Abstract of the Ph.D. thesis

Investigation of antifungal proteins produced by *Penicillium chrysogenum*

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INTRODUCTION

1. Small-size, basic antifungal proteins

The incidence of microbial infections rises steadily in humans from the 1970s. This is either due to the increasing number of patients suffering from immunosuppressive infections or diseases (e.g. AIDS or leukemia) or due to immunosuppressive therapies. On the other hand, pathogenic microorganisms which become resistant to conventional drugs are increasing in number, partly because of intrinsic resistance or secondary resistance developed as a result of prolonged antimicrobial therapies. The development of new antimycotics are badly needed. Antimicrobial, basic proteins from many organisms (human, plants, fungi) bear great potential for the treatment or prevention of infections (Pócsi *et al.*, 2001; Marx *et al.*, 2004).

Antifungal protein produced by *Penicillium chrysogenum* (PAF) is a 55aa protein with 6500 Da molecular mass. The *paf* gene consists of three exons and two introns and its expression is under control of main carbon (CreA) and nitrogen (NRE) regulatory proteins (Marx *et al.*, 1995). The PAF is presented abundantly in the culture medium supplemented with sucrose and nitrate.

PAF inhibits the growth of several filamentous fungi (e.g. *Aspergillus fumigatus*, *A. nidulans*, *A. niger*, *Botrytis cinerea*, *Trichoderma koningii*), reduces the conidial germination and hyphal extension and causes unusual fungal morphology: crippled and distorted hyphae (Kaiserer *et al.*, 2003). In the sensitive fungi PAF induces K^+-efflux and oxidative stress and reduces the metabolic activity of fungal cells. The effect of PAF dramatically decreases in the presence of cations (e.g. Mg^{2+}, K^+, Na^+). The internalization of PAF depends on active cellular metabolism, NaN_3 and KCN inhibited the transport of PAF in to the hyphae (Oberparleiter *et al.*, 2003).

2. Glucose oxidases (GOXs)

Glucose oxidases catalyze the production of H_2O_2 and D-glukono-δ-lactone from D-glucose in the presence of O_2 (Fogarty and Kelly, 1990):

$$\text{D-glucose} + \tfrac{1}{2}\,O_2 \longrightarrow \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

Structurally, GOX is a flavoprotein with 2 FAD coenzymes, it removes two hydrogens from glucose and is itself reduced. The reduced form is then re-oxidized by molecular oxygen. The δ-lactone hydrolyses spontaneously.

This protein is widespread among the fungi, it is produced by e.g. *Aspergillus niger*, *Penicillium amagasakiense*, *P. notatum*, *P. glaucum*, *P. vitale*.

GOX is in most of cases a homodimer enzyme with 155000-192000Da molecular weight (Fogarty and Kelly, 1990) The pH optima of both *Penicillium* and *Aspergillus* enzymes are broad, between pH 4.5 and 7.0. The protein is highly specific for β-D-glucose, the rate of oxidation is about 157 times faster than that of the α-D-glucose, K_m = 11 mM, V_{max} = 15,35 $katkg^{-1}$ (*P. amagasakiense*), K_m = 30 mM, V_{max} = 7,6 $katkg^{-1}$ (*A. niger*) (Witt *et al.*, 1998).

H_2O_2 developed by the enzymatic action of GOX has strong antimicrobial effect. The antimicrobial activity of honey has been attributed by H_2O_2, which is produced by GOX of bees (Taormina *et al.*, 2001). *Helicoverpa zea* GOX produced H_2O_2 is antibacterial and inhibits some oxidative enzymes of plants such as peroxidase and poliphenol oxidase (Eichenseer *et al.*, 1999).

There are some cases for the application of the antimicrobial glucose/GOX system in the food industry, for example to prolong preservation of poultry meat (Jeong *et al.*, 1992). The difference between the GOX tolerance of the species depends on the oxidative stress tolerance, by production of antioxidants, such as catalase and glutathione (Fuglsang *et al.*, 1995). The glucose/GOX/catalase system is used in the food industry because of its oxygen depleting effect, too (Kantt *et al.*, 1993; Dondero *et al.*, 1993), furthermore, the H_2O_2 is decomposed totally preventing the acidification caused by the oxidation of lipids (Fuglsang *et al.*, 1995), therefore, this system is more appropriate for food preservation.

