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Effects of Aflastatin A, an Inhibitor of Aflatoxin Production, on Aflatoxin Biosynthetic Pathway and Glucose Metabolism in *Aspergillus parasiticus*

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Aflastatin A inhibits aflatoxin production by *Aspergillus parasiticus via* an unknown mechanism. We found that aflastatin A clearly inhibited production of norsolorinic acid, an early biosynthetic intermediate of aflatoxin, at a concentration of 0.25 μg/ml. Reverse-transcriptase polymerase chain reaction (RT-PCR), and real-time quantitative PCR (TaqMan PCR) experiments indicated that the transcription of genes encoding aflatoxin biosynthetic enzymes (*pksA*, *ver-1*, and *omtA*) and a gene encoding a regulatory protein for expression of the biosynthetic enzymes (*aflR*) were significantly reduced by the addition of aflastatin A. We also found that aflastatin A elevated the glucose consumption and ethanol accumulation by *A. parasiticus*, and repressed transcription of genes involved in ethanol utilization. These results suggest that aflastatin A inhibits a very early step in aflatoxin biosynthesis prior to the transcription of *aflR* and can influence glucose metabolism in the fungus.

Aflatoxins are a group of mycotoxins produced by some strains of *Aspergillus parasiticus*, *A. flavus*, *A. nomius*, and *A. tamarii*.[1~3] Aflatoxins are potent carcinogens in mammals and can be found as contaminants in a wide variety of food and feed commodities.[4] Thus, the control and management of aflatoxin production are issues of concern.

To protect foods and feeds from aflatoxin contamination, a specific inhibitor of aflatoxin biosynthesis may be useful. Such an inhibitor is expected to reduce aflatoxin contamination without rapid spread of a resistant strain. Recently, we isolated aflastatin A (AsA) from mycelial extracts of *Streptomyces* sp. MRI142 as an aflatoxin production inhibitor.[5~8] AsA has a novel structure and inhibits aflatoxin production by *A. parasiticus* at low concentrations, and could be a lead compound useful in developing effective inhibitors. Unfortunately, no information is available on the molecular mechanism of inhibition of aflatoxin production by AsA.

Many biosynthetic intermediates involved in aflatoxin biosynthesis have been identified.[9,10] In an early step of the pathway, norsolorinic acid (NA)[11] is biosynthesized as an intermediate from one molecule of hexanoyl-CoA and seven molecules of malonyl-CoA in a polyketide manner. After NA formation, there are complex conversion steps to produce aflatoxins. Many genes responsible for the aflatoxin production by *A. parasiticus* or *A. flavus* have also been clarified.[10] They are clustered and include not only the aflatoxin biosynthetic genes but also a regulatory gene, *aflR*, whose product regulates transcription of some enzyme genes for aflatoxin biosynthesis. A study with these genes would provide a very important approach to the investigation of the mode of action of AsA.

Aflatoxin production is affected by environmental and nutritional factors similarly to the cases of other secondary metabolite production by microorganisms.[12] It is known that pH, oxygen, carbon and nitrogen sources, and zinc are all important factors for aflatoxin production.[10] Especially, carbon sources which can induce aflatoxin production are limited to simple carbohydrates such as glucose, sucrose,

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fructose, and maltose.

To clarify the mode of action of AsA on aflatoxin production, we examined the effects of AsA on the biosynthetic pathway of aflatoxin and glucose metabolism in *A. parasiticus*. In this paper, we describe that AsA inhibits a very early step in aflatoxin production and influences glucose consumption and ethanol accumulation.

Materials and Methods

Strains and Growth Media

Aspergillus parasiticus strains NRRL 2999 (ATCC 26691) and ATCC 24690 were obtained from American Type Culture Collection and used as producers of aflatoxin and NA, respectively. These strains were maintained on a potato dextrose agar (PDA, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) medium and subcultured monthly. A spore suspension was prepared from a two-week-old culture, and used as inoculum for this study. All incubations were done at 27°C. Potato dextrose broth (Difco, USA) was prepared according to the protocol supplied by the manufacturer.

