Quantitative Analysis of Anticancer 3-Deoxyanthocyanidins in Infected Sorghum Seedlings

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3-Deoxyanthocyanidins are structurally related to the anthocyanin pigments, which are popular as health-promoting phytochemicals. Here, it is demonstrated that the 3-deoxyanthocyanidins are more cytotoxic on human cancer cells than the 3-hydroxylated anthocyanidin analogues. At 200 μM concentration, luteolinidin reduced the viability of HL-60 and HepG2 cells by 90 and 50%, respectively. Sorghum is a major source of 3-deoxyanthocyanidins, which are present as seed pigments and as phytoalexins responding to pathogen attack. On the basis of the collision-induced dissociation spectra of luteolinidin and apigeninidin, an LC-MS/MS method, operating in multiple-reaction monitoring mode, was developed for the specific detection and accurate quantification of these compounds in complex mixtures, which may be difficult to analyze using absorbance measurements. The results demonstrated that inoculated sorghum seedlings could be utilized for convenient and large-scale production of 3-deoxyanthocyanidins. A quantity of almost 270 μg/g (fresh weight) of luteolinidin was produced 72 h after fungal inoculation of 1-week-old seedlings.

INTRODUCTION

3-Deoxyanthocyanidins are a rare type of flavonoids restricted to a few plant species. They are the major pigments in flowers of sinningia (*Sinningia cardinalis*) (*1*) and are found in silk tissues of certain maize lines (*2*). Sorghum is the only dietary source for 3-deoxyanthocyanidins, which are present in large quantities in the bran of some cultivars (*3*). Many plants use secondary metabolites to protect themselves against pathogen attack. In sorghum, this defense response is an active process resulting in the accumulation of high levels of 3-deoxyanthocyanidin phytoalexins in infected tissues (*4, 5*). Luteolinidin and apigeninidin are the two major 3-deoxyanthocyanidins, and they are structurally related to anthocyanidins (aglycones of anthocyanins) except for the absence of C-3 hydroxylation in the C ring (**Figure 1**).

Flavonoids are increasingly recognized for their range of health benefits, such as reducing the risks of cardiovascular diseases and cancers due to their antioxidant, anti-inflammatory, and chemoprotective properties (*6, 7*). In particular, anthocyanins and anthocyanidins from different sources were shown to suppress proliferation and induce apoptosis in cancer cell lines

Table 1. Major Working Parameters for MRM Analysis

parameter	value
ion spray voltage (V)	4000
source temperature (°C)	500
dwell time per transition (ms)	200
nebulizing gas (gas 1) (arbitrary unit)	45
turbo spray gas (gas 2) (arbitrary unit)	65
curtain gas (gas 1) (arbitrary unit)	50
collision gas (nitrogen) (psi)	10
entrance potential (V)	12
declustering potential (V)	60
focusing potential (V)	350
collision energy (V)	40
collision cell entrance potential (V)	15.3
collision cell exit potential (V)	8

(*8−10*). In a recent study, 3-deoxyanthocyanidins were found to have antioxidant properties similar to those of anthocyanins, but they are more stable to pH, temperature, and light changes, suggesting that they could serve as an alternative source of natural pigments with nutraceutical properties (*3*). Anthocyanins and 3-deoxyanthocyanidins in plant samples are routinely detected through absorbance measurements at visible wavelengths, usually following HPLC separation. These compounds, however, exhibit overlapping absorption spectra and similar retention properties (*11*), making it difficult to obtain distinct elution peaks for individual components, even after long LC separation times (*12, 13*). Previously, we have employed matrix-

