Studies on *Fusarium sporotrichioides* strains producing T-2 toxin

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Abstract

Trichothecene mycotoxins, poisonous secondary metabolites, are produced by plant pathogenic fungi, *Baccharis*, *Myrothecium*, *Stachybotrys*, *Cephalosporium*, *Verticimonosporium*, *Trichothecium* and *Trichoderma*, in addition to *Fusarium*. Contaminations of many cereals such as wheat, corn and rice are due to the trichothecenes, causing serious world-wide problems. The trichothecenes were found at first as trichothesin, an antifungal agent, in 1949. Since Brian isolated DAS (diacetoxyscirpenol) of *Fusarium equiseti* as toxic substance for plants in 1961, the toxin-producing fungi and the metabolites has been isolated and identified. The confirmed derivatives have amounted to as many as two hundred types. Many of these trichothecenes has been isolated from natural contamination. These were moldy corn toxicosis in U.S.A., alimentary toxic aleukia in the former Soviet Union and *Fusarium* toxicosis in Japan. Trichothecenes induce symptoms such as nausea, vomit, diarrhea, mucocutaneous stimulation, leukopenia and aplastic anemia in human and animals. They also induce cytolysis and nuclear destruction in hyperplastic tissue like intestinal epithelium, bone marrow, spleen, testis and ovarium. These toxicities cause inhibition of DNA and proteins syntheses in eucaryocytes. For This effect, it has been considered that the 12,13-epoxytrichothecene ring on the skeleton of trichothecenes may play an important role. The inhibition of protein synthesis is likely to be a result of it's high affinity for ribosome.

Trichothecene is a sesquiterpen containing oxygen atoms and is well studied. Trichothecenes are synthesized following the isoprene rule. It was proven by identification of various intermediates. There are some oxygenation and transfer reactions of the methyl group and hydrogen. So far, the only isolated enzyme that regulates T-2 toxin is trichodiene synthase from

F. sporotricoides.

The trichothecene mycotoxicosis is still wide-spread all over the world. It is quite important to investigate proteins in volved in the biosynthesis of T-2 toxin.

In this dissertation, I will show several N-terminal partial amino acid sequences of novel proteins from *Fusarium* genus in cluding the key enzyme for synthesis of T-2 toxin.

1. Effects of addition of asparagine to the culture medium on the productions of trichothecenes by *F. sporotrichioides* (strain M-1-1 and strain R2301).

Effects of three different concentrations (0.01%, 0.03%, 0.05%) in the medium on the production of T-2 toxin (T-2), diacetoxyscirpenol (DAS) and neosolaniol (NS) were examined for strains M-1-1 and strain R2301 of *F. sporotrichioides.*

Whereas the production of T-2, DAS and NS of strain M-1-1 was increased in proportion to asparagine concentration ($P < 0.05$), in strain R2301, increase of trichothecenes productions were minimal and any stoichiometric relationship between trichothecenes productions and asparagine concentrations were not observed.

2. Effect of asparagine on the fungal growth and fungal protein quantity in strain M-1-1 of *F. sporotrichioides.*

Effect of asparagine addition on the fungal growth and fungal protein quantity in strain M-1-1 was measured from day two to day seven.

Fungal weight increased from the next day after the addition of asparagine ($P < 0.05$). The amount of fungal protein increased in seven-day culture with asparagine.

3. Electrophoresis of the fungal proteins and analyses of their N-terminal amino acid sequences for strain M-1-1 of *F. sporotrichioides*

One thousand two-hundred forty-four spots of *Fusarium* proteins were detected by two-

dimensional electrophoresis and 13 spots of them were identified by their partial amino-terminal sequences.

The partial sequences of the spots 7, 13 and spot 21 show the complete amino acid sequence homologies with those of malaic dehydrogenase of *Saccharomyces cecerevisiae*, the glycer-aldehyde-3-phosphate dehydrogenase of *Trichoderma koningii* and triose-phosphate isomerase of *Schizosaccharomyces pombe*, respectively. The sequences of the spot 1 showed 83.3% sequence homology with the ribosomal S16 protein of *Emericella nidulans*. The homology of the spot 23 with that of phosphopyruvate hydratase of *Saccharomyces cerevisiae* were 72.7%. The sequences of the spot 3 and spot 9 showed 66.7% homology with alcohol dehydrogenase from *S. pombe* and the peptidyl-prolyl isomerase from *N. crassa*, respectively. The sequence of the spot 12 showed the identical sequence as that of spot the 9 but showed a different pI. The spot 2 had 60% sequence homology as compered with that of the ribosomal protein S12 from *Chlamydomonas reinhardtii*. The sequence of the spot 5 showed 58.3% homology with that of serine proteinase from *Coccidioides immitis*. The spot 12 shared 55.6% homology with NADH dehydrogenase chain five from mitochondrion of *Mytilus edulis*. The sequence of the spot 19 showed 46.7% homology with that of hyoscyamine (6β)-hydroxylase from *Hyoscyamus niger*. The spot 6 showed a slight homology (36.8%) with arachin 25K protein of *Arachins hypogaea*.