Effect of AsA on the NA Production

We used an agar dilution method to evaluate the effect of AsA on the growth and the NA production of *A. parasiticus* ATCC 24690. AsA was dissolved in dimethyl sulfoxide and added to PDA, which was used to make assay plates. The final concentration of dimethyl sulfoxide was adjusted to 0.1% (v/v). A spore suspension (10 μl, 2.5×10^4 CFU) of ATCC 24690 strain was inoculated at the center of each plate. After 7 days, the diameter of the colony was measured. The agar plate was extracted with chloroform (60 ml) by using a Waring blender. The chloroform solution was filtered and then evaporated to dryness. The residue was dissolved in methanol (1 ml) and subjected to HPLC analysis for quantification of NA (column: Capcell-Pak C_{18}, 4.6×250 mm, Shiseido, Tokyo, Japan; a gradient elution of 70~95% methanol in 0.1 M acetic acid in 20 minutes and then isocratic elution of 95% methanol in 0.1 M acetic acid; flow rate: 0.8 ml/minute; retention time of NA: 25.5 minutes).

Ethanol and Glucose Assays

Each 100-ml Erlenmeyer flask containing 10 ml of a liquid potato dextrose medium was inoculated with a spore suspension (10 μl, 2.9×10^5 CFU) and cultured statically with or without AsA (1 μg/ml). Supernatant samples (100 μl) were obtained at 24-hour intervals for 8 days. A half of each sample was used for determination of aflatoxin concentration by HPLC.[13] Ethanol concentration in 5 μl of each sample was determined by UV-method using nicotinamide-adenine dinucleotide (NAD), alcohol dehydrogenase, and aldehyde dehydrogenase described by BEUTLER *et al.*[14] The amounts of glucose in the samples were monitored by the spots of orcinol-sulfuric acid reaction on silica gel TLC (0.5 μl of the samples were spotted and developed with 90% acetone in distilled water, Rf 0.4).

Molecular Techniques

Total RNA isolation and RT-PCR analyses for *aflR*, *pksA*, *ver-1*, *omtA*, and β-actin were performed as described previously.[13] Standard molecular techniques were performed as described by SAMBROOK *et al.*[15] We used real-time quantitative PCR (TaqMan PCR)[16] using 7700 Sequence detector (Perkin Elmer Applied Biosystems, Norwalk, Conn.) to analyze *aflR* and *pksA* transcriptions. The primers and TaqMan probes (Table 1) were designed from the *aflR* and *pksA* sequences by using Primer Express software (Perkin Elmer Applied Biosystems). β-Actin was used as a control. For quantitative RT-PCR experiments, the mycelium cultured for 2 days statically with or without AsA (1 μg/ml) was harvested, and then first-strand cDNA was prepared. RT-PCR experiments for *aldA*, *facA*, and *cycA* were performed with the following primer sets and conditions: *aldA*,[21] 5′-TCGGTGGCAAGTCTCCCAAC-AT-3′ and 5′-ATGATACGGTCGAACTGGAGCT-3′ (94°C, 30 seconds; 56°C, 30 seconds; 72°C, 30 seconds for 23 cycles); *facA*,[22] 5′-TTCTGCGGTGGTGATGTCGGCT-3′ and 5′-TCGGTTTGCCAGTAGGTGTCGCA-3′ (94°C, 30 seconds; 56°C, 30 seconds; 72°C, 30 seconds for 25 cycles); *cycA*,[23] 5′-AAGGGTGCTAAGCTCTTCGAG-3′ and 5′-CTTGAGACCACCGAAAGCCATCT-3′ (94°C, 30 seconds; 56°C, 30 seconds; 72°C, 30 seconds for 23 cycles). The resulting amplified DNA fragments were resolved on 5% polyacrylamide gel electrophoresis with SYBR® Green I nucleic acid stain (FMC BioProducts, Rockland, USA), and were quantitated using FLA-3000 and Image Gauge software (FUJIFILM, Tokyo, Japan). The amount of amplified DNA fragment of each gene was normalized by that of β-actin from each sample.

Results

Effects of AsA on the NA Production

A. parasiticus ATCC 24690, a mutant blocked in aflatoxin biosynthesis, can still produce NA. AsA reduced

Table 1. Specific primers and TaqMan-probes for TaqMan PCR.