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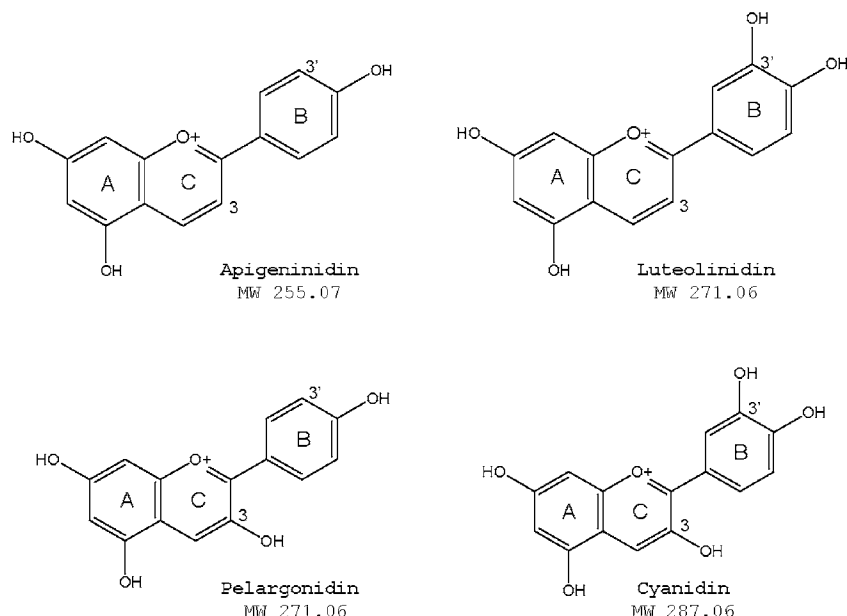3-Deoxyanthocyanidins in Infected Sorghum

J. Agric. Food Chem., Vol. 55, No. 2, 2007 **255**

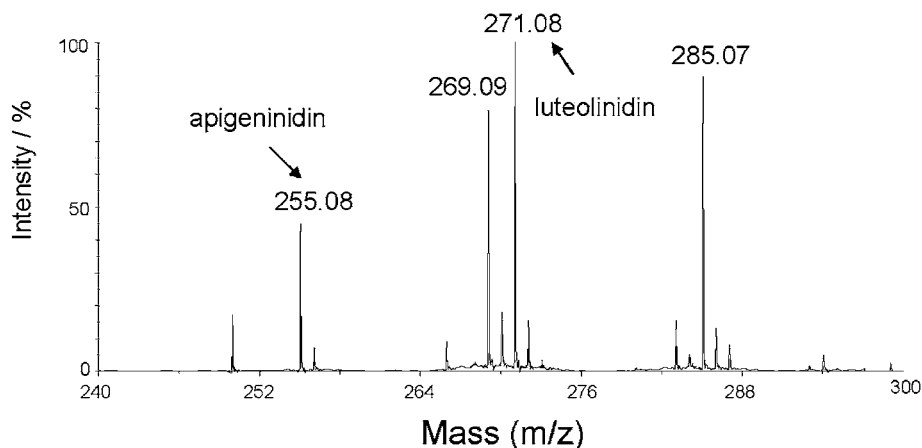**Figure 1.** Structures of 3-deoxyanthocyanidins and anthocyanidins described in this study.

Figure 2. MALDI-TOF analysis of a methanol extract prepared from inoculated sorghum seedlings. Major ions correspond to the MWs of apigeninidin (255) and luteolinidin (271). The ions at *m/z* 269 and 285 are believed to be methylated derivatives of apigeninidin and luteolinidin, respectively.

assisted laser desorption coupled with time-of-flight (MALDI-TOF) to detect these flavonoid pigments in crude plant extracts (*14*).

To further explore the biological activities of 3-deoxyanthocyanidins, cytotoxic activities of luteolinidin and apigeninidin on two human cancer cell lines, in comparison to the anthocyanidins, were investigated in this study. In addition, we developed a simple, sensitive, and specific method using LC coupled with tandem mass spectrometry (LC-MS/MS) in multiple-reaction monitoring (MRM) mode for accurate detection and quantification of 3-deoxyanthocyanidins in complex mixtures derived from inoculated sorghum seedlings. The LC-MS/MS method will be particularly useful for the analysis of these pigmented compounds in colored biological matrices that may affect analyses performed using common absorbance techniques.

MATERIALS AND METHODS

Plant and Fungal Materials. Sorghum (*Sorghum bicolor*) hybrid seeds and fungal strains used were kindly provided by R. Nicholson (Purdue University, West Lafayette, IN). The sorghum cultivars DK18 and DK46 have brown pericarp, nonpigmented testa, and white

endosperm. They were well characterized in their anthocyanin accumulation and 3-deoxyanthocyanin phytoalexin response in mesocotyl tissues (*5*, *11*). Seeds were planted in rolls of germination paper and kept in the dark for 4 days at 28 °C. Etiolated seedlings with elongated mesocotyls were inoculated with conidial suspensions of *Colletotrichum sublineolum* isolate TX430BB (1.0 × 10⁶ conidia/mL) or *Cochliobolus heterotrophus* isolate Hmo-120 (1.0 × 10⁵ conidia/mL). Infected seedlings were then incubated at 100% relative humidity under constant light at room temperature.

