Inhibition of Aflatoxin B_1 Biosynthesis in *Aspergillus flavus* by Anthocyanidins and Related Flavonoids

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Anthocyanidins and precursors or related flavonoids were tested at concentrations from 0.3 to 9.7 mM (~0.1−3.0 mg/mL) for activity against growth and aflatoxin B_1 biosynthesis by *Aspergillus flavus* Link:Fr. NRRL 3357. Aflatoxin B_1 production was inhibited by all anthocyanidins tested, and 3-hydroxy compounds were more active than 3-deoxy forms. Monoglycosides of cyanidin were 40% less inhibitory than the aglycon, whereas a monoglucoside and a diglucoside of pelargonidin were 80 and 5%, respectively, as active as the aglycon. Of eight flavonoids tested, only kaempferol was moderately active, whereas luteolin and catechin were weakly inhibitory. Binary combinations of delphinidin and three other aflatoxin inhibitors acted independently of each other. Results with an aflatoxin pathway mutant indicated that anthocyanidin inhibition occurred before norsolorinic acid synthesis.

Keywords: *Aspergillus flavus; aflatoxin B_1 inhibition; anthocyanins; anthocyanidins; flavonoids*

INTRODUCTION

Aspergillus flavus Link:Fr. and *Aspergillus parasiticus* Speare are preharvest pathogens of several important food crops including maize, cotton, peanuts, and several tree nut crops (Farr et al., 1989). Many strains of *A. flavus*, and nearly all strains of *A. parasiticus*, produce aflatoxin B_1 (AFB_1) (Klich and Pitt, 1988), a potent hepatotoxin (Campbell and Stoloff, 1974) and carcinogen (Squire, 1981). Contamination of maize is endemic in many growing regions and can be severe, if sporadic, in others. Outbreaks result in economic losses and can affect public health in areas of the world where regulatory action levels are not enforced. Consequently, there is interest in developing maize lines that are either resistant to *A. flavus* infection or inhibit aflatoxin production (Davis et al., 1985). However, constraints on resistance factors in food and plant parts used as feed are greater than for nonedible parts. Therefore, if well-known naturally occurring components generally regarded as safe could be shown to control infection or toxin production, there would be less chance of encountering unexpected side effects than if compounds with a shorter history of use were to be introduced. Work by others has shown that some flavonoids are biologically active against *A. flavus* and *A. parasiticus* (DeLucca et al., 1987; Mallozzi et al., 1996), and anthocyanins may be involved in maize resistance to corn earworm (Wilson et al., 1995), anthracnose (Hammerschmidt and Nicholson, 1977), and inhibition of *Fusarium solani* spore germination (Kraft, 1977). Although anthocyanins are present in kernels of some maize lines, no examination of their effect on aflatoxin has been reported. The following studies were undertaken to assess the possibility of using kernel components, or their precursors, as part of a strategy for AFB_1 control and to provide additional information on structure/activity requirements for aflatoxin inhibition. Results showing the effect on growth and AFB_1 production of anthocyanidins (anthocyanin aglycons) representative glycosides (anthocyanins) and precursors or structurally related flavonoids are presented.

EXPERIMENTAL PROCEDURES

Compounds. Anthocyanidins, anthocyanins, eriodictyol, taxifolin, and luteolin were obtained from Indofine Chemical Co., Inc., Somerville, NJ, and were 94−100% pure as determined by HPLC by the supplier. Remaining compounds were obtained from Sigma Chemical Co. (St. Louis, MO) and had a similar range of purity. 4-ABOA was a gift from Dr. David Miller (McGill University). All compounds were used as supplied and stored at −80 °C upon receipt and when not in use.