Gene	Primer and probe sequences 5'-3' sense antisense TaqMan-probe ([FAM]-5'-3'-[TAMRA])	Reference
aflR	AGTCAATGGAACACGGAAACC	17,18
	AAACGCGTCGAAAAGACTCC	
	TGCCGATTTCTTGGCTGAGTCGA	
pksA	AAATCTCTACGCCGGTCTGTG	19,20
	ATGAACTGTCCCTTAGCATGGC	
	CCGAGTACAAGACCCACATTCGTCAGTTAC	
β-actin	CGCGGATACACCTTCTCCACTA	
	ACGTAGCAGAGCTTCTCCTTGA	
	CGCTGAGCGTGAAATTGTCCGTGA	

Table 2. Effect of AsA on norsolorinic acid production by *A. parasiticus* ATCC 24690 on agar plate.

AsA (mg/ml)	[a] Norsolorinic acid conc. (mg/ml)	[a] Diameter of colony (mm)
0	36	65
	41	67
0.05	11	62
	15	63
0.1	9.5	63
	7.0	63
0.25	0.7	50
	N.D. [b]	50

[a] Observed values in duplicate experiments.

[b] Not detected (<0.2 mg/ml)

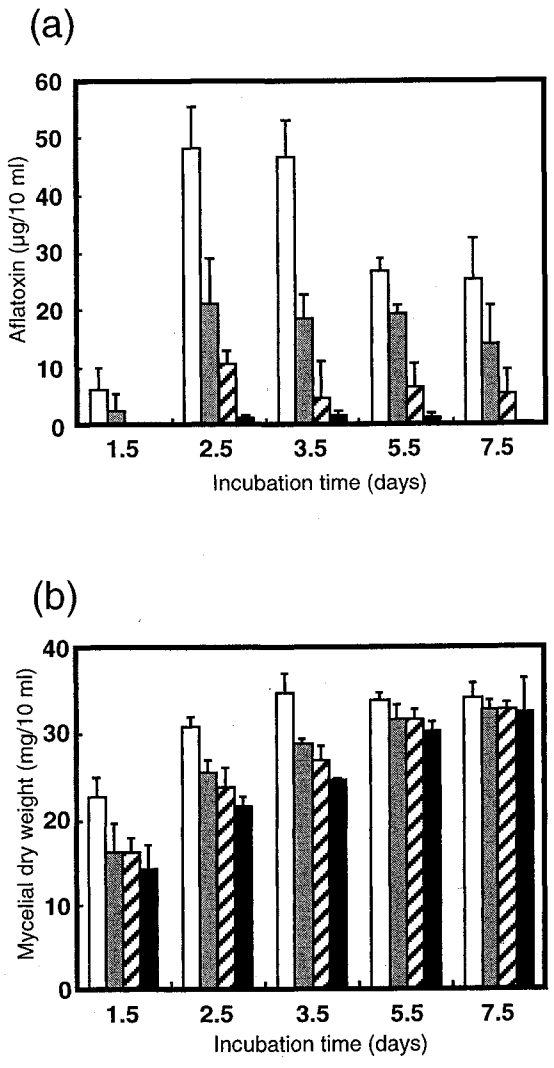
NA production by this strain to less than a half of that of the control at a concentration of 0.05 μg/ml in the agar medium, while the growth of this strain was not significantly affected. When the concentration of AsA was increased to 0.25 μg/ml, NA production was clearly inhibited (Table 2).

Effects of AsA on the Transcription of Genes Responsible for Aflatoxin Biosynthesis

Next, we examined the effect of AsA on the transcription of genes involved in aflatoxin biosynthesis. *A. parasiticus* NRRL 2999 was cultured in a liquid medium with or without AsA. The culture broth was filtered to separate the filtrate and mycelia, which were used for aflatoxin and total RNA isolation, respectively. Aflatoxin in each filtrate was quantified by HPLC, affording the time course of aflatoxin production as shown in Figure 1. The fungus began to produce aflatoxin at around 1.5 days of cultivation and the amount reached a maximum after 2.5 days of cultivation. Aflatoxin production was inhibited dose-dependently at all incubation times when AsA was added to the culture (Figure 1a). AsA almost completely inhibited aflatoxin production at the concentration of 1.0 μg/ml under the culture conditions. AsA delayed the mycelial growth of the fungus dose-dependently (Figure 1b). After 7.5 days of cultivation, however, the mycelial weight of the fungus was not affected by AsA as reported previously.[6]

Fig. 1. Time course of aflatoxin production (a) and mycelial weight (b).

