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OBSAH/CONTENT

HYDROLYTIC ENZYMES OF CARNIVOROUS PLANTS AS A PROMISING ANTIFUNGAL AGENS

HYDROLYTICKÉ ENZÝMY MÄSOŽRAVÝCH RASTLÍN AKO SĽUBNÉ ANTIFUNGÁLNE AGENSI

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ABSTRACT

Recombinant chitinase from the carnivorous plant *Drosera rotundifolia* and recombinant β-1,3-glucanase from *Drosera binata* were expressed in the *E. coli* expression system, purified and tested for their capability to inhibit the biomass growth of filamentous fungi *Trichoderma viride, Alternaria solani, Rhizoctonia solani,* and *Fusarium poae.* Chitinase significantly inhibited the growth of *T. viride* (32,2%), *A. solani* (30,8%), and *F. poae* (23,3%). β-1,3-glucanase showed inhibition of biomass growth of *F. poae* (24,1%), *A. solani* (14,3%), and *R. solani* (17,6%). Differences between the inhibition capabilities of two tested hydrolytic enzymes suggest potential synergistic application with aim of inhibition of a wider range of filamentous fungi species.

Keywords: chitinase, β-1,3-glucanase, antifungal effect, carnivorous plants

INTRODUCTION

The production of proteins with an antifungal effect in response to the invasion of fungal pathogens is one of the main defensive mechanisms of plants. A group of plant proteins with increased expression rates during the effect of various stress factors, named "pathogenesis-related (PR) proteins", not only initiate a plant defensive response, but their expression can lead to the increased resistance of a plant organism against a pathogenic attack (Bhardwaj et al., 2021). In general, PR proteins reduce the growth, multiplication and differentiation of pathogens and their effectivity is highly dependent on the plant species from which they originate. Nowadays, PR proteins are divided into 17 families, based on their enzymatic properties and primary structure homology.

Among them, hydrolytic enzymes β-1,3-glucanases and chitinases, exhibit strong antifungal activity, which can hypothetically lead up to hyphal lysis (Sandhu et al., 2017).

Plant chitinases (family 3, 4, 8, and 11 pathogenesis-related proteins) are hydrolytic enzymes with molecular mass in the range of 15–43 kDa, which are able to cleave chitin, an N-acetylglucosamine polysaccharide, present in fungal cell walls, into chitooligosaccharides, dimers, and monomers of N-acetylglucosamine by degradation of its glycosidic bonds. Plant β-1,3-glucanases (family 2 pathogenesis-related proteins), with molecular mass in the range of 33–44 kDa can hydrolyze β-1,3-D-glycosidic linkages in β-1,3-glucans. β-1,3-glucanases can degrade fungal cell walls by disrupting hyphal tips, releasing oligosaccharides, which induce the production of other PR proteins and the cascade of consecutive defensive mechanisms (Perrot et al., 2022). Besides their roles in plant defensive mechanisms, both groups of enzymes are involved in a wide range of physiological and biochemical processes, such as plant growth, development, seed germination, and pollination (Ohnuma et al., 2011; Balasubramanian et al., 2012). Carnivorous plants stand in a special place because their chitinases and β-1,3-glucanases evolved analogues with similar hydrolytic enzymes other plant species, but published studies confirmed their involvement in the prey digestion. This fact suggests their high potential as a gene source of hydrolytic enzymes for molecular biology (Jopcik et al., 2017; Schulze et al., 2012).

Modern-day crop production aims for cultivars with in-built genetic resistance against the most dangerous pathogens if possible, but there is a high risk of overcoming resistance from a virulent phytopathogen (Cornelissen and Schram, 2000). Plant breeding methods have many limitations, in particular, applicability only to crossbreed species and the inevitable transfer of undesirable genes and therefore unwanted traits. Methods of molecular biology and genetic engineering can surpass these limitations and introduce genes from any genetic source responsible for the production of signaling molecules or an increased resistance against pathogens into any plant species (Sandhu et al., 2017).