AIM OF THIS WORK

It has already been noted, that the number of resistant microorganisms is sharply rising. The production of novel antimicrobial agents is sorely needed, for example by the exploitation of naturally occuring antimicrobial proteins. Our choice fell on the antifungal protein produced by *Penicillium chrysogenum* (PAF), which was earlier described by Dr. Marx *et al.* (1995), but its antimicrobial spectrum and mechanism of action had not revealed.

In the early experiments I investigated the effect of the culture media of *Penicillium chrysogenum* NCAIM 00237 to the growth inhibition of *Candida albicans*. During the protein purification, we found that the anticandidal effect was attributed to glucose oxidase, but we also isolated the small, basic PAF from the culture medium finding no activity against *Candida albicans*.

In 2003 some information on PAF became known, in collaboration with Dr. Florentine Marx and colleagues (Department of Molecular Biology, Medical University of Innsbruck, Innsbruck, Austria). We proved, that PAF has antifungal effect on filamentous fungi resulted in atypical branching in the sensitive species. PAF induces e.g. oxidative stress in *A. niger* and K^+-efflux in *A. nidulans* (Kaiserer *et al.*, 2003). It is also reported, that PAF enters by active transport the fungal cells and localizes mainly in the citoplasm.
Based on these results I studied the following questions:

(1) Can PAF change the membrane potential, which most likely elicits the atypical morphology in the sensitive fungi?

(2) As PAF enters the cells via active transport, it can be assumed, that PAF binds to a specific receptor and induces G-protein mediated signal transduction. To verify this statement I used *A. nidulans* mutants in G-protein signaling and I also made G-protein inhibitory experiments.

(3) It is well known, that PAF induces production of ROS. Therefore, is it possible, that ROS which accumulate mainly in the mitochondria cause damage of them?

(4) Can oxidative stress cause programmed cell death?

(5) Finally, I checked the possibility of application of PAF by experiments on its cytotoxicity on different mammalian cells.

We also investigated the purified glucose oxidase and characterized in the following relations:

(1) Are there any differences of molecular weight, kinetic parameters of glucose oxidase produced by *P. chrysogenum* compared to other species (e.g. *A. niger*)?

(2) How much are the differences of the sensitivity against oxidative stress and glucose oxidase among the microorganisms?

(3) Do the antioxidants, such as catalase, vitamin C counteract the effect of glucose oxidase?

(4) What kind of applications of GOX are possible in the food industry (as due to its cytotoxic effect it can not be applied in human therapies)?

MATERIALS AND METHODS

1. Purification, characterization of the proteins

To produce antifungal proteins, *Penicillium chrysogenum* NCAIM 00237 strain was grown on minimal medium (2 % sucrose and 0,3 % $NaNO_3$). We purified the proteins from a 72 h culture filtrates with the following procedure: ultrafiltration, ion exchange chromatography or cromatofocusing and gel filtration. The purity of the proteins were checked by SDS-PAGE (Laemmli, 1970). N-terminal protein sequencing of GOX was performed by Edman degradation and homology search was carried out using NCBI protein-protein BLAST (http://www.ncbi.nlm.nih.gov/BLAST). GOX activities were measured by a modification of the D-glucose rate assay of Leary *et al.* (1992). In kinetic experiments D-glucose concentration varied between 0.5-5 mM and K_m and V_{max} parameters were calculated with Grafit, version 2.1 software (Erithacus Software LTD., Horley, UK). D-xylose, D-fructose, D-galactose and D-arabinose were also tested.

2. Determination of the antimicrobial spectra of the proteins

All microorganisms were cultivated in YPD medium (20 g peptone, 10 g yeast extract and 20 g glucose per litre) in 96-microtiter plates according to Lee *at al.* (1999). To each exponential growth phase microculture (V=100 µl) 10-20 µl aliquots of samples were added. From the purification steps 0.1-50 μgml^{-1} proteins were used. In experiments regarding the determination of antimicrobial spectra 0.1-40 μgml^{-1} GOX was tested. To investigate the mechanism of action of PAF and GOX 0.1-50 μgml^{-1} PAF, 5-150 mM H_2O_2, 0.004-1 U GOX, 4-200 U catalase and 1 $mgml^{-1}$ vitamin C were checked.

To determine the antimicrobial effect, 10 µl aliquots of 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) solution (5.0 $mgml^{-1}$ MTT dissolved in phosphate-buffered saline, pH 7.4) were added to each well, and then the plates were incubated further at 37 ^oC for 4 h. The formazan crystals were solubilised with the addition of 30 µl 20 % (w/v) SDS solution also containing 20 mM HCl and the reaction mixtures were incubated at 37 ^oC for 16 h (Lee et al. 1999). The turbidity in each well was read at 570 nm on a BIO-TEK (Winooski, VT, USA) EL 340 Microplate Bio Kinetics Reader (Winooski, VT, USA).