Chemicals and Reagents. Pure authentic standards of 3-deoxyanthocyanidin (luteolinidin and apigeninidin) and anthocyanidin standards (cyanidin and pelargonidin) were purchased from Extrasynthase (Genay, France) and Alexis (Carlsbad, CA), respectively.

MALDI-TOF and MS-MS Analysis. Mesocotyl tissues of inoculated plants were excised and placed in HPLC-grade methanol. The compounds were allowed to leach into the methanol overnight at 4 °C. The methanol extracts were analyzed by a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems Perceptive, Framingham, MA) operating in positive mode with a nitrogen laser (337 nm) at an accelerating voltage of 2 kV. The matrix used was α-cyano-4-hydroxycinamic acid (10 mg/mL in 50% ACN, 50% ddH₂O with 0.1% acetic acid). Samples were diluted 10-fold and mixed with the matrix solution in a ratio of 1:1. After MALDI-TOF analysis, selected ion species were subjected to MS/MS fragmentation in a QSTAR XL

256 *J. Agric. Food Chem.,* Vol. 55, No. 2, 2007

Shih et al.

quadrupole/TOF hybrid mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, ON, Canada) equipped with an o-MALDI source. Collision-induced dissociation (CID) spectra were acquired using argon as the collision gas under collision energies of 30 V. High-purity nitrogen gas (99.995%) was used as curtain gas.

Cell Cultures and Cytotoxicity Assays. Human leukemia HL-60 and hepatoma HepG2 cell lines (ATCC, Rockville, MD) cells were grown in RPMI-1640 medium containing 0.2% sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (GibcoBRL, Gaithersburg, MD). Test compounds, dissolved in DMSO, were added to the culture medium to achieve the desired concentrations. The final concentration of DMSO in the medium was 0.2%, and it did not affect the viability of the two cell lines under investigation. All culture plates were incubated in a humidified incubator at 37 °C under 5% CO_2. HL-60 cells were inoculated at a density of 5×10^4 cells/mL in the culture medium supplemented with test samples in 96-well round plates. After 48 h of incubation, aliquots of the treated cells were counted with a hemocytometer under a microscope. Cell viability was estimated using the trypan blue dye exclusion method. The adherent HepG2 cells were first inoculated at a density of 2.5×10^4 cells/mL in 96-well flat bottom plates for 24 h, after which the test samples were added. After 48 h of incubation, cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 5 h of incubation with MTT (5 mg/mL) (Sigma, St. Louis, MO), the blue formazan crystals formed were dissolved by isopropanol in 0.04 N HCl. Absorbance of the blue formazan solution was measured by a microplate reader at 570 nm.

LC-MRM Analysis. An LC-MS/MS system operated in multiple-reaction monitoring (MRM) mode was performed for quantification of luteolinidin and apigeninidin in the plant extracts. Filtered samples (20 μL) were injected into a Perkin-Elmer HPLC system (PE200 micropump) equipped with a Zorbax Eclipse XDB-C18 column (5 μm, 150 × 2.1 mm; Agilent Technologies). Elution was performed using a mobile phase of 0.5% (v/v) formic acid (A) and 100% methanol (B), with a linear gradient of 20−65% B over 15 min, followed by an increase to 100% B over 5 min, which was then held at 100% B for 5 min. The flow rate was maintained at 0.2 mL/min, and elution was analyzed by monitoring the transition reactions of both apigeninidin (m/z 255→171) and luteolinidin (m/z 271→187). MRM experiments were conducted on the heated Turbo Ion Spray interface of an API-2000 triple-quadrupole mass spectrometer (Applied Biosystems) operated in positive ion mode. MS conditions were optimized to achieve maximum sensitivity (**Table 1**). Quantification of apigeninidin and luteolinidin was based on the LC-MRM peak area. Calibration curves ($R^2 > 0.99$) over the range of 0.5−10 μg/mL for apigeninidin and 10−160 μg/mL for luteolinidin were used for calculations. Data acquisition, peak integration, and calculation were interfaced to a computer workstation running the Analyst 1.3.1 software (Applied Biosystems).