In our study, purified chitinase from the carnivorous plant *Drosera rotundifolia* and purified β-1,3-glucanase from *D. binata,* produced in a bacterial expression system, were tested for their potential antimicrobial effect against various filamentous fungi. Antifungal properties were evaluated against *Trichoderma viride, Alternaria solani, Rhizoctonia solani,* and *Fusarium poae.* Cell walls of these fungi contain chitin as well as β-1,3-glucan, substrates of tested hydrolytic enzymes. *T. viride* was used as a model organism, while other species represent important crop pathogens (Schlumbaum et al., 1986; Timberlake and Marshall, 1989).

MATERIAL AND METHODS

E. coli BL21-CodonPlus(DE3)-RIL cells with pET32a-chitinase or pET32a-β-1,3-glucanase were incubated overnight at 37°C with shaking at 220 rpm in LB broth with supplemented antibiotics (100 $\mu g.ml^{-1}$ ampicillin, 100 $\mu g.ml^{-1}$ kanamycin, 50 $\mu g.ml^{-1}$ chloramphenicol). The cell suspension was transferred into fresh LB broth without antibiotic selection with the final concentration of 5% (*v/v*) and incubated at 37°C with shaking at 220 rpm for approx. 2 hours (OD_{600} = 0.6). Next, IPTG inductor was added with the final concentration of 1 mM, and cells were incubated for another 2 hours. After incubation, cells were harvested by centrifugation at 4000 rpm for 15 minutes and frozen at -80°C until purification.

Purification of expressed proteins was performed by metal affinity chromatography. Briefly, collected induced cells were lysed in 5 ml of SDS lysis buffer [50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole; 2% SDS (*w/v*), (pH 8.0)] and another 5 ml of lysis buffer without SDS was added and gently homogenized. The sample was stored on ice for 30 minutes and centrifuged twice at 4°C at 4000 rpm for 15 minutes. The supernatant was loaded on Ni-NTA agarose (Qiagen) and unbound proteins were eluted with 20 ml of washing buffer [50 mM NaH_2PO_4; 300 mM NaCl; 20 mM imidazole, (pH 8.0)]. Recombinant proteins were eluted with 1,2 ml of elution buffer [50 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole, (pH 8.0)]. Obtained purified protein samples and crude protein extract were separated on 12% mini-gels (*w/v*) by SDS-PAGE (Mini-Protean Cell, Bio-Rad) according to Laemmli (1970).

Antifungal activity assay was performed by a modified protocol of Broekaert et al., (1990). Spores of filamentous fungi *T. viride, A. solani, R. solani,* and *F. poae* with a final concentration of 10^5 spores/ml in potato-dextrose broth (PDB) were incubated in 25 mM acetate buffer (pH 5,2) with 20 μg of purified chitinase or β-1,3-glucanase at 25°C for 24 hours (*T. viride, F. poae*), 34 hours (*R. solani*) and 40 hours (*A. solani*). After incubation, absorbance at 595 nm was measured on Synergy H1 microplate reader (BioTek, Winooski), and the percentage of inhibition was calculated as A595 of the sample ÷ A595 of the control × 100. The antifungal assay was performed in five biological replicates and three technical replicates.

RESULTS AND DISCUSSION

Recombinant chitinase from *D. rotundifolia* and β-1,3-glucanase from *D. binata* were successfully produced in bacterial *E. coli* BL21-CodonPlus(DE3)-RIL expression strain. Detection of recombinant protein was performed by protein separation with SDS-PAGE. After visualization and comparison of total protein content from induced and non-induced cells, recombinant chitinase with the predicted molecular mass of 49 kDa and recombinant β-1,3-glucanase with the calculated molecular mass of 50,7 kDa were visible and distinguishable and their predicted molecular mass correlates with mass detected by gel separation. After purification under native conditions on Ni-NTA agarose, obtained samples were also separated with SDS-PAGE, and purified recombinant enzymes were detected.

