PH.D. THESIS

# STUDY OF THE CATALASE ACTIVITY IN THE OPPORTUNISTIC PATHOGEN ZYGOMYCETES FUNGUS, *RHIZOPUS ORYZAE*

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## Introduction

Among zygomycetes, members of the order *Mucorales* are saprophytic organisms which can be frequently isolated from soil and plant and animal organic materials. Several species are used as model-organisms in the study of various biological processes such as the fungal sexual process or the morphogenesis. Zygomycetes fungi have a great importance in medical, industrial, biotechnological and agricultural aspects. Some species are considered opportunistic pathogens causing zygomycosis which is an invasive, rapidly evolving and often fatal infection in patients having immunocompromised health condition.

Although zygomycoses are relatively rare, recently an increase in the number of cases can be observed among immunocompromised patients due to the application of advanced medical techniques (e.g. bone marrow and organ transplantation, chemotherapy, steroid therapy). Such infections may rarely occur in patients with healthy immune system under specific health conditions, e.g. long-term use antibiotic or tissue injury.

The risk of zygomycosis increases in diabetics, particularly in those associated with ketoacidosis. Although zygomycosis is relatively rare, it is highly progressive and the treatment is difficult. These opportunistic, mainly rhinocerebral fungal infections are of increasing clinical interest due to the high mortality rate (75-90%). The therapy generally involves an early diagnosis followed by aggressive surgical and antifungal treatment. Moreover, these fungi have an intrinsic resistance against the majority of the recently available antifungal agents. All these aspects raise the need of a deeper understanding of the molecular, genetic and physiological background of the pathogenicity of zygomycetes fungi.

Two basic factors contribute to the increased risks of zygomycosis on the host side: the lack of inhibition of the spore germination and the failure of the immune response against the intensive mycelial growth. In a healthy body, the macrophages and the neutrophil cells play an important role in the defence against the invasive fungal infections. The latter effectively kills the fungal hyphae with hydrogen peroxide. The first phase of the defence is the macrophage phagocytosis and oxidative destruction of the spores (in addition, the normal serum also has an inhibitory effect on the spore germination). In the second phase, the neutrophil cells play a crucial role, reaching the surface of the growing hyphae by chemotaxis and damaging the hyphae by cytotoxic oxidative processes without phagocytosis. In the above-mentioned major risk groups, both protective phases are generally poor. Therefore, the microbial catalase production against hydrogen peroxide of the host may be regarded as a possible virulence factor especially in patients with impaired neutrophil function because the neutrophil-mediated hyphal damage by hydrogen peroxide effectively killing fungal hyphae is strongly blocked by catalase. Consequently, in a future treatment, the functionality of catalases of *R. oryzae* may also be targeted, which raises the need for their detailed investigation.

Catalases (EC 1.11.1.6., Hydrogen peroxide oxidoreductase) constitute one of the most common groups of enzymes. Their function is to eliminate hydrogen peroxide by the catalase dismutation reaction. Catalases are widespread in the aerobic living world and can be found in bacteria as well as in plants and animals.



**Aims**

The aims of the present work are the study of the catalase genes of *Rhizopus oryzae*, which is the fungus most frequently isolated from zygomycosis, and the structural characterization of the encoded proteins. To reach these aims, study of the four catalase genes (*cat1*, *cat2*, *cat3*, *cat4*) and the investigation of the structure of the encoded proteins (CAT1, CAT2, CAT3, CAT4) creating their three dimensional model were planned and the following specific objectives were defined:

1. **Examination of the sensitivity of *R. oryzae* against oxidative stress under different culturing conditions and during the life cycle of the fungus.**
2. **Phylogenetic and sequence analysis of the catalases and the encoding genes.**
3. **Construction of the 3D molecular model of the *R. oryzae* catalase proteins.**
4. **Analysis of the expression of the isolated genes:**
   - Analysis of the transcription analysis of the catalase genes under different oxidative conditions and in different developmental stages the fungus by quantitative PCR (qPCR);
   - Isoenzyme analysis to study the expression of catalases.
5. **Systemic infections caused by *R. oryzae* and *R. microsporus* in healthy and immunocompromised animal models.**
6. **Construction of plasmids useful for deletion of the four catalase genes.**

**Methods**

DNA and RNA based techniques:

