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 mg/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 β-glucanase, 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 6-helices and three anti-parallel β-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 found in complex with glycosylceramides, while defensin DmAMP1 from *Diitlia 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 defense-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 defense-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 (Janetcki, 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
through 0.22μm 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 incubated 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 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, 5μg of purified proteins was incubated on the wheel with 40μl 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 matrices 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 ββββ fold, which is comprised of 6-helix and three anti-parallel β-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 0.35-0.65 M NaCl, and P2 peak – at salt concentration of 0.6-0.8 M (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 25μg/ml in the agar, while P2 protein had the same effect at lower concentration (10μg/ml). Further analysis indicated that the inhibition of hyphae elongation to 70% occurs at 1 μg/ml (P2) and 3 μg/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.
Defensins from this group inhibit 6-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 4\textsuperscript{th} group which were isolated from the leaves of \textit{Spinacia oleracea}, differs significantly from others by their amino acid content and combin functional properties of the 2\textsuperscript{nd} and the 3\textsuperscript{rd} groups [16].

To classify the isolated \textit{P.sylvestris} seedlings defensins, the microscopic investigation of mycelium \textit{F.oxysporum}, grown in the presence of P1 and P2 proteins, was per-
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 Picea abies [9].

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 (P. sylvestris) [3] and Hs-AFP1 from Heuchera sanguinea [5].

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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References


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