Assays. Compounds were tested using suspended disk cultures prepared and grown under conditions previously described (Norton, 1997) with a medium pH of 4.7. Briefly, the culture system consisted of a 20 mL scintillation vial with an open top cap fitted with a Teflon-coated septum. The septum was pierced by a pin to which a 6 mm glass fiber disk was affixed. The disk, containing the test compound, medium, and inoculum, was placed in a vial that was humidified with 1 mL of sterile water in the bottom. Flavonoids were dissolved in ethanol, filter sterilized, and then pipetted onto the disks inside a sterile biological hood. Disks were then placed in a sterile desiccator, and the ethanol was removed under reduced pressure. Each disk received 29 μL of medium, containing 10000 spores/mL, that was applied to the top and bottom of the disk. Glycosides were dissolved in DMSO for application and evaporated under reduced pressure in a sterile desiccator containing Drierite; 4-ABOA was dissolved in acetone and the solvent removed as for ethanol. Anthocyanidins were dissolved in medium and filter sterilized and then spores added. Except as noted, each experiment is the average of 10 replicates involving two separate incubations of 5 replicates at each concentration. Each incubation used conidia from different subcultures of fungus. Concentrations were selected on the basis of results from preliminary experiments with pelargonidin, which showed the range for no effect and maximum activity. All other compounds were then tested at the same molar concentrations with equal percentage changes between

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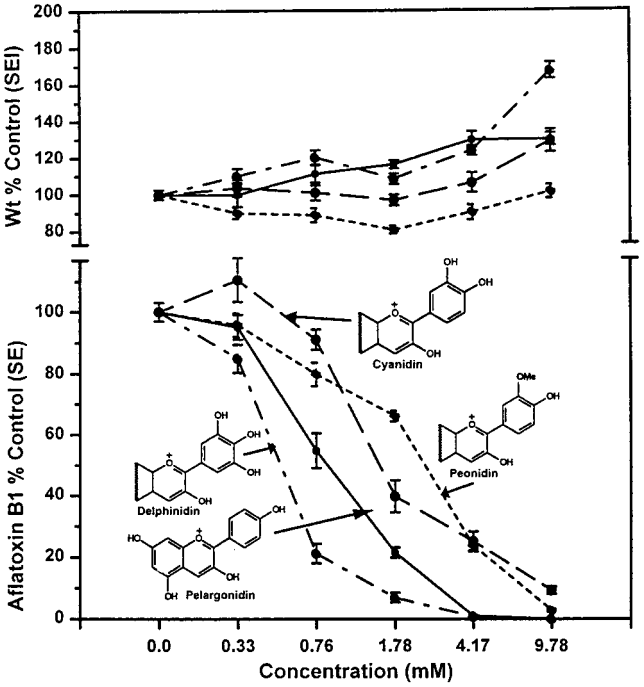

Figure 1. Effect of anthocyanidins on growth and AFB_1 production by *A. flavus* NRRL 3357. Concentration is a log scale except for the control point (0.0). Vertical lines represent standard error. Vertical lines represent 95% confidence interval. Controls were 149.7 and 197.2 μg/mL of medium for incubations 1 and 2, respectively.

each level so they could be plotted as a log plot on an abscissa with an equal interval scale. Because aflatoxin levels may vary appreciably from incubation to incubation, results were reported as a percentage of the control (0 concentration) for each experiment. *A. parasiticus* SRRC 162, an aflatoxin pathway mutant that accumulates norsolorinic acid, was obtained from Dr. Nancy Keller, Texas A&M University, College Station, TX. *A. flavus* NRRL 3357 was obtained from the USDA-ARS, National Center for Agricultural Utilization Research, Peoria, IL.

Aflatoxin Analysis. Aflatoxins were extracted and quantitated by HPLC as described previously (Norton, 1995), except that analysis was isocratic using water/acetonitrile (69:31, v/v). Although *A. flavus* NRRL 3357 produced small amounts of AFB_2 in addition to AFB_1, these data would not have affected the interpretation of the data and were not tabulated. Norsolorinic acid was extracted and analyzed as described previously (Norton, 1997).

RESULTS AND DISCUSSION

The effect of anthocyanidins on AFB_1 production and growth is shown in Figures 1 and 2. Inhibition of AFB_1 was similar at the highest levels for pelargonidin and delphinidin, where nearly complete inhibition occurred; however, at lower levels delphinidin was more inhibitory. Activity was less for cyanidin, which was similar to malvidin. Highest inhibition was obtained with 3-OH compounds, which were ~3 times more active than the related 3-deoxy compounds. The presence of one B-ring *O*-methyl group on peonidin resulted in inhibition generally similar to that of cyanidin; however, malvidin (with two methoxy groups) was significantly less active than delphinidin. Growth was stimulated by the highest levels of most of the 3-OH compounds but not by the 3-deoxy compounds.