- DNA extraction
- RNA extraction and cDNA synthesis
- Polymerase chain reaction, real-time quantitative PCR (qPCR)
- Cloning of DNA fragments
- DNA sequencing
- Plasmid construction
- Transformation of bacteria
- Plasmid DNA extraction

Analysis of the nucleotide and the amino acid sequence data:

- DNA sequence comparison
- Nucleotide sequence analysis (BLAST, FASTA)
- Deduction of the amino acid sequences from nucleotide sequences
- Nucleotide and amino acid alignment
- Amino acid sequence analysis
- Phylogenetic analysis

Electrophoretic techniques:

- Agarose gel electrophoresis
- Polyacrylamide Gel Electrophoresis

Protein-based techniques:

- Protein assay
- Isoenzyme analysis

Three-dimensional protein modelling:

- Homology modelling (MODELLER program package)

Animal model:

- Immunosuppression
- Infection
- Infection study

Genetic transformation of fungi:

- Generation of fungal protoplast
- PEG-mediated protoplast transformation



**Results**

- The aim of our study was to investigate the effect of $H_2O_2$ induced oxidative stress on the growth of *R. oryzae in vitro*. This effect was examined in solid and liquid medium and at different developmental stages of the fungus. It was found that the oxidative stress generated by 1.5 mM $H_2O_2$ caused about 50% growth inhibition. Lower concentrations of $H_2O_2$ caused only a slight growth inhibition, while higher concentrations of $H_2O_2$ (>2.5 mM) led to total blockade of the fungal growth in minimal (YNB) and complete (YEG) media. According to these findings, 1.5 mM $H_2O_2$ was used to generate oxidative stress in the further experiments.
- At the beginning of the functional characterization of the catalase genes, we did not have information on the dynamics of spore germination. We determined the time of the spore germination of the *R. oryzae* uracil auxotrophic strain in the presence and absence of $H_2O_2$. Under optimal growth conditions, the germination of sporangiospores started at 4 hours after inoculation, while this time shifted to 6 hours under oxidative stress. This experiment served important information for further studies about the time intervals of sampling.
- The total enzyme activity of the catalases determined by a Catalase Assay Kit showed variations in the different developmental stages of the fungus and under oxidative stress conditions.
- Phylogenetic analysis of the *R. oryzae* catalase genes. We found four catalase homologous regions in the genome database of the *R. oryzae*. Several catalase genes have been identified in other filamentous fungi and yeasts. The amino acid sequences of the corresponding proteins were compared to those of the four *R. oryzae* catalases. Based on these amino acid sequences, a phylogenetic tree was inferred. Topology of this

phylogenetic tree corresponded well to that presented previously by Klotz at al (2003). Catalases of *R. oryzae* situated in the clades 2 and 3 of the heme-containing monofunctional catalases on this tree. CAT1 seems to be a member of clade 3 and shows great similarity to the peroxisomal catalases, while CAT2, CAT3 and CAT4 belong to clade 2.

- The 3D structure of catalases of *R. oryzae* was built by homology modelling based on experimental structures of other catalases. Templates for the homology modelling were obtained by sequence alignment of the *R. oryzae* sequences against the pdb database of NCBI by PSI-BLAST search. Proteins above a reliable alignment score were selected.. Although it is well known that catalases function in homotetrameric form, a preliminary ranking of the templates was performed by homology modelling for later tetramer modelling using the protein monomeric chains. Each monomer contains a *hem* b fused ring in its active centrum, thus the homology modelling was performed with not only the protein chains but also using the *hem* molecules in place. The presence of *hem* caused a great improvement in the quality of the models. Further refinement of the models was observed by applying a second loop modelling step. The templates were ranked by the percent structural overlap values between the monomer models and the corresponding templates and the best scored templates were used in the final multichain-multitemplate homology modelling. In these models, the active centres were found to be highly conserved containing the key amino acid histidine. The mutation of this residue was found to demolish the activity of the catalase in *P. mirabilis*.
- After isolation of the catalase genes, these results make possible to determine the site of the point mutations, which may result in fungal strains with decreased defence ability in the described experimental conditions. The final verification of these hypotheses could be performed by X-ray



diffraction of crystals of the proteins obtained from expression the genes in proper cells.

