings on growth of *F.oxysporum* mycelium in potato-dextrose agar.

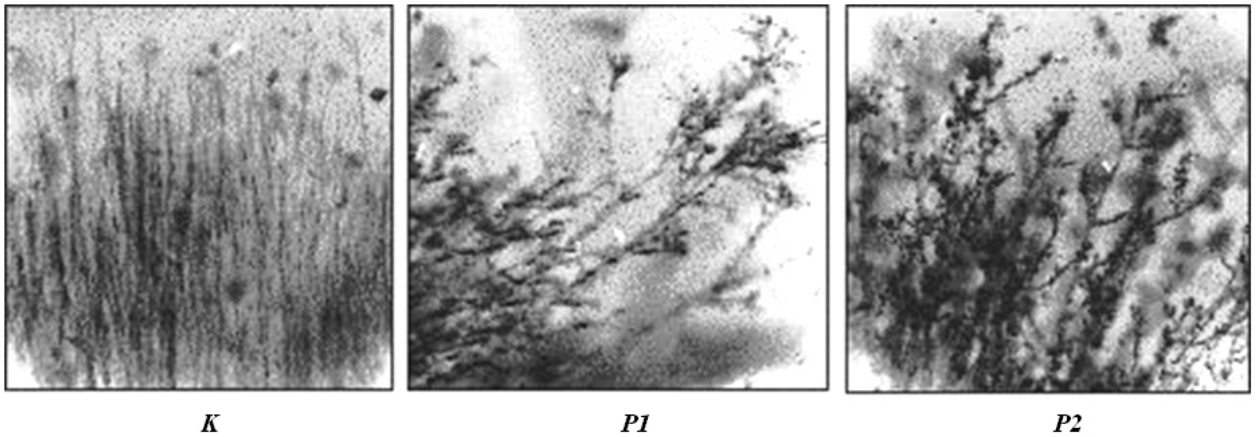

Fig.3 The effect of P1(3 mg/ml) and P2(1 mg/ml) preparations from Scotch pine seedlings on the morphology of mycelium pathogenic fungus *F.oxysporum*.

Defensins from this group inhibit б-amilases and proteases *in vitro*, the synthesis of proteins in a cell-free system, but do not reveal any fungistatic properties. The defensins of the 4th group which were isolated from the leaves of *Spinacia oleracea*, differs significantly from others by their amino acid content and combin functional properties of the 2nd and the 3rd groups [16].

To classify the isolated *P.sylvestris* seedlings defensins, the microscopic investigation of mycelium *F.oxysporum,* grown in the presence of P1 and P2 proteins, was per-

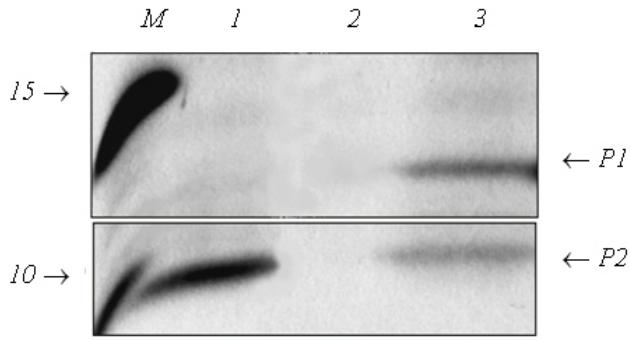

Fig.4 Analysis of binding specificities of P1 and P2 preparations from Scotch pine seedlings towards phosphotyrosine-sepharose. Specifically-associated proteins were separated by SDS-PAGE and visualized by silver staining): *1* – pTyr-sepharose; *2* – Tyr-sepharose; *3* – P1 and P2 protein preparations; *M* – molecular weight markers.

formed. It was shown (Fig.3), that both proteins cause morphological changes of fungi mycelium, *i.e.* thickening of hyphae and the increase of their gemmation. Taking this into account, defensin-like proteins from P1 and P2 fractions exhibit functional similarity to proteins form morphogenic group of defensins, such as Rs-AFP2 from *Raphanus sativus* [3] and Hs-AFP1 from *Heuchera sanguinea* [5].

Previously, we isolated several proteins from the extracts of *P.sylvestris* seedlings by phosphotyrosine affinity chromatography (pTyr-Sepharose). The identity of purified proteins was obtained by mass spectrometry. One of the purified proteins (10kDa) was found to be highly homologous to defensin SPI1 from *Picea abies*). Therefore, it was appropriate to study the relationship between P1 and P2 proteins purified by conventional approaches and a 10kDa protein isolated by affinity purification on phosphotyrosine column. To do so, we applied P1 and P2 fractions on phosphotyrosine-Sepahrose. As shown in Fig. 4, specific interaction with phosphotyrosine matrix was only observed with **P2** protein which has molecular weight of 9.8kDa. This analysis indicates that P2 protein and previously isolated and mass spectrometry identified 10kDa proteins has similar biochemical properties and may represent the same protein. The mass spectrometry of P1 and P2 proteins will confirm this suggestion.

