Ph.D. thesis

EXAMINATION OF MYCOTOXIN DETOXIFICATION ABILITY OF MICROSCOPICAL FUNGI

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Introduction

Mycotoxins are secondary metabolites of fungi, which may cause diseases in animals or humans. Mycotoxin contamination of agricultural products is a serious health hazard throughout the world. One of the most important mycotoxins is ochratoxin A (OTA), which is produced by several *Aspergillus* and some *Penicillium* species. Maximum levels for OTA in commodities have been set by Commission Regulation (EC). Furthermore, there are also national laws and regulations in the European Union. The occurrence of OTA in several commodities (feeds, foods and beverages) is considered as a serious health hazard in view of its nephrotoxic, teratogenic, hepatotoxic and carcinogenic properties. Several strategies are available for the detoxification of mycotoxins. These can be classified as physical, chemical, physicochemical and (micro)biological approaches. Microbes or their enzymes could be applied for mycotoxin detoxification; such biological approaches are now being widely studied.

Aims

We concentrated on (micro)biological approaches, which could be applied for mycotoxin detoxification. We examined OTA degrading activity of several zygomycetes and astaxanthin-producing yeast isolates. Furthermore, kinetics of OTA degradation and adsorption were also examined in some selected isolates (*Rhizopus stolonifer, Rh. microsporus, Phaffia rhodozyma*). We tried to clarify what kind of enzyme is responsible for OTA degradation in *Ph. rhodozyma* CBS 5909. We set the following aims:

- Examination of OTA degradation of *Ph. rhodozyma*.
- Effects of temperature on OTA degradation and adsorption caused by *Ph. rhodozyma*.

- OTA adsorption assay
- Analysis of OTA degradation with high performance liquid chromatography (HPLC)
- Effects of protease and carboxypeptidase inhibitors on OTA degradation caused by *Ph. rhodozyma*.

We supposed that a carboxypeptidase is responsible for OTA degradation in *A. niger* CBS 120.49. Accordingly we started to identify and clone the *cpa* gene in *A. niger*. We set the following aims:

- Expression of *cpa* gene by *Pichia pastoris*.
- Transformation of atoxigenic *Aspergillus* isolates with a transformation vector which contains the isolated *cpa* gene.

Methods

Analytical methods for the detection of OTA:
- Thin-layer chromatography (TLC)
- High performance liguid chromatography (HPLC)

DNA based techniques:
- DNA extraction
- Polymerase chain reaction (PCR, Inverse PCR)
- Cloning of DNA fragments
- DNA sequencing
- Plasmid construction
- Transformation of bacteria
- Plasmid DNA extraction

Analysis of the nucleotide and the amino acid sequence data:
- Checking of the nucleotide sequences
- Analysis and comparison of the nucleotide sequences, deduction of the amino acid sequences from nucleotide sequences
- Alignment of the nucleotide and the amino acid sequences

Application of *Pichia pastoris* expression system:
Protein extraction, SDS-poliacrylamide gel electrophoresis of proteins
Genetic transformation of *Aspergillus sp.*:
- Optimization of hygromycin B selection
- Generation of protoplasts
- Integrative transformation using protoplasts

Results

Examining of *Rhizopus* isolates for mycotoxin degrading activities. (J. VARGA et al. 2005)

In this study, isolates belonging the genus *Rhizopus* were examined for their ability to degrade OTA in a liquid medium. Kinetics of OTA degradation was examined in some selected isolates, and model experiments using moistened wheat as substrate have also been set up to examine the applicability of the isolates for OTA degradation in cereals. A total of 51 *Rhizopus* isolates were tested for OTA degradation. A number of *Rhizopus* isolates were able to degrade OTA in liquid medium. In the further studies we concentrated on the *Rh. stolonifer* TJM 8A8 (Rh 5) and *R. microsporus* NRRL 2710 (Rh 31) isolates. The kinetics of OTA degradation of *Rhizopus* isolates have been examined in liquid medium, and compared to that of *A. niger* CBS 120.49 examined previously by Varga et al. 2000. TLC analysis indicated that *A. niger* could degrade more than 90 % of OTA after a 6-day incubation, while the *Rhizopus* isolates could degrade about 90 % of OTA in about 12 days. We could not observe significant differences between OTA degradation kinetics of the two *Rhizopus* isolates. In the model experiments, two *Rhizopus* isolates (*Rh. stolonifer* TJM 8A8, *Rh. microsporus* NRRL 2710) and *A. niger* CBS 120.49 were inoculated to moistened wheat grains spiked with OTA to examine whether they can degrade OTA under more natural conditions. Only the *Rh. stolonifer* isolate could degrade about 95 % of OTA on wheat. The other isolates were unable to degrade OTA under these conditions possibly because they were unable to use wheat as substrate for growth, or because this substrate did not induce the expression of the enzymes necessary for OTA decomposition. The observation that a *Rh. stolonifer* isolate is able to degrade OTA on wheat grains is promising

