SUMMARY OF PH.D DISSERTATION

Investigation of biologically active peptaibol compounds produced by members of the filamentous fungal genus *Trichoderma*

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INTRODUCTION

Trichoderma species are ecologically and agriculturally beneficial, but certain species can cause damage in the commercial production of mushrooms or may be the causal agents of human infections. Among the bioactive secondary metabolites produced by *Trichoderma* species, studying the peptaibols is important for the better understanding of the processes that occur during their interactions with plant pathogens throughout their biocontrol activity, or during infections of mushrooms, or even humans.

Trichoderma species are the main producers of peptaibols, that are short peptides, usually 5–20 amino acid residues long, containing α-aminoisobutyric acid (Aib) and isovaline, as well as acetylated N-terminus and 1,2-amino alcohols at the C-terminus. The name 'peptaibol' is formed from the word **pept**ide and the two most characteristic residues, **Aib** and the C-terminal amino alcoh**ol.** They are membrane-active compounds forming several hydrophobic transmembrane helices surrounding a central pore with the ability to aggregate and establish ion channels in lipid bilayer membranes.

Species of the *Trichoderma* clade Viride, including *T. koningiopsis* and *T. gamsii* are beneficial organisms of industrial, agricultural, and medicinal fields. *T. aggressivum* f. *europaeum* and *T. pleuroti* belong to clade Harzianum/Virens and can cause green mould diseases in the commercial production of white button mushroom (*A. bisporus*) and oyster mushroom (*P. ostreatus*), respectively. Clade Longibrachiatum contains species such as *T. longibrachiatum*, *T. bissettii* or *T. citrinoviride*, which can cause infections in immunocompromised humans. Certain species of this clade are beneficial and widely used in the industry, e.g. *T. reesei* for its cellulase production. This clade is ecologically highly versatile, some of its species can be found worldwide like the opportunistic human pathogens *T. longibrachiatum* and *T. bissettii*, whereas others are ecologically restricted, like the biotechnologically important cellulase producer *T. reesei*.

In this study the peptaibol profiles and related bioactivities of *T. koningiopsis*, *T. gamsii, T. aggressivum* f. *europaeum*, *T. pleuroti,* as well as 17 species from clade Longibrachiatum were examined, in order to broaden our knowledge in the field of *Trichoderma* peptaibiomics.

AIMS

Along with the increasing number of described *Trichoderma* species, the chances to find novel, yet undescribed peptaibol compounds or even new peptaibol families are promising. Furthermore, peptaibols are bioactive compounds, they can play role in interactions with plant pathogens or during the infection of mushroom crops or humans, therefore the changes in the peptaibol profiles and their bioactivities are important to study. During this work we intended to focus on the following topics:

- Optimization of the high performance liquid chromatography (HPLC) - mass spectrometry (MS) to identify peptaibol compounds
- Investigation of the peptaibol production of *Trichoderma* species with potential biocontrol abilities
- Investigation of the peptaibol production of *Trichoderma* species causing green mould disease of cultivated mushrooms
- Comparative investigation of the peptaibol profiles of species from the clinically relevant clade Longibrachiatum
- Examination of the bioactivity of peptaibols on bacteria, fungi, plants and mammalian cells

METHODS

Culturing methods:

- Solid phase cultivation in Petri-dishes
- Solid phase cultivation in large plates (40 × 40 cm)

Separation techniques:

- Extraction of peptaibols
- Analysis of peptaibols using HPLC-MS
- Purification of peptaibols using semi-preparative HPLC

Quantitative and qualitative investigation of peptaibols:

- Primary structure determination using mass spectrometry
- Peptaibol profile determination using mass spectrometry
- Peptaibol profile analysis using cluster analysis in the ClustVis web tool

Bioassays:

- Testing the effect of peptaibol extracts on bacteria and yeasts
- Testing the effect of peptaibol extracts on filamentous fungi

- *In vitro* confrontation assays
- Bioassays for peptaibol toxicity on mushroom mycelia
- Bioassays for peptaibol toxicity on filamentous fungi
- Bioassays for peptaibol effects on plants (*Arabidopsis thaliana*)
- Peptaibol bioactivity assays against mammalian cells (porcine kidney tubular epithelial cell line (PK-15) and boar sperm)

RESULTS

1. Optimization of HPLC-MS to identify peptaibol compounds

Based on using alamethicin (ALM) as standard, the HPLC-MS Agilent 1100 system and the HPLC-Orbitrap-MS, Dionex UltiMate 3000 instrument were optimized to identify peptaibol compounds.

2. Peptaibols from *T. gamsii* and *T. koningiopsis* (clade Viride)

The study of the peptaibiome composition of *T. koningiopsis* and *T. gamsii* from clade Viride of the genus *Trichoderma* revealed a total of 30 peptaibol sequences (26 new and 4 known). The novel peptaibol group named koningiopsins was described with 11 new 19-residue-long sequences, which were produced by *T. koningiopsis*.

3. Peptaibol production of *Trichoderma* species causing mushroom green mould diseases

During the study of the peptaibol production of *Trichoderma* species causing green mould disease, 20 new and 5 already known hypomurocin-like compounds were detected from *T. aggressivum* f. *europaeum*. In the case of *T. pleuroti*, 24 novel, 18-residue-long peptaibol compounds were identified and named as tripleurins.