- Quantitative PCR was used to determine the change in the gene expression during the growth of the fungus and in the presence of oxidative stress. The strongest activity was measured for the *cat1* gene in each developmental stage under normal growth conditions, followed by the gene expression activity of *cat2*. Under oxidative stress conditions, the *cat2* gene showed the highest expression level. The *cat4* gene was activated by oxidative stress in the spore and by internal stress under normal conditions in later periods of cultivation.
- Isoenzyme analysis revealed changes in the intensity of the expression of the different catalases during the fungal growth.
- Studies using both healthy and immunosuppressed animal models were used to study the virulence of *R. oryzae* and *R. microsporus*. Animal models of *R. oryzae* mycosis developed in mice, in which immunosuppression was induced by cyclophosphamid, has not been reported before. In these experiments *R. oryzae* infection frequently caused rhinocerebral zygomycosis causing a 90% mortality of the infected animals within 3 days following the infection. *R. microsporus* caused a slower course of infection with milder consequences than *R. oryzae* and affected particularly the gastrointestinal areas. The survival rate was about 50% at 7.5 days after infection.
- The genome database of *R. oryzae* contains four catalase homologous genes. Specific primers were designed to the upstream and downstream regions of the genes. Then the genes were amplified from the genomic DNA of the fungus and cloned into plasmid. To investigate the role of the four catalases in the pathogenicity, transformation vectors were constructed for deletion of the genes. The vectors carried the *pyrG* selection

marker gene inserted in the catalase genes. The transformation experiments using the deletion vectors are currently in progress.

**Summary**

The results are summarized as follows:

1. Using *in vitro* tests we determined that the oxidative stress induced by 1.5 mM hydrogen peroxide caused around 50% growth inhibition both in *R. oryzae* and *R. microsporus* strain. At higher concentrations of hydrogen peroxide *R. microsporus* strains proved to be more sensitive than *R. oryzae* strains in complete and minimal medium as well.
2. We determined the dynamics of the spore germination in the presence and absence of hydrogen peroxide.
3. Total catalase activity of both *R. oryzae* and *R. microsporus* in different develiopmental stages of the fungus and under oxidative stress conditions was determined. In all cases, the catalase activity of *R. oryzae* was higher than that of *R. microsporus*.
4. Phylogenetic analyses of catalases revealed that CAT1 belongs to calde 3, the monofunctional, mainly peroxisomal, small-subunit catalases, while CAT2, CAT3, CAT4 proteins belong to clade 2, the monofunctional large-subunit catalases.
5. Sequence analysis of the catalase proteins identified the conserved amino acid positions for *hem* binding and the absence of those required for NADPH binding.
6. Homology modelling:
   - Modelling strategy was developed by modelling the monomer units. The presence of constraints such as the prosthetic group and loop modelling improved the model. The quality of the model was measured by the percent structural overlap values between the model and the corresponding template.



   - In many cases, modelling the tetramers with symmetry conditions as additional constraint further improved the model. This was especially enhanced for the long chain target proteins modelled with shorter chain templates as if the chains in the tetramer would arrange each other.
   - We have created 1008 homology models based on single templates.
   - In the final tetramer model, the modelling strategy used as follows: the presence of *hem*, loopmodelling, multiple chain template (tetrameric), symmetry conditions and multiple templates.
   - The amino acids in the conserved positions can be found in the same positions in the homology models.
   - The resulting models contain the key His in their active centre, whose mutation may result in non-functional enzyme.
7. The phylogenetic relationships were determined by the homology models: the best models rated by the percent structural overlap values were obtained when the template and the modelled catalase were at the close in the phylogenetic tree.
8. Real-time PCR was used to detect the transcription level of the genes during the fungal growth and in the presence or absence of oxidative stress.
9. Isoenzyme analysis was used to determine the enzymatic activity of the catalases in the different development stages.
10. Pathogenicity of *R. oryzae* and *R. microsporus* was investigated in healthy and immunocompromised animal models. The immunosuppression of the mice was induced by cyclophosphamide. The infection caused by *R. oryzae* was more severe than that caused by *R. microsporus*.
11. To analyze the pathogenicity of the different catalases, transforming vectors for the deletion of the catalase genes were constructed. The transformation experiments are in progress.

**Papers taken into account in the Ph. D. process:**

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