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Organic acids suppress aflatoxin production via lowering expression of aflatoxin biosynthesis-related genes in *Aspergillus flavus*

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ABSTRACT

Aflatoxins are naturally occurring carcinogens. Humans and animals, such as turkeys, are highly susceptible to aflatoxin-induced diseases. In this study, we investigated the inhibitory effects of 5 food additives on the growth and aflatoxin production in *Aspergillus flavus* ATCC 22546. Propionic acid completely inhibited fungal growth at a concentration of 0.5%. Furthermore, no fungal growth was observed when the growth medium was treated with 0.05% benzoic acid, 0.1% sorbic acid, 0.5% acetic acid, or 0.5% butyric acid. In comparison to propionic acid, other food additives showed weak antifungal activities. Propionic acid, butyric acid, benzoic acid, and sorbic acid also exhibited potent antiaflatoxigenic activities at a concentration of 0.1%. However, addition of 0.1% acetic acid did not inhibit aflatoxin production. At a concentration of 0.05%, propionic acid lost its antiaflatoxigenic activity, whereas at the same concentration, benzoic acid, butyric acid, and sorbic acid showed potent antiaflatoxigenic activities with a 95% inhibition of aflatoxin production. Benzoic acid drastically inhibited the expression of genes related to aflatoxin biosynthesis, whereas sorbic acid only inhibited the expression of a transcription gene, *yab*. Collectively, our results suggest that benzoic acid is promising alternative to propionic acid as food preservatives.

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1. Introduction

Aflatoxins are well-known mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* that show strong carcinogenic effects in humans and induce susceptibility to aflatoxicosis in most animals (Peraica, Radić, Lucić, & Pavlović, 1999). Their presence in foods and animal feedstuff needs to be eliminated or maintained below the acceptable maximum residue level on a daily basis (Asters et al., 2014; Bui-Klimke, Guclu, Kensler, Yuan, & Wu, 2014; Madbouly, Ibrahim, Sehab, & Abdel-Wahhab, 2012; Siripatrawan & Makino, 2015).

Governmental agencies and industries have developed strategies to reduce aflatoxin contamination in order to mitigate its adverse effects on the public community and industrial food manufacturers (Wu & Khlangwiset, 2010). There are three main routes for aflatoxin contamination based on time differences: preharvest, post-harvest (including storage), and food processing. Minimizing aflatoxin contamination is considered at the time when contamination of aflatoxins occurs. Consequently, several countries have developed methods for reducing aflatoxin contamination concomitant with the periodic monitoring of aflatoxin contamination in cereals (Baquião et al., 2013; Khan, Asghar, Iqbal, Ahmed, & Shamsuddin, 2014; Lee, Her, & Lee, 2015). Chemical treatment of *Aspergillus* infection using man-made fungicides (Lee, Mahoney, & Campbell, 2002) is one of the most efficient tools for controlling mycotoxigenic fungi and eliminating mycotoxin contamination (Santos, Marin, Sanchis, & Ramos, 2011). However, fungal resistance to fungicides and its consequent threat to food safety and public health have been well-documented (Price, Parker, Warrilow, Kelly, & Kelly, 2015; Shao, Zhang, Ren, & Chen, 2015). Recent studies report that *Aspergillus fumigatus* has acquired resistance to azole drugs, such as itraconazole, voriconazole, posaconazole, and isavuconazole and that the resistance had been caused by environmental exposure. Amino acid substitution in Cyp51A and the overexpression of the ABC transporter, Cdr1B, have been reported in the resistant strain of *A. fumigatus* (Hagiwara, Watanabe, Kamei, & Goldman, 2016). *Aspergillus* has also acquired other resistance mechanisms, such as the overexpression of the target proteins with

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reduced affinity and increased degradation rate of azole compounds (Hagiwara et al., 2016). In addition, Cai et al. (2015) have demonstrated that M233I mutation in the β-tubulin of *Botrytis cinerea* causes it resistance to zoxamide (Cai et al., 2015). In their study, three types of phenotypes were isolated from 161 *B. cinerea* strains: strains sensitive to zoxamide and carbendazim, strains sensitive to zoxamide and resistant to carbendazim, and strains resistant to zoxamide and carbendazim (Cai et al., 2015).

Fungal resistance to currently used fungicides has highlighted the development of new chemical agents to treat fungal infections. Naturally occurring compounds, including essential oils, can be used to replace fungicides that are currently being used (Kazemi, 2015; Kedia, Prakash, Mishra, & Dubey, 2014). Piperidine alkaloids isolated from *Piper longum* L. (including piperlongumine, piperine, pipernonaline, and piperoctadecalidine) have exhibited potent antifungal and antiaflatoxigenic activities against *A. flavus* WRRC 3-90-42. Methylenedioxy-containing compounds, including piperonal, have shown specific antiaflatoxigenic activity (Lee et al., 2002; Moon et al., 2016; Park, Bae, Kim, & Lee, 2016). These methylenedioxy-containing compounds act by inhibiting cytochrome P450 activity; one of these synthetic compounds, in particular, has shown the down-regulation of five genes: *aflD*, *aflK*, *aflQ*, *aflR*, and *aflS* (Dinger, Meyer, & Maurer, 2016; Moon et al., 2016; Reen & Singh, 1991). With natural products, a fumigant, ethanedinitrile, also showed potent inhibitory effect on the fungal growth and aflatoxin production of *A. flavus* (Choi et al., 2017).