RESULTS AND DISCUSSION

MALDI-TOF and MS-MS Analysis of Sorghum 3-Deoxyanthocyanidins. Sorghum is a major source of the 3-deoxyanthocyandins, a unique class of flavonoids. The presence of flavylium cation in the C rings of anthocyanidins and 3-deoxyanthocyanidins allows these compounds to be detected readily with their actual MWs recorded when using positive mode mass spectrometry (*15*). Previously, we demonstrated that MALDI-TOF, which employs a soft ionization technique widely used in current proteome studies, was sensitive for the detection of anthocyanidins and 3-deoxyanthcyanidins in crude plant extracts (*14*). In this study, MALDI-TOF data (**Figure 2**) for crude extracts from inoculated sorghum seedlings revealed the MWs for apigeninidin (m/z 255) and luteolinidin (m/z 271). The ions at m/z 269 and 285 are believed to represent the methyl ethers of apigeninidin and luteolinidin, respectively (*11*). To positively confirm the identities of apigeninidin and luteolinidin in the sorghum extracts, selected ions detected in the MALDI-TOF
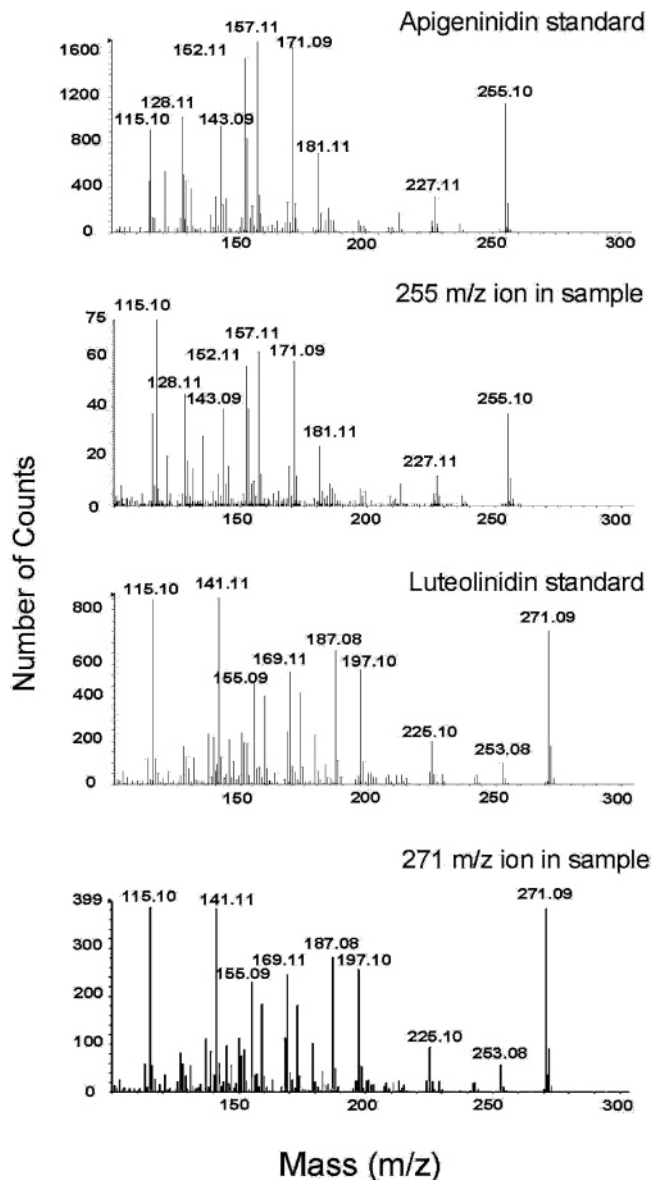

Figure 3. MS/MS CID spectra for 3-deoxyanthocyanidin standards and the selected ion species (m/z 255 and 271) detected in the methanol extracts prepared from infected sorghum seedlings.

spectrum were subjected to CID to obtain their product ion profiles. The CID spectra for the ions at m/z 255 and 271 were essentially identical to those obtained with authentic standards of apigeninidin and luteolinidin, respectively (**Figure 3**). Luteolinidin differs from apigeninidin by the presence of an additional 3′-hydroxylation group on the B ring (**Figure 1**). Consistent with this, a number of the major product ions in the luteolinidin CID spectrum are heavier (by m/z 16) than those in the apigeninidin CID spectrum, suggesting that they were readily generated from the B ring during low-energy CID.