3. Determination of the mechanism of action

The effect of PAF on the membrane potential of *A. nidulans* was monitored with di-8-ANEPPS voltage sensitive dye and the hyphae were visualized by laser confocal microscope (Zeiss, LSM 510; Zeiss, Jena, Germany). In the experiments with G-protein signaling *A. nidulans fadA*^{G203R} and *A. nidulans ΔflbA* mutants and guanidine nucleotide analogs (GTPγS, GDPβS és ATPγS) were applied. To detect the formation of ROS, PAF-treated hyphae were stained with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and mitochondria with MitoTracker Green (Molecular Probes, Eugene, USA). For fluorescence microscopy laser confocal microscope were used (in collaboration with Marx *et al.*). Apoptosis markers were tested on protoplasts (Vágvölgyi and Ferenczy, 1991). Phosphatidyl-serine exposure was determined with Alexa Fluor 488 annexin V, DNA fragmentation was investigated by DAPI and TUNEL assay. Ultrastructural analysis was performed by transmission electron microscopy (in collaboration with Marx *et al.*). The investigation of the cytotoxic effect of PAF was carried out on different mammalian cells.

The localization of GOX were studied by indirect immunofluorescence staining on *A. nidulans*. In the GOX/catalase system the steady-state H_2O_2 was calculated by the method of Keleti (1985).

RESULTS AND DISCUSSION

1. The antifungal protein produced by *Penicillium chrysogenum* (PAF)

The *Penicillium chrysogenum* NCAIM 00237 industrial strain produces a small-size, basic antifungal protein (PAF) in a medium supplemented with 2 % sucrose and 0,3 % $NaNO_3$ with 15 % yield of the extracellular total protein.

This protein induces hyperpolarization in the sensitive *A. nidulans*, which is detected even 40. minutes after the incubation with PAF.

The mechanism of action of PAF is most likely connected with G-protein mediated signal transduction pathway. This was proved by using of *A. nidulans* mutants in G-protein signaling and with guanidin nucleotide analogs.

PAF evokes ROS accumulation in mitochondria in *A. nidulans* causing their desintegration (Marx *et al.*).

PAF induces programmed cell death in *A. nidulans* and we found the following typical apoptotic markers in a presence of protein:

- the fragmentation of the DNA
- the distortion of nuclei
- the translocation of phosphatidyl-serine from the inner to the outer leaflet of the cell membrane
- the shrinkage, blebbing of the cell membrane, appearance of vacuoles (Marx *et al.*).

PAF does not induce the production of inflammatory proteins in human blood cells, either the level of IL-6, IL-8 or TNF-α do not increase after PAF treatment.

According to the observations of our colleagues PAF is not cytotoxic to any mammalian vascular endothel cells, neuronal cells and muscle cells investigated. Therefore, PAF can be a potential drug in human treatments and in the agriculture against plant diseases.

2. Glucose oxidase from *Penicillium chrysogenum*

The *P. chrsogenum* NCAIM 00237 industrial strain produces an antimicrobial enzyme, glucose oxidase in medium supplemented with 2 % sucrose and 0,3 % $NaNO_3$, whose antimicrobial activity was the highest at 72 h fermentation time.

The molecular weight of glucose oxidase of *P. chrysogenum* differs from those produced by other *Penicillium* species, but its kinetic parameters are very similar to them.

Although the enzyme applied in a concentration of 40 μg/ml was effective against all of the test-organisms, significant species-specific differences in GOX-sensitivity were recorded. E.g. the GOX-sensitivity of human pathogenic *Candida* species increased in the order of *C. albicans*≅*C. dubliniensis*<*C. parapsilosis*<*C. glabrata*≅*C. krusei*. The sensitivity of *Aspergillus* species also showed species-specific differences: *A. giganteus*<*A. niger*≅*A. terreus*<*A. nidulans*. The GOX sensitivity of test organisms was dependent on their inherent antioxidant potential.

GOX does not attach to the cell wall of target cells and the steady-state H_2O_2 was too low in the GOX/catalase system. Most likely, the low oxygen tension controlled by the oxygen-consuming GOX/catalase system resulted in a secondary growth inhibition replacing the primary inhibition exerted by H_2O_2.

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