A. parasiticus was cultured for 1.5 to 7.5 days with or without AsA. Open bars, control; gray, shaded and solid bars; cultured with 0.25, 0.5, 1.0 μg/ml of AsA, respectively. Each value represents the mean of triplicated experiments and the vertical bar represents standard deviation.



Among the four known genes associated with the aflatoxin production, *pksA*, *ver-1*, *omtA*, and *aflR*, were chosen to analyze the transcriptional levels of their mRNA. RT-PCR was used for estimating mRNA levels of these genes transcribed in the mycelia cultured with or without AsA. When AsA was added to the culture, the transcription of all the aflatoxin-related genes was repressed in a dose-dependent manner (Figure 2a).

We confirmed the effect of AsA on aflatoxin biosynthesis by measuring mRNA of *pksA* and *aflR* with the TaqMan PCR method (Figure 2b). The mRNA levels of both *pksA* and *aflR* were dose-dependently reduced in the cultures with AsA and transcription of *pksA* was more severely affected than that of *aflR*.

Glucose Metabolism of *A. parasiticus* in the Presence of AsA

Next, we examined the effect of AsA on glucose metabolism of *A. parasiticus*. *A. parasiticus* NRRL 2999 was cultured in a potato dextrose medium containing glucose at a concentration of 2% (w/v) with or without AsA. The amounts of aflatoxin and ethanol in the samples were determined by HPLC and UV-method,[14] respectively, and glucose concentration in the samples was also monitored by silica gel thin layer chromatography (Figure 3). In the control experiment, a small peak of ethanol accumulation was observed at 2 days cultivation around which time aflatoxin was produced most vigorously (Figure 3a and b). On the other hand, in the presence of AsA, ethanol concentration increased up to 6 days cultivation, and then quickly dropped (Figure 3b). As shown in Figure 3c, glucose was metabolized faster in the presence of AsA than in the control without AsA. When glucose was almost completely metabolized after 6 days cultivation in the presence of AsA, ethanol concentration rapidly declined to the control level (Figure 3c).

Fig. 2. Transcriptional patterns of aflatoxin biosynthetic genes.

(a) RT-PCR analyses. Total RNA was isolated from fungal mycelia cultured for 1.5 to 3.5 days with or without AsA (0, 0.25, 0.5 or 1.0 μg/ml). Transcriptions of *pksA*, *ver-1*, *omtA*, *aflR* and actin genes were analyzed by RT-PCR.[13] (b) Transcription of *aflR* and *pksA* measured with TaqMan PCR method. Open bars, control; gray, shaded and solid bars; cultured with 0.25, 0.5 or 1.0 μg/ml of AsA. The amount of *aflR* or *pksA* mRNA was normalized by the amount of β-actin mRNA in each sample.