Cytotoxic Activities on Human Cancer Cells. A number of flavonoids, including anthocyanins, have been shown to exhibit a variety of health benefits, such as chemoprotective properties. In this study, the 3-deoxyanthocyanidins were tested, for the first time, on their cytotoxicity toward two human cancer cell lines at various concentrations. The HL-60 cell line, derived from a patient with acute leukemia, is a commonly used in vitro model for investigations of anticancer agents. As shown in **Figure 4**, both luteolinidin and apigeninidin exhibited stronger cytotoxicity on the HL-60 cells when compared to their
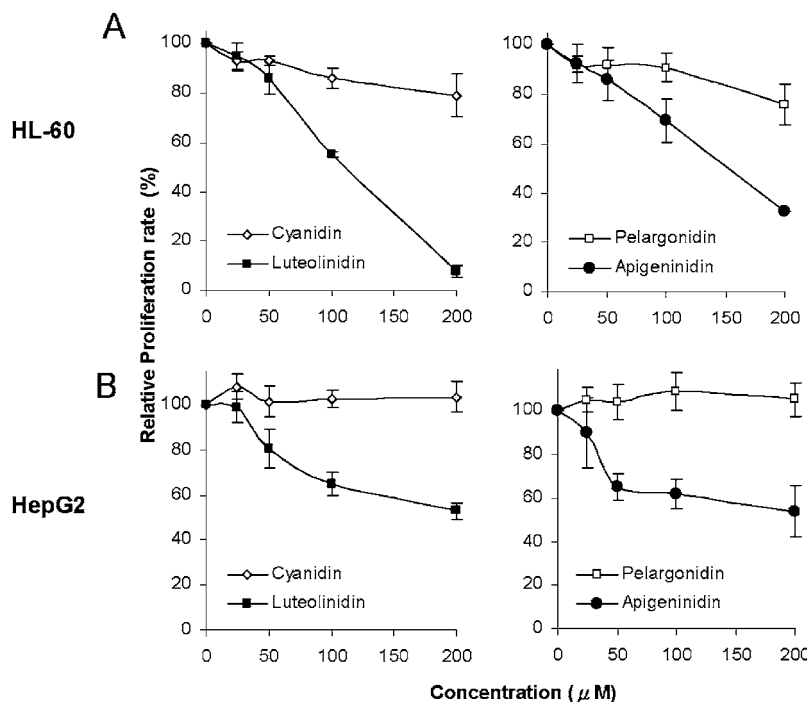
Figure 4. Cytotoxic effects of 3-deoxyanthocyanidins and anthocyanidins. HL-60 (**A**) and HepG2 (**B**) cells were incubated with different concentrations of the test compounds for 48 h. The 3-deoxyanthocyanidins (luteolinidin and apigeninidin) were more effective in reducing the viability of the human cancer cells than the anthocyanidins (cyanidin and pelargonidin). Control cultures without treatments were regarded as 100% proliferation. Note the dose-dependent reduction of viability of HL-60 cells by the 3-deoxyanthocyanidins. Data are expressed as mean ± SD of triplicate experiments.

Table 2. Amounts of Luteolinidin (L) and Apigeninidin (A) Accumulated in Sorghum DK18 and DK46 Seedlings Following Fungal Inoculations[a]

	time after inoculation with *C. sublineolum*		time after inoculation with *C. heterotrophus*	
	72 h	96 h	48 h	72 h
DK18	161.8 ± 29.0 (L); 13.1 ± 2.8 (A)	177.5 ± 18.6 (L); 13.6 ± 1.2 (A)	90.2 ± 7.6 (L); 16.7 ± 1.1 (A)	268.1 ± 27.9 (L); 35.0 ± 2.8 (A)
DK46	116.2 ± 8.4 (L); 10.25 ± 1.4 (A)	111.9 ± 30.0 (L); 8.34 ± 0.7 (A)		

[a] DK18 plants were infected with two different pathogens. Values are expressed as mean ± SD, μg/g of fresh weight (triplicate measurements).