Twelve peptides had been blocked at their amino termini.

4. Decomposition by anhydrous hydrazine of N-terminal blocked amino acids and amino acid sequencing of the blocked proteins of strain M-1-1 of *F. sporotrichioides* fungal protein

Among anhydrous hydrazine decomposition and amino acid sequencing of proteins of twelve blocked at N-terminal, the protein spot 15 was blocked by pyrrolidone carboxylic acid group, and the amino terminal 15 residues were determined. The N-terminal amino acid sequence was Glu-Thr-Val- Ser-X-Met-Arg-Leu-X-X-X-Val-X-Asp-Asn.

5. Changes of biosynthesis of two protein species caused by the asparagine addition

Of the two-dimensional electrophoresis of proteins from strain M-1-1 of *F. sporotrichioides*, two species of proteins, spot 9 (7.0, 19.7 kDa) showed recognizable increases by asparagine addition and the N-terminal amino acid sequence of one of them (spot 9) was determined up to the 41 step (the number 41 residue) by o-phthalaldialdehede (OPA) reaction. As a result, the spot was identified as a component of peptidyl-prolyl cis-trans isomerase (PPIase).

The protein of spot 13 (6.2, 42.6kDa) was sequenced to the 24th residue, and this sequences was searched for their amino acid sequence homology by using PIR-international protein sequence database. The result revealed that this protein was glyceraldehyde-3-phosphate dehydrogenase (GPDase).

PPIase activity was the highest at the day three and four of asparagine-added incubation periods, with the strongest relation being observed between fungal weight and T-2 production.

I think GPDase might be an inducer of the mevalonic acid in the course of trichothecene synthesis.

This study, stanting from the asparagine addition experiment using strain M-1-1 of *F. spororichioides* which lead to an active production of T-2 toxin, was put into operation by introducing two-dimensional polyacrylamide gel electrophoresis. The results of preceding examination showed that asparagine addition on Strain M-1-1 augumented production of fungal protein and promoted fungal growth, and thus resulted in an increase of trichothecenes (T-2, DAS, NS) production. This study also showed that the enhanced protein synthesis activity induced enzymes and proteins involved in the biosynthesis of trichothecenes.

Existence of cellular proteins of approximately 1,244 species in strain M-1-1 of *F. sporotrichioides* have been proved by introducing two-dimensional polyacrylamide gel electrophoresis for the first time for the genus *Fusarium* proteins. *Fusarium* proteins of 25 species were subjected to amino-terminal sequencing, and amino terminal partial sequences of

cellular proteins of 13 species were newly determined. The *Fusarium* protein showing 46.7% homology with hyoscyamine (6β)-hydroxylase (H6H) from *Hyoscyamus niger* suggests a possibility that it is related to the formation of the early phase of 12,13-epoxy- trichothecene ring of active center on trichothecenes skeleton.

One protein in the two species of proteins which was recognizably increased by asparagine addition was identified as PPIase a (7.0, 19.7 kDa) and PPIase b (6.4, 19.7 kDa) and was the first proteins for *F. sporotrichioides*, with the finding that PPIase a was minor type. Also, amino acid sequences from the N-terminal to 41th residue for strain M-1-1 were the first reports of partial amino acid sequences of PPIase of the genus *Fusarium*. On the basis of existence of PPIase in *Fusarium*, I think that the growth of fungal plant body is regulated through the chemical interaction between PPIase and cyclosporin A. It was shown for the first time for the genus *Fusarium* that the second protein found to increase was glyceraldehyde-3-phosphate dehydrogenase (GPDase). Its amino acid sequence from N-terminal to the 24th residue for strain M-1-1 was the first report as partial amino acid sequences of GPDase of *Fusarium*. These increases of PPIase and GPDase by asparagine addition are proportionally related to the increase in fungal production T-2. It is likely that GPDase activity is a starting material in biosynthsis of mevalonic acid pathway at the early phase of trichothecene biosynthsis, thus the three new proteins (H6H, PPIase, GPDase) were indicated to be related to trichothecene biosynthsis. These observations are not only effectual clues for studying the mechanisms of T-2 biosynthsis,in the future, but also are very useful for searching specific genes that regulate the expression of the enzyme system that are involved in T-2 production and its chemical and biological nature. If we can identify the specific genes controlling T-2 production, it will become possible to analyze, by using similar method, for the many other poisonous fungal species and strains. It will also be useful for diagnosis and prevention of mycotoxicosis of domestic animals and fowls.