Antifungal activity assay was tested against filamentous fungi *T. viride, A. solani, R. solani,* and *F. poae*. As shown in Figure 2, both purified recombinant hydrolytic enzymes were able to exhibit an antifungal effect on three of four tested fungi. Biomass growth of *F. poae* and *A. solani* was significantly inhibited in presence of active purified chitinase (inhibition 23,3%) and β-1,3-glucanase (inhibition 24,1%) in comparison to the growth of these fungi in presence of inactivated heat-treated enzymes. The inhibition effect on the growth of *T. viride* was significant only in presence of chitinase (inhibition 32,2%), β-1,3-glucanase did not show an effect on growth.

On the other hand, *R. solani* was not affected by the presence of active purified chitinase, but β-1,3-glucanase significantly slowed its biomass gain (inhibition 17,6%).

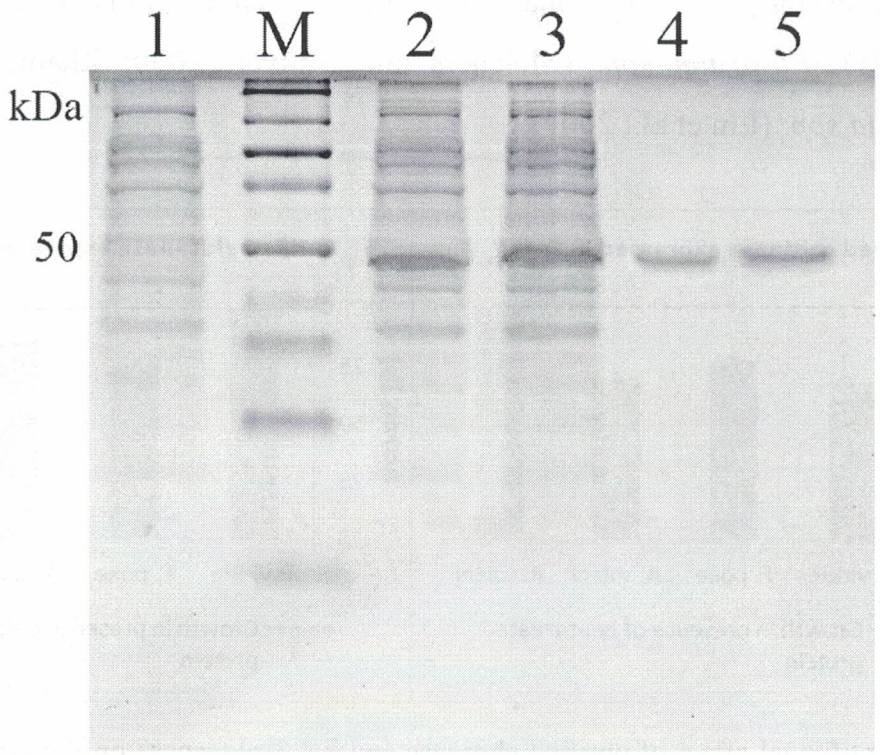

Figure 1: SDS-PAGE analysis of recombinant chitinase and β-1,3-glucanase from crude protein extracts and after Ni-NTA purification. Lane 1 – crude protein extract of non-induced *E. coli* BL21-CodonPlus(DE3)-RIL cells, lane 2 – expressed recombinant chitinase in crude protein extract of induced cells, lane 3 – expressed recombinant β-1,3-glucanase in crude protein extract of induced cells, lane 4 – purified recombinant chitinase, lane 5 – purified recombinant β-1,3-glucanase, lane M - Spectra™ Multicolor Broad Range Protein Ladder (ThermoFisher Scientific)

Cell walls of all tested fungi species have incorporated chitin, chitosan as well as β-1,3-glucans, which are digestive substrates of tested purified enzymes. Loss of any of these cell wall components can drastically influence the morphology, growth, and viability of influenced fungus. However, the amount, composition, and incorporation of these elements into cell walls differ between fungi species. This is a key factor, which influences the antifungal effectivity of tested enzymes. The content of chitin in filamentous fungi represents up to 15% of cell wall mass, β-1,3-glucan as a major component of the cell wall contributes from 30% up to 80% to total cell wall mass (Free, 2013). Chitinases and β-1,3-glucanases from carnivorous plants with prey-digestive functions are not well tested for their antifungal properties, but they possess

strong enzymatic activity analogous to similar isoenzymes from a group of PR proteins in plants. Transgenic expression of genes encoding chitinases and β-1,3-glucanases was already successfully used for enhancing plant resistance against *R. solani*, (Brogue et al., 1991), *Fusarium spp.* (Melchers and Stuiver, 2000; Zhang et al., 2019) or *Alternaria spp.* (Liu et al., 2009).