because it might allow the biological elimination of this mycotoxin. These fungi may provide a source of enzymes which could be used for detoxification of OTA in contaminated agricultural products. Accordingly, further studies are in progress to determine which enzymes take part in the detoxification process in *Rhizopus* isolates, and to identify the genes necessary for OTA degradation.

Ochratoxin degradation and adsorption caused by astaxanthin-producing yeasts (Z. PETERI et al. 2007)

In this study, we examined OTA degrading and adsorbing activities of astaxanthin-producing yeast isolates (*Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous*). The data presented indicate that besides producing astaxanthin, *Ph. rhodozyma* is also able both to detoxify and to adsorb OTA at temperatures well above the temperature optimum for growth of *Phaffia* cells. One *Ph. rhodozyma* and two *X. dendrorhous* isolates have been tested for OTA degradation. All of them were able to degrade OTA in a liquid medium (PM) after less than 10 days. In the further studies, we concentrated on OTA degrading or adsorbing activities of isolate CBS 5905 of *Ph. rhodozyma*. This *Phaffia* isolate could degrade more than 90% of OTA in about 7 days at 20°C. Interestingly, a significant amount of OTA was found to be bound by the cells after two days, indicating that OTA is also adsorbed by the cells. The ferment broth of either induced or uninduced cells was unable to degrade OTA. These observations indicate that the enzyme(s) responsible for OTA degradation is not excreted into the ferment broth, so the enzyme responsible for OTA degradation is cell-bound. Our data indicate that *Ph. rhodozyma* is able to convert OTA to ochratoxin α, and this conversion is possibly mediated by an enzyme related to carboxypeptidases. Chelating agents like EDTA and 1,10-phenanthroline

inhibited OTA degradation caused by *Ph. rhodozyma* indicating that the OTA degrading enzyme is a metalloprotease, similarly to carboxypeptidase A. The temperature optimum of this enzyme was found to be above 30°C, which is much higher than the temperature optimum for growth of *Ph. rhodozyma* cells, which is around 20°C. Both viable and heat-treated (dead) *Ph. rhodozyma* cells were also able to adsorb significant amounts of OTA. Further studies are in progress to identify the enzyme responsible for OTA degradation in *Ph. rhodozyma*.

Isolation and characterization of *Aspergillus niger cpa* gene

In the course of our work, we started to identify the enzyme responsible for OTA degradation in *A. niger* CBS 120.49, which has been examined previously by Varga et al. (2000) for the kinetics of OTA degradation. We have supposed that the enzyme involved in the reaction is possibly a carboxypeptidase, as carboxpeptidase A can convert OTA to ochratoxin α. The entire *cpa* gene in *A. niger* was identified and the promoter and terminal regions of this gene were also determined. The whole *cpa* gene has been cloned and analyzed: a 3287 bp long sequence was determined. This sequence contains a 1939 bp long open reading frame and a 72 bp long intron. This ORF encodes a 621 amino acid long protein. Besides the coding region, the 648 bp long promoter region and the 700 bp long terminal region were determined as well.

Expression of *cpa* gene by *Pichia pastoris* and atoxigenic *Aspergillus* isolates.

We have supposed that a carboxypeptidase is responsible for OTA degradation in *A. niger,* and tried to verify this with two transformation experiments.

1. Transformation of *Pichia pastoris* isolate: In this experiment, the isolated *cpa* gene was inserted into the pPCIZα vector, which was used in the transformation of the *Pichia pastoris* KM71H isolate. Before the transformation process, we tested that *Pichia pastoris* isolates have ability of OTA degradation. The results showed that these isolates were unable to degrade OTA in liquid medium, but they had low ability of OTA adsorption. We carried out the expression of *cpa* gene in tranformant *Pichia pastoris* isolates. Then we have tested whether the carboxypeptidase protein was secreted into the ferment broth and whether it was able to degrade OTA. The results showed that this protein was not secreted into the ferment broth or was present only in low quantities.