Anthocyanidins are normally not found in maize; if these pigments are present, then they nearly always occur as glycosides (i.e., as anthocyanins). There are two

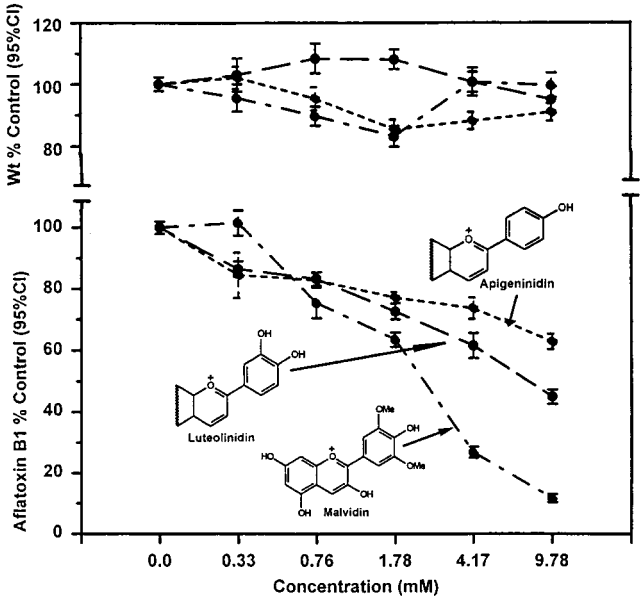

Figure 2. Effect of malvidin and 3-deoxy anthocyanidins on growth and AFB_1 production by *A. flavus* NRRL 3357. Concentration is a log scale except for the control point (0.0). Vertical lines represent 95% confidence interval. Controls were 171.6 and 197.8 μg/mL for incubations 1 and 2, respectively.

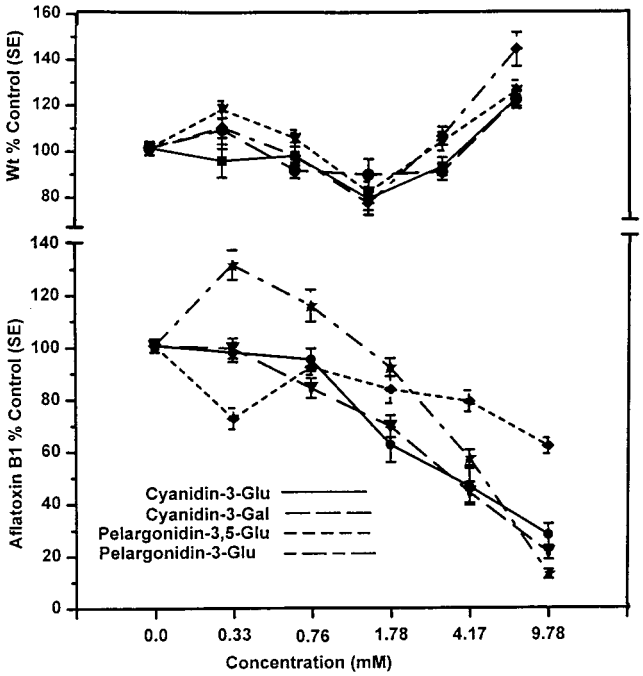

Figure 3. Effect of glycosylated pelargonidin and cyanidin on AFB_1 production by *A. flavus* NRRL 3357. Concentration is a log scale except for the control point (0.0). Vertical lines represent standard error. Controls were 91.1 and 113.4 μg/mL for incubations 1 and 2, respectively.

other anthocyanins found in some maize lines, cyanidin 3-mono- and 3-dimalonylglucosides (Harborne and Self, 1987), but the levels and activity of these are unknown. To assess the relevance of the inhibition found for the aglycons, the two main glycosides that occur in maize, pelargonidin and cyanidin 3-glucosides (Harborne and Gavazzi, 1969), were tested for activity. In addition, a mono- and diglycoside not reported in maize kernels was tested (Figure 3). The monoglycosides of cyanidin were similar in activity, indicating that the specific sugar, glucose or galactose, made little difference. Pelargonidin