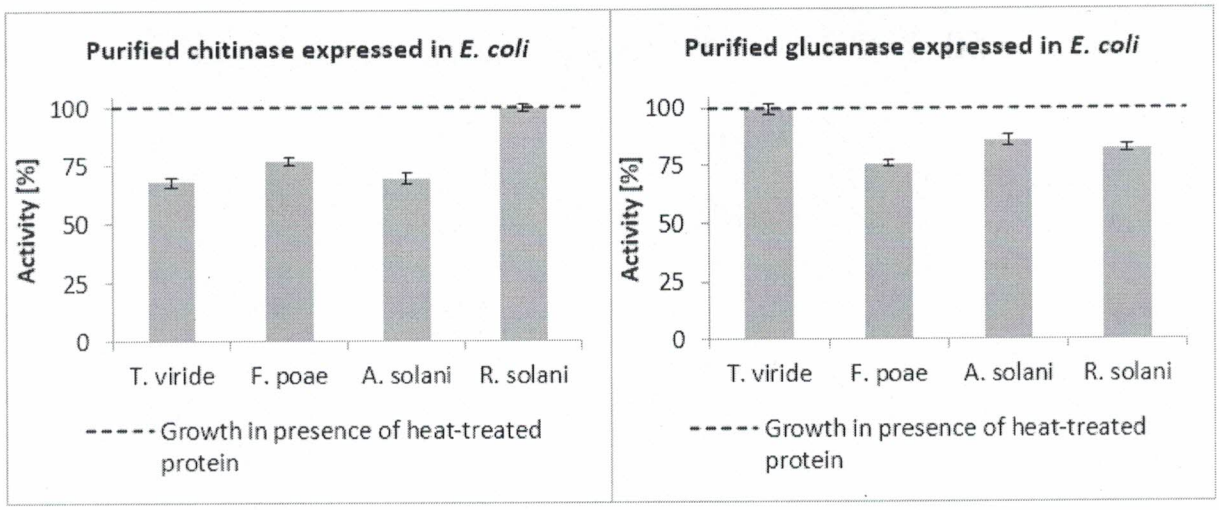

Figure 2: Antifungal effect of purified chitinase and β-1,3-glucanase on the growth of selected filamentous fungi

Based on obtained data for the inhibition effect of tested purified chitinase and β-1,3-glucanase in "*in vitro*" conditions, the potential of incorporation of these transgenes with aim of improving crop resistance to fungal pathogens is a very promising way for further investigations. Further investigation should be aimed at discovering a potential synergistic effect on antifungal properties when chitinase and β-1,3-glucanase are used simultaneously. Chitin and β-1,3-glucan on the apex of growing hyphae of filamentous fungi are synthesized simultaneously, therefore mixed action of both hydrolases can lead to better digestion of mixed structures of chitin and glucan polymers (Sandhu et al., 2017).

CONCLUSIONS

This study was aimed at the investigating antifungal effect of purified recombinant chitinase and β-1,3-glucanase on the growth of four filamentous fungi, *T. viride, A. solani, R. solani,* and *F. poae*. After successful expression and subsequent

purification on Ni-NTA agarose, which was confirmed by the detection of expressed transgenic proteins by SDS-PAGE, antifungal activity was investigated under "*in vitro*" conditions. Purified chitinase significantly inhibited the growth of *F. poae* (23,3%), *A. solani* (30,8%), and *T. viride* (32,2%), recombinant β-1,3-glucanase showed significant inhibition in the case of *F. poae* (24,1%), *A. solani* (14,3%) and *R. solani* (17,6%). Further investigation is needed for the evaluation of the potential syne.

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