Detoxification and decomposition of pre-existing aflatoxins using organic acids can be used to clean residual aflatoxins (Aiko, Edamana, & Mehta, 2016; Lee et al., 2015). Detoxification of aflatoxin using ozone was successfully introduced in corn flour by increasing the exposure time (Luo et al., 2014). Enzymes such as laccases and manganese peroxidases have also been used for the detoxification process (Yehia, 2014; Zeinvand-Lorestani et al., 2015; Loi et al., 2016). Laccases are multicopper oxidases that show 23% of aflatoxin B1 degradation after occurrence (Loi et al., 2016).

Physical treatments using gamma rays can efficiently eliminate the presence of aflatoxins in foodstuffs (Di Stefano, Pitonzo, Cicero, & D'Oca, 2014). Microbiological degradation of pre-existing aflatoxins has been extensively studied using *Rhodococcus* strains and yeasts (Cserháti et al., 2013; Rependkiene, Levinskaite, Paskevicius, & Raudoniene, 2013). Bacterial biocontrol agents have been used to treat fungal growth and they possibly affect aflatoxin production by inhibiting the expression of aflatoxin biosynthetic genes, such as *aflD* and *aflR* (Al-Saad, Al-Badran, Al-Jumayli, Magan, & Rodriguez, 2016; Hua, Hernlem, Yokoyama, & Sarreal, 2015; Kachouri, Ksontini, & Hamdi, 2014). Aflatoxins undergoing the detoxification process may produce more toxic metabolites in comparison to the parent molecules and this process may be followed by the structural determination of major metabolites (Diao et al., 2012).

In recent times, the biosynthesis of aflatoxins in *Aspergillus* has been well-documented (Yu, 2012). The aflatoxin biosynthesis starts with acetate and malonyl-CoA functions as the extender. The first step is catalyzed by acetyl-CoA synthase and the elongated β-keto acyl chain is formed from β-keto polyketide catalyzed by polyketide synthases (Yu, 2012). Therefore, the first step in the inhibition of aflatoxin biosynthesis is the inhibition of the fatty acid synthase.

The aim of this study was to understand the inhibitory effects of organic acids on the growth and aflatoxin biosynthesis in *A. flavus* to use them as food additive to protect mycotoxins from foods and feedstuffs. The mode of inhibitory action exhibited by the tested organic acids was determined using RT-PCR based on the expression of genes involved in aflatoxin biosynthesis. These results provided us to prove scientific clues how organic acids differently inhibited on the aflatoxin production. And this study might give an idea to use lower amount of organic acids in foods and feedstuffs in comparison to the currently used disinfectant, propionic acid.

2. Materials and methods

2.1. Chemicals

Acetic acid, adipic acid, benzoic acid, butyric acid, citric acid, ferulic acid, formic acid, fumaric acid, gluconic acid, itaconic acid, lauric acid, DL-malic acid, oxalic acid, palmitic acid, propionic acid, sorbic acid, succinic acid, and tartaric acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals used in this study were of reagent grade quality.

2.2. Microorganisms

In this study, *A. flavus* ATCC 22546 was obtained from the Korea Culture Center of Microorganisms (Seoul, Korea). Malt extract agar (MEA; Difco, Franklin Lake, NJ) was used for the subcultures. Potato dextrose broth (PDB; Difco) was used for liquid culture media.

2.3. Measurement of antifungal activities of test compounds

A. flavus spores (equivalent to 10^6) were inoculated into 25 ml of PDB liquid culture media, followed by the addition of the test compounds. After the addition of fungal mycelia and the test compounds, the culture was incubated by shaking at 25 °C for 5 days. There were at least three replicates for each concentration of the test compound. After incubation for 5 days, the growth rates were measured by determining the mycelial and sclerotial dry weights (Moon et al., 2016). The control consisted of only solvent. pH was measured after 5 days of incubation. Arithmetic means of the three replicates were calculated and analyzed for any significant difference in comparison to the control using one-way ANOVA followed by Tukey's test, and a value of $p < 0.05$ was used to indicate a significance difference. The statistical analysis was done by using SPSS Statics for Windows V13.0 software.

2.4. Aflatoxin analysis by high-performance liquid chromatography (HPLC)

Analyses of aflatoxin B- and G-types were conducted using HPLC (Lee et al., 2002). The conditions for HPLC analysis are described previously (Lee et al., 2002).

2.5. Isolation of total RNA and RT-qPCR

A. flavus mycelia in liquid cultures were carefully harvested by filtering using a cell strainer (SPL Life Sciences Co., Ltd., Gyeonggi-do, Korea). The harvested mycelia were placed in a mortar and ground to a fine powder with an appropriate amount of liquid nitrogen. Total RNA was extracted from the *A. flavus* mycelia using QIAzol Lysis reagent (QIAGEN Inc., Hilden, Germany). Extracted RNAs were quantified by determining the absorbance at 260 and 280 nm using a μDrop™ Plate (Thermo Fisher Scientific Inc., Waltham, MA). Then, the RNA was qualitatively evaluated using agarose gel electrophoresis (1%) with ethidium bromide. Complementary DNA (cDNA) was prepared using a Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc.). The extracted RNAs (2 μg) were used for compound synthesis.

A Rotor-Gene SYBR Green PCR kit (QIAGEN Inc.) with 100 ng of cDNA was used for RT-qPCR analysis. Specific primers synthesized by Genotech (Daejeon, Korea) were used in this study to understand the relationship between aflatoxin biosynthesis and the test chemical. The primers for the genes involved in aflatoxin biosynthesis (*yap*, *aflC*, *aflD*, *aflE*, *aflG*, *aflK*, *aflL*, *aflO*, *aflQ*, *aflR*, *aflS*, and 18S

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