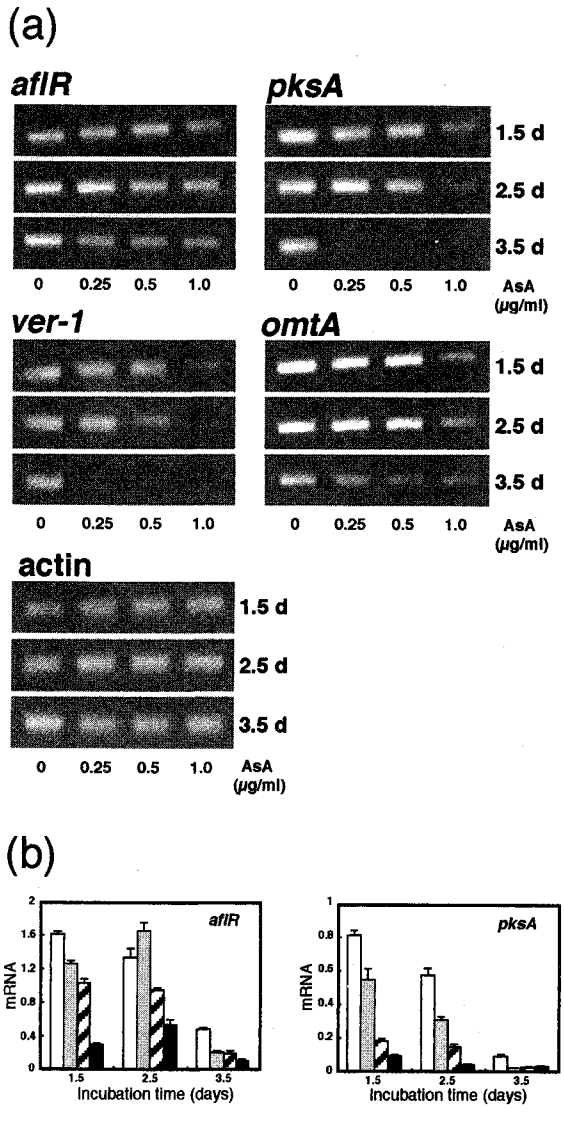
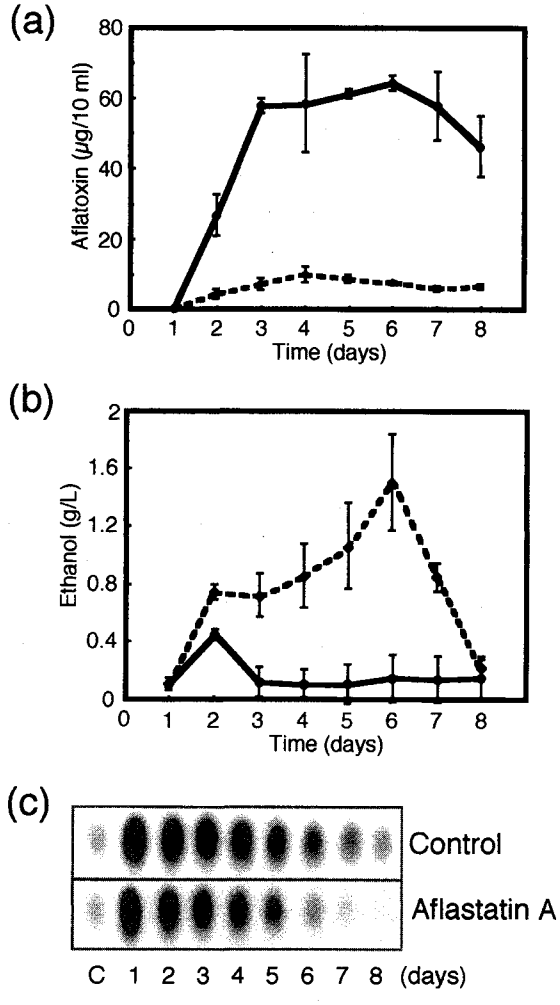


Fig. 3. Time course of concentrations of aflatoxin (a), ethanol (b), and glucose (c).

A. parasiticus was cultured for 8 days with or without AsA (1 μg/ml) and the concentration of aflatoxin, ethanol, and glucose in the culture fluid was monitored by HPLC, UV-method and silica-gel TLC, respectively. Solid and dotted lines represent 'control' and '+AsA', respectively. 'C' means an authentic sample of glucose (0.1% glucose, w/v).

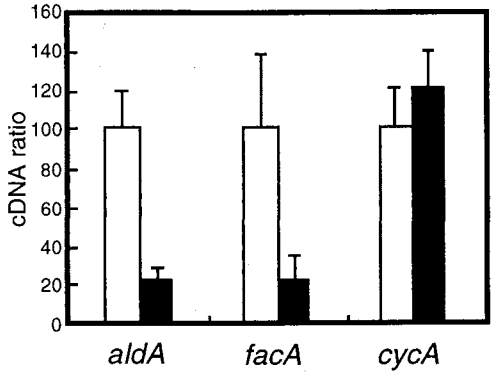


Effects of AsA on the Genes Responsible for Ethanol Utilization and Respiration

We next examined effects of AsA on the transcription of genes involved in ethanol utilization (*aldA* and *facA*, encoding aldehyde dehydrogenase and acetyl-CoA synthetase, respectively)[21,22] and respiration (*cycA*, encoding cytchrome *c*).[23] Because these genes of *A. parasiticus* had not been cloned, oligonucleotide primers based on the sequence of *A. nidulans* were used to construct a quantitative RT-PCR assay for gene transcription. Since each sequence of the amplified DNA fragment obtained from *A. parasiticus* showed high homology to that of the corresponding part in each gene of *A. nidulans* (85% for *aldA*, 81% for *facA*, 78% for *cycA*),