3-hydroxylated anthocyanidin analogues (i.e., cyanidin and pelargonidin, respectively). Luteolinidin reduced the viability of HL-60 cells by >90% at 200 μM, whereas cyanidin reduced the viability by only around 20% at the same concentration. Apigeninidin was less effective than luteolinidin (around 70% reduction at 200 μM), but it was still stronger than pelargonidin in reducing the viability of the HL-60 cells (**Figure 4**). It is interesting to note that as a phytoalexin, luteolinidin was found to be more toxic than apigeninidin toward a number of phytopathogenic fungi (*5*, *16*). HepG2 is an adherent cell line derived from liver tissues of a hepatocarcinoma patient. Overall, cytotoxic activities of the 3-deoxyanthocyanidins on the HepG2 cells were not as effective as they were on the HL-60 cells. At 200 μM, luteolinidin and apigeninidin reduced the viability of the cancer cells by around 40% (**Figure 4**). On the other hand, cyanidin and pelargonidin did not show cytotoxicity on the HepG2 cells at all concentrations tested. Taken together, our results suggested that the 3-deoxyanthocyanidins are more effective than anthocyanidins in reducing the viability of the human cancer cell lines tested in this study. The lower cytotoxicity of anthocyanidins could result from the presence of C-3 hydroxylation, which is known to cause instability in these pigments (*17*).

LC-SRM Analysis and Quantification. There are increasing interests in natural colorants with functional properties (*3*), and the 3-deoxyanthocyanidins represent good candidates with potential anticancer activities useful in nutraceutical applications.

Sorghum produces significant amounts of 3-deoxyanthocyanidins following pathogen attack, in addition to accumulation in the bran. For example, microspectrophometric analysis revealed that luteolinidin accumulated to levels of 0.48−1.20 ng per infected cell (*18*). Here, we were intrigued to determine accurately the amounts of the anticancer 3-deoxyanthocyanidins that could be produced in our inoculated mesocotyl system.

Pigmentations (and hence absorbance) of anthocyanins and 3-deoxyanthocyanidins is highly pH-dependent (*3*). In addition, HPLC separation of a mixture of these compounds usually does not result in distinct elution peaks (*12*, *13*). Thus, accurate quantification of individual pigments in crude extracts may become difficult with the use of LC coupled with absorbance measurements. In recent years, the use of LC coupled with MS operating in MRM mode has emerged as a simple and sensitive means for the detection of specific metabolites in complex extracts (*19−21*). On the basis of their CID spectra (**Figure 3**), we selected the key reactions m/z 255→171 and 271→187 for MRM detection of apigeninidin and luteolinidin, respectively, in our plant extracts. Representative LC-MRM profiles obtained for the authentic standards are shown in **Figure 5**. Sorghum cultivar DK18 does not accumulate anthocyanin pigments in mesocotyls but synthesizes 3-deoxyanthocyanidin phytoalexins as a defense response. Following inoculation with fungal pathogens, both luteolinidin and apigeninidin were detected in the LC-MRM profile obtained for the crude plant extract (**Figure 5**). In cultivar DK46, both anthocyanin pigments and 3-deoxy-

258 *J. Agric. Food Chem.,* Vol. 55, No. 2, 2007
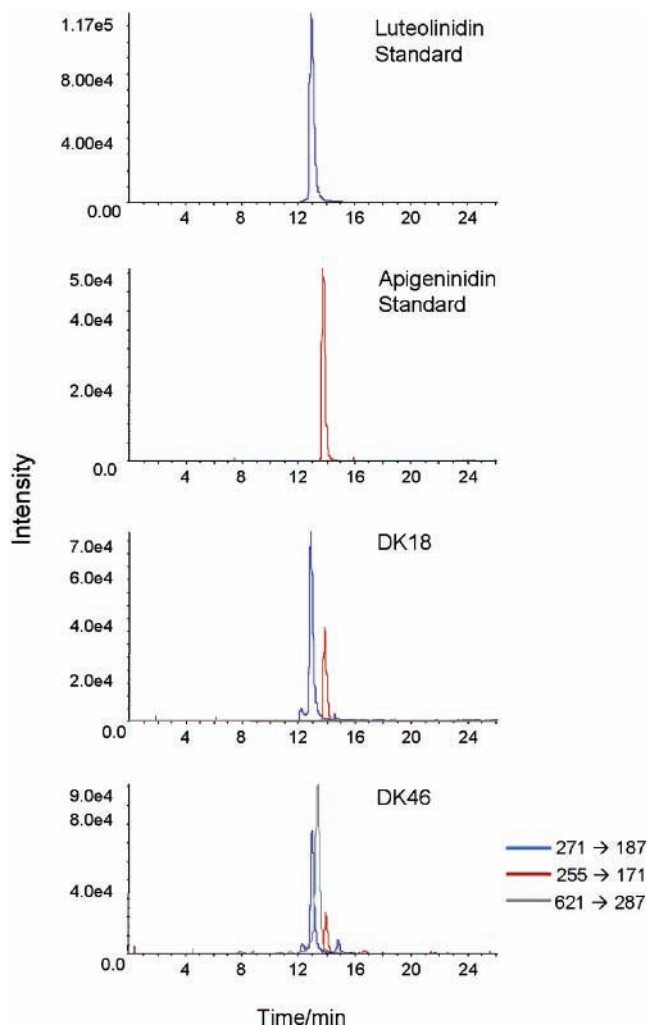
Shih et al.