4. Peptaibol production of *Trichoderma* species from clade Longibrachiatum

Structural diversity examination of peptaibol compounds produced by *Trichoderma* species from clade Longibrachiatum revealed a total of 128 20-residue-long peptaibols from the 17 species examined (*T. aethiopicum, T. andinense, T. capillare, T. citrinoviride, T. effusum, T. flagellatum, T. ghanense, T. konilangbra, T. longibrachiatum, T. novae-zelandiae, T. pinnatum, T. parareesei, T. pseudokoningii, T. reesei, T. saturnisporum, T. sinensis* and *T. orientale*), including 53 new and 75

recurrent compounds. Furthermore, 8 novel 19-residue-long sequences were identified from the extracts of *T. flagellatum*, *T. sinensis* and *T. parareesei* and named as brevicelsins. The peptaibols from clade Longibrachiatum could be categorized into groups A, B and C based on their primary structure, where groups A and B consist of only 20-residue-long peptaibols, while group C comprises exclusively of the new, 19-residue-long brevicelsins.

5. Phylogenetic relations based on peptaibol production

Although, we found that the phylogenetic relationships between the producer species within clade Longibrachiatum are less reflected by the clustering based on peptaibol profiles than based on gene sequences, the peptaibol profiles of the species from this clade may still provide insights into their evolution. Our results suggest that the production of group A peptaibols may be an ancestral trait of clade Longibrachiatum, while the switch to the production of group B peptaibols might have occurred multiple times and seems therefore to be the result of convergent evolution. This switch has not been completed fully in certain species, which are able to produce some group A compounds in addition to group B peptaibols.

6. Bioactivity of peptaibols produced by *T. gamsii* and *T. koningiopsis*

The examination of the antibiotic activity of *T. gamsii* and *T. koningiopsis* against a broad spectrum of different microorganisms showed that Gram-positive bacteria were strongly inhibited, while Gram-negative bacteria seemed to be less sensitive to the peptaibol treatment. No inhibitory effects of the studied peptaibol extracts could be observed on yeasts, while filamentous fungi showed considerable sensitivity, suggesting that the type of the host cell wall may play a role in the sensitivity to peptaibols.

7. Bioactivity of peptaibols produced by *T. aggressivum* f. *europaeum* and *T. pleuroti*

The peptaibols produced by *T. aggressivum* f. *europaeum* and *T. pleuroti* were found to be potential growth inhibitors of mushroom mycelia, therefore their contribution to the antagonistic arsenal of mushroom green mould species can be suggested. The changes detected in the quantity of peptaibol compounds during *in vitro* confrontation

of *Trichoderma* species with *Agaricus* and *Pleurotus* implicate the impact of the host mushroom on *Trichoderma* peptaibol metabolism.

8. Bioactivity of *T. reesei* peptaibols

Although, peptaibols are toxic to bacteria and fungi, no negative effects could be detected on *A. thaliana* plants at concentrations of *T. reesei* peptaibols below 0.1 mg ml^{-1}. The 0.1 mg ml^{-1} concentration of the peptaibol extract is still toxic to plant pathogenic filamentous fungi. This extract is also toxic to PK-15 and boar sperm cells, but it needs direct contact with cell membranes, like in the case of common amphiphilic detergents. These findings may be useful in tropical agricultural environments, as peptaibol solutions with biocontrol capabilities may replace the use of thermotolerant *Trichoderma* strains with the potential of causing opportunistic infections in humans.

Altogether, this work highlights the peptaibiome diversity within the genus *Trichoderma*, the biological activity of peptaibols towards various organisms and opens an avenue to their potential exploitation in plant disease management.

SUMMARY

- The HPLC-MS instruments were optimized to identify peptaibols from *Trichoderma* extracts.
- We identified 26 new and 4 known peptaibol compounds from *T. gamsii* and *T. koningiopsis,* including the 11 new 19-residue-long sequences which were named as koningiopsins.
- We detected 20 new and 5 known hypomurocin-like compounds in *T. aggressivum* f. *europaeum* extracts, and 24 novel, 18-residue-long peptaibol compounds were identified and named as tripleurins from *T. pleuroti* extracts.
- A total of 128 20-residue-long peptaibols from the 17 examined species belonging to clade Longibrachiatum were identified, including 53 new and 75 recurrent compounds, as well as 8 novel 19-residue-long sequences, which were named as brevicelsins.
- The peptaibol extracts of *T. gamsii* and *T. koningiopsis* strongly inhibited the growth of Gram-positive bacteria and filamentous fungi, while Gram-

negative bacteria seemed to be less sensitive and no inhibitory effects of the studied peptaibol extracts could be detected on yeasts.

- The peptaibols produced by *T. aggressivum* f. *europaeum* and *T. pleuroti* are potential growth inhibitors of mushroom mycelia. We detected changes in the quantity of peptaibol compounds during *in vitro* confrontation of *Trichoderma* species with *Agaricus* and *Pleurotus*.
- No negative effects could be detected on *A. thaliana* plants at concentrations of *T. reesei* peptaibols below 0.1 mg ml^{-1}. The 0.1 mg ml^{-1} concentration of peptaibol extract is still toxic to plant pathogenic filamentous fungi.
- The 0.1 mg ml^{-1} concentration of *T. reesei* peptaibol extract is toxic to PK-15 and boar sperm cells.

LIST OF PUBLICATIONS RELATED TO THIS THESIS

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