2. Transformation of atoxigenic *A. niger* (JHC 607) and *A. nidulans* (SZMC 0552) isolates: We have created a transformation vector (pANCPA), which contains the *cpa* gene and the hygromycin B resistance gene as selection marker. Along with the *cpa* gene, *trpC* promoter and *trpC* terminal regions were inserted to the transformation vector. Before the transformation process, the minimal inhibitory concentration (MIC) values for hygromycin B of *A. niger* JHC 607 and *A. nidulans* SZMC 0552 isolates were determined. These hygromycin B concentrations were used in the transformation system. After the successful transformation, we tested these isolates (*A. niger* T55L, *A. nidulans* T55C) whether they were able to degrade OTA in liquid medium. The result showed that these transformants were unable to degrade OTA in liquid medium. We suppose that the *cpa* gene was not integrated into the genom of *A. niger* and *A. nidulans*, or it was not expressed in these isolates.

We initially supposed that a carboxypeptidase is responsible for OTA degradation in *A. niger* CBS 120.49. This hypothesis was not confirmed or disproved. We are planning further experiments to confirm that this enzyme is responsible for OTA degradation.

The results summarized in the Ph. D. thesis were published in the following articles:

Varga, J., **Péteri, Zs**., Tábori, K., Téren, J., Vágvölgyi, Cs. (2005) Degradation of ochratoxin A and other mycotoxins by *Rhizopus* isolates. *International Journal of Food Microbiology* 99,321-328.

Péteri Zs., Téren J., Vágvölgyi Cs. and Varga J. (2007) Ochratoxin adsorption caused by astaxanthin-producing yeasts. *Food Microbiology* 24: 205-210.

Posters summarizing the results of the Ph. D. thesis:

Varga, J., Rigó, K., **Péteri, Zs**., Tábori, K., and Vágvölgyi, Cs. (2002) Degradation of mycotoxins by filamentous fungi. Croatian, Hungarian and Slovenian Symp. Ind. Microbiol. Microbiol. Ecol., Opatija, Abstracts 55.

Varga, J., Rigó, K., **Péteri, Zs**., Tábori, K., Téren, J., and Vágvölgy, Cs. (2002) Degradation of mycotoxins by *Rhizopus* and *Aspergillus* isolates. MMT 2002. Balatonfüred, Abstracts.

Varga, J., Rigó, K., **Péteri, Zs**., Tábori, K., Téren, J., and Vágvölgy, Cs. (2004) Ochratoxin degradation caused by *Rhizopus* and *Aspergillus* isolates. 2nd CEFOOD, Budapest, Abstracts.

Péteri, Zs., Varga, J., Vágvölgyi, Cs., Tábori, K., Téren J. (2005) Zygomycetes as microbial detoxifiers of ochratoxin A. Third Hungarian Mycological Conference 2005 Mátraháza, Abstracts.

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Other publications:

Varga, J, Rigó, K., **Péteri, Zs.**, Tábori, K., Téren, J., Vágvölgyi, C. (2004) Ochratoxin degradation caused by Rhizopus and Aspergillus isolates. *Proceedings of the 2nd Central European Food Congress,* CD-ROM, P-S-07, pp. 1-6.

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Varga J, Kocsube S, **Peteri Z**, Samson RA (2009) An Overview of ochratoxin research. In Applied mycology (Rai M, Bridge P, eds) CABI Publishers, 38-55.

Participation on congresses:

Varga, J., Rigó, K., **Péteri, Zs**., Tábori, K., Téren, J., and Vágvölgyi, C. (2003): Ochratoxin producing fungi ni green coffee and other retail products in Hungary. Workshop on Mycotoxins and Food Safety: An Overview on Toxigenic Fungi and Mycotoxins in Europe. Martina Franca, 24-25 October 2003.

Péteri, Zs., Barta, K., Csernetics, Á., Vágvölgyi Cs. and Papp T. (2007) Cloning and partial sequence analysis of the *Gilbertella persicaria* farnezyl pyrophosphate synthase gene. MMT 2007 Budapest, Abstracts.