3-glucoside was less inhibitory than cyanidin 3-glucoside except at the highest level, in contrast to the results found for the aglycons, where it was more active (Figure 1). The concentration producing 50% inhibition for both cyanidin monoglycosides was ~3.5 mM as compared to 1.5 mM for the aglycon. For the mono- and diglucosides of pelargonidin 50% inhibition values were 4.8 mM and, by extrapolation, 17 mM. For the aglycon this value was 0.9 mM. The ratios between the aglycon and the monoglucoside and between the monoglucoside and the diglucoside were similar—indicating that the addition of each sugar decreased the activity of the compounds by 50 and 80% for cyanidin and pelargonidin, respectively. Mycelium incubated with the aglycons or the glycosides was intensely dark colored after extraction with chloroform and then autoclaving in water. Additional extractions with ethanol and methanol had no effect on color. In addition, at the end of the incubation the mycelium was considerably darker in color than the disks were at the beginning of the experiment. Areas on the disk without growth were colorless—suggesting either that the compound was concentrated within the mycelium or that the compound in solution had equilibrated into colorless forms. These observations suggest that the glycosides were taken up by the mycelium but converted to an insoluble form which was considerably less active.

The decrease in inhibition for the glycosides suggests that hydrolysis of the sugars to produce the more active aglycon occurred slowly, if at all, during the time of aflatoxin biosynthesis. It also suggests that the 3,5-positions were approximately similar in susceptibility to hydrolysis, because the decreases in inhibition were similar for each position, that is, 80%. The change in inhibition for the 3-glucosides of cyanidin and pelargonidin relative to the aglycons further indicates that hydrolysis did not occur to a significant extent.

The large differences in activity found for the anthocyanidins and the effect of B-ring hydroxylation suggested an examination of 3′-, and 3′,4′-OH compounds with either a saturated or unsaturated heterocyclic ring. Several of these compounds are intermediates in anthocyanidin biosynthesis, and if any showed activity against *A. flavus* or aflatoxin synthesis, then corn lines accumulating the compound(s) could be useful for control. The results for these flavonoids are shown in Figures 4 and 5. Of the eight compounds tested, only kaempferol was moderately inhibitory and had activity similar to that of malvidin. Luteolin and catechin were weak inhibitors, but none of the other compounds in this group inhibited aflatoxin appreciably, although some showed moderate inhibition of growth. Kaempferol and narigenin were reported by Mallozzi et al. (1996) to inhibit both growth and AFB_1 in *A. flavus* NRRL 6513, but at concentrations ~$^1/_{20}$ of those found here. Quercetin was tested by Mallozzi et al. (1996), and they also found no activity. Paster et al. (1988) evaluated catechin at concentrations up to 10 mmol/L and found little effect on an *A. flavus* strain. DeLucca et al. (1987) reported no activity for eriodictyol against *A. parasiticus*; however, they found that luteolin inhibited aflatoxin. Weidenborner et al. (1990) reported little activity for quercetin and naringenin, although they did report activity for other flavonoids against a range of fungi, including *A. flavus*, and suggested that high activity was correlated with a lack of polar hydroxy groups. However, this concept does not appear to explain the results found here: apigenin and naringenin have the fewest hydroxy groups but were inactive. Combinations of eriodictyol, lutein, and 5,7-dihydroxychromone were found by DeLucca et al. (1987) to be more active than single compounds against aflatoxin formation by *A. parasiticus*, and synergistic effects between other flavonoids were also reported by Weidenborner and Jha (1993). These investigators concluded that mixtures of polar and nonpolar flavonoids might be more active than single compounds. In the present work, the only clear structure/activity correlation for flavonoids seems to be that flavones as a group are inactive—regardless of