Fig. 4. Transcript levels of genes involved in ethanol utilization.

A. parasiticus was cultured for 2 days with or without AsA. Open bars; control, solid bars; cultured with 1.0 μg/ml of AsA. mRNA of the genes were quantitated by quantitative RT-PCR analysis using FLA-3000 and Image Gauge software.



each DNA fragment was judged to be involved in the gene encoding *aldA*, *facA*, or *cycA* in *A. parasiticus*. RT-PCR experiments revealed that transcription of both of *aldA* and *facA* was significantly repressed by the addition of AsA, while that of *cycA* was not affected by AsA (Figure 4).

Discussion

Various substances are known to inhibit aflatoxin production.[24] Organophosphorous insecticides with choline esterase inhibitory activity, such as dichlorvos, inhibit an esterase that hydrolyzes versiconal acetate to versiconal.[25] Tricyclazole and chlobenthiazone appear to inhibit a reductase involved in the biosynthetic step from versicolorin A to demethylsterigmatocystin.[26] 2, 3, 4, 5, 6-Pentachlorobenzyl alcohol, phthalide and pyroquilon target an early step of aflatoxin biosynthesis before the synthesis of NA[27,28] since exogenous NA can be converted to aflatoxin in their presence. However, the mode of action of these three substances has not been verified.

Our results show that the mode of action of AsA is different from that of these other inhibitors. AsA inhibits NA production and also affects other steps in the aflatoxin biosynthetic pathway. For example, the mRNA levels of *pksA*, *ver-1*, *omtA* and *aflR* were clearly reduced by AsA. PKSA, VER-1, and OMTA are biosynthetic enzymes involved in the conversion steps from hexanoyl-CoA to NA, from versicolorin A to demethylsterigmatocystin, and from sterigmatocystin to *O*-methylsterigmatocystin, respectively. AFLR activates the transcription of these three genes.[29~32] Therefore, the repression of *aflR* transcription by AsA may be the most important for inhibition of aflatoxin biosynthesis. Our results suggest that AsA does not simply inhibit an enzyme in the aflatoxin biosynthetic pathway, but may inhibit a very early step prior to the expression of *aflR*.

We found that AsA elevated glucose consumption and ethanol accumulation in *A. parasiticus*. This suggested that AsA can influence carbon metabolism in the fungi, which was supported by the fact that the transcription of *aldA* and *facA* was repressed by AsA. A close relationship of glucose metabolism or ethanol utilization with aflatoxin production in aflatoxigenic fungi has been suggested from phenomena observed in aflatoxin-producing cultures such as degraded mitochondria,[33] ethanol accumulation[34] or increased expression of alcohol dehydrogenase gene (*adh1*).[35] Therefore, the changes in carbon metabolism caused by the addition of AsA may affect *aflR* transcription, leading to inhibition of aflatoxin production, or AsA may influence a regulatory system that regulates both of carbon metabolism and aflatoxin production. However, the possibility that AsA's target leading to the change of carbon metabolism is different from that leading to reduction of *aflR* expression can not be ruled out.

Secondary metabolite production by fungi usually begins during a stationary phase and is regulated by an unknown mechanism. Although several environmental and nutritional factors appear to play important roles in this regulation, they do not seem to be specific for secondary metabolite production. In this sense, aflatoxin is typical of fungal secondary metabolites. AsA appears to act on the early regulatory system, which may lead to the expression of *aflR*. Therefore, it is very important to clarify AsA's mode of action, not only for developing new inhibitors of aflatoxin biosynthesis but also to better characterize broader regulatory mechanisms of secondary metabolite production in fungi.

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