The proteins **P1** and **P2,** isolated from *P.sylvestris* seedlings, have very similar antifungal activity, but different in their binding potential to pTyr-Sepharose. It is known that defensins from *Arabidopsis thaliana* genome form a multigene family, which includes 13 genes. Two defensins were isolated from Pythium dimorphum-infected spruce roots, SPI1 and SPI1B (9). Sequence analysis indicated that their primary protein sequences are highly homologous (difference in three amino acid residues) [9]. It is possible that **P1** and **P2** proteins are the products of different genes, which encode defensine-like proteins in pine. We can not exclude that they belong to different AMP groups. On other hand, **P1** protein might be the product of posttranslational modification of **P2.** It is important to note that purified defensin-like proteins (mol. weight 9.8 and 11.3 kDa) have higher molecular weight when compared with classical plant defensins (5-7kDa). Although, in *Arabidopsis* genome, 2 genes were identified, coding proteins with 122 and 129 amino acid residues with gamma-thionin domain (typical for plants), and signal peptides [6]. Further sequence analysis of P1 and P2 isolated proteins would allow us to define their place among plant's antifungal proteins.

In conclusion, we describe for the first time the isolation and partial functional characterization of two defensin-like proteins from *P.sylvestris* seedlings. The anti-fungal activity of these proteins towards pathogenic fungus *F.oxysporum* opens for us the opportunity to develop new approaches for increasing innate resistance of conifers to infectious diseases.

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Характеристика дефензиноподобных белков из проростков сосны обыкновенной

Резюме

Два дефензиноподобных белка с молекулярными массами 11,3 и 9,8 кДа выделены из семидневных проростков сосны (Pinus sylvestris L.) методом ионообменной хроматографии на фосфоцеллюлозе. Эти белки ингибировали рост мицелия фитопатогенного гриба Fusarium oxysporum в концентрации 1 мкг/мл, а также вызывали морфологические изменения гифов.

Ключевые слова: сосна обыкновенная, дефензин, Fusarium oxysporum.

REFERENCES

1. Boman H.G. Peptide antibiotics and their role in innate immunity. // Annu. Rev. Immunol. 1995. **13**. P. 61-92.
2. Selitrennikoff C.P. Antifungal proteins. // Appl. Environ. Microb. 2001. **67.** P. 2883-2894.
3. Terras FRG, Eggermont K, Kovaleva V, Raikhel NV, Osborn RW, Kester A, Rees SB, Torrekens S, Van Leuven F, Vanderleyden J, Cammue BPA, Broekaert WF. Small cysteine-rich antifunga1 proteins from radish *(R*aphanus sativus L.). Their role in host defense // Plant Cell. 1995. **7**. P. 573-588.
4. Cammue B.P.A., De Bolle M.F.C., Terras F.R.G., Proost P., Damme J.V., Rees S.B., Vanderleyden J., Broekaert W.F. Isolation and characterization of a novel class of plant antimicrobial peptides from Mirabilis jalapa L. seeds. // J.Biol.Chem. 1992. **267**. N 4. P. 2228-2233.
5. Osborn R.W., Desamblanx G.W., Thevissen K., Goderis I., Torrekenes S., vanLeuven F., Attenborough S., Rees S.B.,

Broekaert W.F. Isolation and characterization of plant defensins from seeds of Asteracea, Fabaceae, Hippocastanaceae and Saxifragaceae // FEBS Lett. 1995. **368**. P. 257—262.
6. Thomma B.P.H.J., Cammue B.P.A., Thevissen K. Plant defensins. // Planta. 2002. **216.** P. 193-202.
7. Thevissen K, Ghazi A, DeSamblanx GW, Brownlee C, Osborn RW, Broekaert WF. Fungal membrane responses induced by plant defensins and thionins // J. Biol. Chem. 1996. **271**. P. 15018—15025.
8. Thevissen K, Ferket KKA, Franqois IEJA, Cammue BPA. Interaction of antifungal plant defensins with fungal membrane components // Peptides. 2003. **24**. P. 1705— 712.
9. Fossdal CG, Nagy NE, Sharma P, Lцnneborg A. The putative gymnosperm plant defensin polypeptide (SPI1) accumulates after seed germination, is not readily released, and the SPI1 levels are reduced in Pythium dimorphum-infected spruce roots. // Plant Mol. Biol. 2003. **52**. P. 291—302.
10. Kovalyova V.A., Cramer R., Gout I.T., Gout R.T. The analysis of tyrosine phosphorylation in the process of seed germination of Scots pine. // Укр. Биохім. Журн. 2005. **77.** №2. С. 119-120.
11. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. // Nature. 1970. **15**. P. 680-685.
12. Shevchenko A., Wilm M., Vorm O., Mann M. **Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels.** // Anal. Chem. 1996. **68**. P. 850- 858.
13. Lam Y.W., Wang H.X., Ng T.B. **A robust cysteine-deficient chitinase-like antifungal protein from inner shoots of the edible chive Allium tuberosum.** // Biochem. Biophys. Res. Commun. 2000. **279.** P.74-80.
14. Sundberg, L., J. Porath. Preparation of adsorbents for biospecific affinity chromatography: attachment of group-containing ligands to insoluble polymers by means of bifunctional oxiranes. // J. Chromatogr. 1974. **90**. P. 87-98.
15. Fant F., Vranken W., Broekaert W., Borremans F. Determination of the three-dimensional solution structure of Raphanus sativus antifunfal protein 1 by 1H NMR. // J Mol. Biol. 1998. **279.** P. 257—270.
16. Ozaki Y., Wada K., Hase T., Matsubara H., Natanishi T., Yoshizumi H. Amino acid sequence of a purothionin analogue of barley flour.// J.Biochem. 1980. **87**. P. 549-555.
17. Segura A, Moreno M, Molina A, Garcia-Olmedo F. Novel defensin subfamily from spinach (Spinacia oleracea) // FEBS Lett. 1998. **435**. P. 159—162.