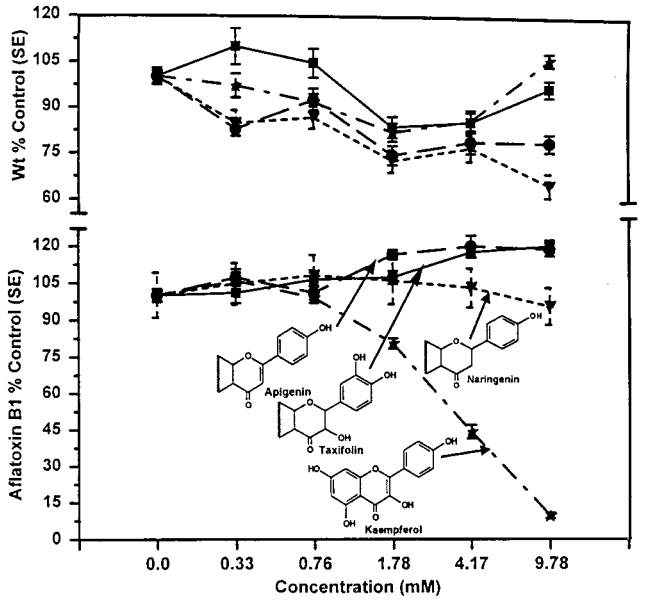

Figure 4. Effect of taxifolin and 4′-hydroxy flavonoids on growth and AFB_1 production by *A. flavus* NRRL 3357. Concentration is a log scale except for the control point (0.0). Vertical lines represent standard error. Controls were 148.2 and 150.9 μg/mL for incubations 1 and 2, respectively.

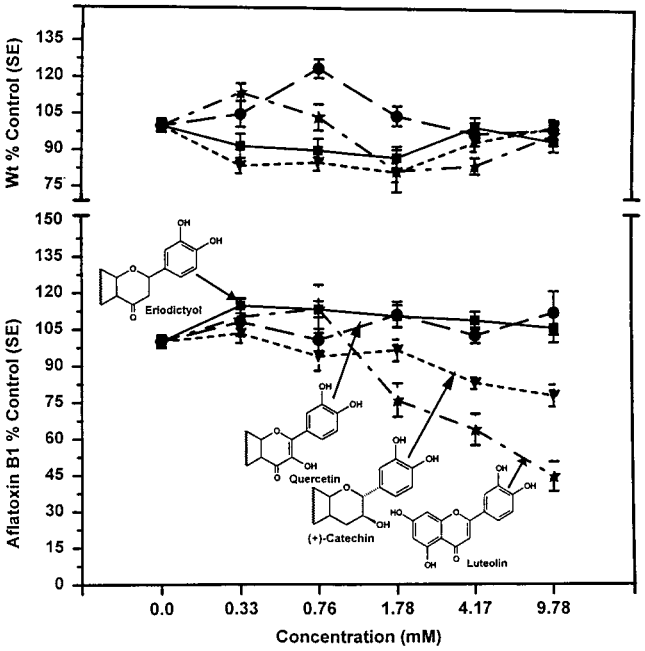

Figure 5. Effect of 3′,4′-dihydroxy flavonoids on growth and AFB_1 production by *A. flavus* NRRL 3357. Concentration is a log scale except for the control point (0.0). Vertical lines represent standard error. Controls were 135.0 and 150.8 μg/mL for incubations 1 and 2, respectively.

Table 1. Effect of Combined Aflatoxin Inhibitors on *A. flavus* NRRL 3357

compound	concn (μg/mL)	AFB_1[a] % ctl	expect $A \times B$[b]	wt[c] % ctl	expect $(A+B)/2$[d]
Experiment 1[e]					
β-carotene (β-C)	50	60		119	
pelargonidin (Pel)	230	33		124	
kaempferol (K)	1200	62		97	
β-C + Pel		18	20	115	122
β-C + K		33	37	102	108
Pel + K		14	21	110	110
Experiment 2					
β-C	50	41		81	
4-ABOA[f]	100	19		102	
Pel	230	40		83	
K	1200	57		75	
β-C + 4-ABOA		9.5	7.9	109	92
K + 4-ABOA		15.3	11.0	80	88
Pel + 4-ABOA		10.0	7.9	99	93

[a] Control was 91.7 μg/mL for experiment 1 and 113.2 μg/mL for experiment 2. [b] Expected value using the product of the individual results. [c] Control was 4.13 mg/mL for experiment 1 and 3.22 mg/mL for experiment 2. [d] Expected value using the average of the individual results. [e] Each experiment is the average for 10 replicates for each compound or pair. [f] 4-Acteylbenzoxazolin-2-one.