Figure 5. Representative LC-MRM chromatograms of 3-deoxyanthocyanidin standards and sorghum extracts. Methanol extracts were prepared from inoculated seedlings of cultivars DK18 and DK46. Elution from LC separation was monitored by MS/MS in positive ion MRM mode for the key reactions m/z 255→171 (apigeninidin), m/z 271→187 (luteolinidin), and m/z 621→287 (cyanidin 3-dimalonyl glucoside).

anthocyanidin phytoalexins accumulated when the seedlings were inoculated and exposed to light. During LC separation, the anthocyanin pigment and the 3-deoxyanthocyanidins had similar retention times, leading to overlapping elution peaks when detected by a visible lamp (*11*). In complex mixtures containing these flavonoid metabolites, we were able to obtain distinct peaks for the individual pigment compounds by LC-MRM analysis (**Figure 5**). The anthocyanin pigment accumulated in sorghum mesocotyl is cyanidin 3-diamalonyl glucoside with a MW of 621 (*22*). We used the transition reaction m/z 621→287, representing the loss of the sugar unit, to monitor the elution of the cyanidin anthocyanin. As shown in **Figure 5**, this pigment eluted after 13.37 min, which was only 0.4 min later than luteolinidin. These two peaks were not resolved when the elution was monitored using absorbance measurements (*11*).

Luteolinidin represents the predominant 3-deoxyanthocyanidin accumulated in the infected seedlings, indicating that most of the precursors for 3-deoxyanthocyanidin biosynthesis were 3′-hydroxylated. When DK18 plants were inoculated by the anthracnose pathogen *C. sublineolum*, up to 178 μg/g (fresh weight) of luteolinidin was detected after 96 h (**Table 2**). Inoculated DK46 plants accumulated <120 μg/g (fresh weight)

during the same time period. In both cases, the amounts of luteolinidin accumulated were >10-fold the amounts of apigeninidin accumulated. The lower levels of overall 3-deoxyanthocyanidin production in DK46 plants could be explained by metabolic competition with the anthocyanin pathway as revealed previously (*11*). We further challenged the DK18 plants with *C. heterostrophus*, a nonpathogen of sorghum. This maize pathogen was known to induce a rapid nonhost defense response in sorghum (*16*). Using the LC-MRM quantification method, the inoculated DK18 plants were found to produce as much as 268 μg/g (fresh weight) of luteolinidin 72 h after inoculation.

In summary, 3-deoxyanthocyanidins were demonstrated to be more effective in reducing the viability of human cancer cells than anthocyanidins, which are more widely distributed in nature. So far, sorghum remains the major source for these unique flavonoid pigments, which have promising potentials for development into a food colorant or supplement exhibiting health benefits. In contrast to the time and efforts required for growing sorghum crops and processing grains into bran, significant amounts of luteolinidin could be conveniently produced in inoculated seedlings within 3 days (**Table 2**). Thus, our mesocotyl inoculation system could be utilized for large-scale production of 3-deoxyanthocyanidins for pharmacological investigations and potential nutraceutical applications. In addition, the LC-MRM method allows specific detection and accurate quantification of individual 3-deoxyanthocyanidins in complex plant extracts containing other pigments. This method will also be useful for pharmacokinetics studies of 3-deoxyanthocyanidins in colored biological matrices (e.g., blood samples) that would otherwise affect analysis using common absorbance-based measurements.

ABBREVIATIONS USED

MRM, multiple-reaction monitoring; MALDI-TOF, matrix-assisted laser desorption ionization−time-of-flight; RPMI-1640 medium, Roswell Park Memorial Institute 1640 medium

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3-Deoxyanthocyanidins in Infected Sorghum

J. Agric. Food Chem., Vol. 55, No. 2, 2007 **259**

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