B-ring hydroxylation. Comparison of the activities for cyanidin (Figure 1), luteolinidin (Figure 2), and eriodictyol (Figure 5) indicates that the most active structures result from isomerization after deprotonation of the flavylium nucleus. Interactions between anthocyanins, copigments, and metal ions have been reported (Asen et al., 1972; Goto et al., 1976) and might enhance the activity of these compounds in maize, but these studies have not been done.

The possibility of interactions between flavonoids, anthocyanidins, and other compounds found in maize kernels that inhibit aflatoxin was investigated by examining kaempferol and delphinidin and two other AFB_1 inhibitors from maize: 4-acetylbenzoxazolin-2-one (4-ABOA) (Miller et al., 1996) and β-carotene (Norton, 1997). Each compound was tested alone, at a concentration expected to give ~50% inhibition, and with each of the other compounds. The number of combinations required that the experiment be done in two parts with the single compounds (except 4-ABOA) common to both groups. These results are shown in Table 1. The expected level of AFB_1 was calculated as the product of the individual inhibitions, that is, with no interaction assumed, and the expected effect on growth was calculated as the average of the two values. Most values were within 10% of the expected value for AFB_1 inhibition, indicating that there was little interaction. Growth was also generally within 10% of the expected value, although growth was affected much less than toxin synthesis.

If an aflatoxin inhibitor is to be of potential value in a food crop, it should act at the beginning of or early in the biosynthetic pathway because several of the intermediates are also toxic. We recently used an *A. parasiticus* aflatoxin pathway mutant partially blocked at norsolorinic acid (NA) to show that α-carotene inhibited NA and AFB_1 to similar extents (Norton, 1997), suggesting that inhibition was at or before the polyketide synthase step. NA is the first isolatable product of the aflatoxin pathway (Townsend, 1986). A similar experiment was done with two of the inhibitors found in the present study, delphinidin and kaempferol (Table 2). Delphinidin was similar to α-carotene in that it strongly inhibited both NA and aflatoxin, suggesting that inhibition occurs before NA synthesis. Kaempferol, however, stimulated aflatoxin synthesis in this fungus strain.

Table 2. Effect of Delphinidin, α-Carotene, and Kaempferol on Aflatoxins and Norsolorinic Acid Production by *A. parasiticus* Mutant SRRC 162[a]

	NA[b] (% ctl[c])	AFB_1 (% ctl)	AFB_2 (% ctl)	AFG_1 (% ctl)	AFG_2 (% ctl)
delphinidin[d]	2	10	0.6	1	nd[e]
α-carotene	8	22	35	3.5	7
kaempferol	110	157	259	124	148

[a] Each value is the average for five replicates. [b] Norsolorinic acid. [c] Aflatoxin B_1 control = 9.2 μg/mL. [d] Delphinidin concentration = 4.2 mg/mL, α-carotene = 1 mg/mL, and kaempferol = 9.8 mg/mL. [e] Not detected.

Only at a pH <2 do anthocyanins and their aglycons exist in solution as the flavylium cation form (AH^+) shown in Figures 1 and 2; at higher pH values the cation occurs in equilibrium with the deprotonated purple quinoid (A) and the colorless carbinol pseudobase (water 2−adduct, B) and chalcone (C) forms (Mazza and Miniati, 1993). Can an inference be drawn about which of the possible forms was most likely involved in the inhibition shown above? The equilibrium distribution depends primarily on the hydroxyl, glycosyl, and methoxyl pattern of the flavylium nucleus, pH, temperature, and copigments (Mazza and Miniati, 1993). The pH of the culture medium was >2; therefore, there would have been a mixture of related compounds present. The temperature and pH of the medium were 25 °C and 4.7, respectively, and the only likely copigment present at levels sufficient to affect color was 76 mM asparagine in the medium. Asen et al. (1972) has shown that some amino acids can act as copigments and that the copigment concentration, molar ratio to pigment, and pH affect the equilibrium mixture of forms. These investigators found that as pH increased, absorbance decreased, but copigment offset part of the decrease. Absorbance in the pH range 4.15−5.1 with copigment was ~25% of the absorbance at pH 3.16, with most of the absorbance from the stabilized quinoid form. Absorbance also increased as the copigment/pigment ratio and pigment concentration increased. The ratio in our medium would have been from 7.8 (9.8 mM pigment) to 230 (0.33 mM). The increase in absorbance by the amino acids tested by Asen et al. (1972), which did not include asparagine, ranged from 3 to 25% at a molar ratio of 3:1. We observed that the color of the solutions faded as they dried on the assay disks but returned upon addition of medium. There probably was, then, a copigment effect from the asparagine in the medium, and this would have tended to increase the concentration of the colored quinoid form.

Variation in substitution patterns for the compounds is the other variable that would determine the equilibrium of the different forms of the test compounds. Of the anthocyanidins tested, an equilibrium distribution of the various forms was found in the literature only for apigeninidin and the related compounds cyanidin 3,5-diglucoside and malvidin 3-glucoside. Assuming a similar distribution for the concentration and medium used for the present work, at pH 4.7 the percentages for apigeninidin would be as follows: (AH^+) 13%, (A) 53%, (B) 8%, and (C) 26% (Brouillard et al., 1982). For cyanidin diglucoside the distributions are as follows: (AH^+) <1%, (A) 6.5%, (B) 80%, and (C) 13.5% (Mazza and Brouillard, 1987). For malvidin 3-glucoside the

distributions are (AH^+) 2.3%, (A) 3.7%, (B) 83%, and (C) 11% (Brouillard and Delaporte, 1978). If these values are comparable for the glycosides tested here, and given that 3-hydroxy anthocyanidins are less stable (levels of the colored forms are lower) than the structurally related 3-deoxy forms (Mazza and Miniati, 1993) and that asparagine in the medium probably increased the concentration of the quinoid form, then the cation is unlikely to be the active form; otherwise, apigeninidin would have shown more activity. A similar argument would hold for the quinoid, which should be the principle form for apigeninidin. Malvidin and cyanidin were similar in activity, but cyanidin 3-glycoside inhibition was less than for the aglycon. However, glycosylation tends to stabilize the quinoid form and prevents isomerization to the α-diketone. Therefore, the data suggest that the colorless carbinol (B), or possibly the α-diketone (Timberlake and Bridle, 1975), was most likely the active form.

In conclusion, pelargonidin and cyanidin 3-glucosides were the most active of the compounds tested that are known to occur in maize kernels. Are these compounds found in maize at levels that could affect aflatoxin levels by themselves? The combined concentration of these two compounds in aleurone tissue of mature maize kernels calculated from data by Larson et al. (1986) yields a range of 17.3−29.5 μM. Inspection of Figure 3 shows that at this concentration little, if any, inhibition would be expected. However, other *A. flavus* strains might be more sensitive [cf. β-carotene (Norton, 1997) and kaempferol (Malozzi et al., 1996)]. Aleurone tissue accounts for only ∼5% of the kernel weight. If we assume toxin is produced proportionately in this layer, then the anticipated effect would have to be small in any event. In general, tissue-specific toxin inhibitors (as opposed to fungal growth or spore germination inhibitors) would not be expected to have a significant effect except in germ and, secondarily, endosperm tissue. Assuming that the concentration of the active molecular form(s) must have been lower than the level tested, then inhibition by the active form would have been higher than shown. The relatively high activity for delphinidin and pelargonidin, coupled with the indication that these compounds act at the beginning of the pathway, and their general acceptance in foods suggest that further investigation is warranted on the mode of action of these compounds as well as possible interactions with copigments and other flavonoids which might enhance their activity.

ABBREVIATIONS USED

AFB_1, aflatoxin B_1; 4-ABOA, 4-acetylbenzoxazolin-2-one; NA, norsolorinic acid.

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