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Detection of Cyanidin in Different-colored Peanut Testae and Identification of Peanut Cyanidin 3-sambubioside

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Peanut testae are potent sources of polyphenols. When the water extracts and acid hydrolysates of five different-colored testae were analyzed by HPLC, chromatograms monitored at 280 nm varied remarkably, whereas two major peaks in the chromatograms monitored at 530 nm were detected only in kernels having completely or partially black color. After acid hydrolysis of the extracts, cyanidin was detected in each of the hydrolysates. By respectively subjecting the black testae of raw and roasted (175 °C for 20 min) kernels of a black colored cultivar to water extraction and HPLC analysis, a prominent peak was detected in both extracts. The structure of the substance under those peaks was identified by mass and NMR spectrometry as cyanidin 3-sambubioside in peanut testae for the first time. Subjection of cyanidin 3-sambubioside to antioxidation and anti-inflammation assessments revealed that it was a potent antioxidant and inhibitor of nitric oxide production.

KEYWORDS: Peanut; peanut testa; cyanidin; ^{1}H and ^{13}C NMR spectroscopy; cyanidin 3-sambubioside; nitric oxide

INTRODUCTION

Peanut is one of the most popular foods consumed worldwide. As viewed from peanut kernel appearance, the color of kernel testae varies widely. Five color groups are readily distinguishable: white, tan, red, purple, and wine (*1*). Normally, the peanut testae are either ingested with the kernels or discarded in the blanching (deskinning) process. Because peanut testae contribute about 3% of the kernel weight (*2*), it is worthwhile to assess whether there is any profit to using the testae for some value-added product development. From the food science point of view, in addition to the search for potent sources of natural colorants, further investigations addressing nutritional impact or toxicological concern of the peanut testae after ingestion are warranted. In recent years, researchers have found polyphenols and other related chemicals in the peanut testae (*2*−*6*). The massive tonnage of testae generated as waste byproduct in peanut-blanching plants is thus worthy of consideration for extraction of these bioactive phytochemicals.

In addition to many known nutritional and sensory contributions of peanut-containing foods, the effects of chronic peanut consumption on energy balance and hedonics have been reported (*7*). In Taiwan, peanuts with different kernel colors are consumed, and a black seeded cultivar, locally named Black Kingkong, is widely grown and popularly consumed. Some people believe that peanuts with black testae are good for their health, whereas some think otherwise. In this study, testae were respectively collected from five different-colored peanut kernels and subjected to water extraction and/or acid hydrolysis followed by analyses with high-performance liquid chromatography (HPLC) for anthocyanin characterization. The goal of this study was to evaluate different-colored peanut kernels for the presence of beneficial chemical compounds and to characterize their structures and bioactivities.

MATERIALS AND METHODS

Seed Materials. Four different-colored peanut kernels of *Arachis hypogaea* L. of the 2008 fall crop were obtained from Taiwan Agricultural Research Institute (Taichung, Taiwan). Additionally, kernels of a black-seeded cultivar, locally named Black Kingkong, were obtained by growing the plants as fall crops of 2006 and 2008 at a peanut farm in Chiayi, Taiwan. In total, kernels of five distinguishably different colors, namely, black (Black Kingkong), dark red (accession VA 10), red (accession NI 2), pink (accession NI 17), and black-pink mix (accession NI 54) (**Figure 1**), were used in this study. All harvested pods were dried under sunshine and shelled manually, and the kernels were sorted, deposited in polyethylene (PE) plastic bags, and stored at −20 °C until used. Testae were manually removed, crushed into flour by a coffee mill, and subjected to water extraction and HPLC analyses.

Aqueous Extraction and HPLC Analysis. Testae were weighed (1 g) and deposited into a 50 mL beaker to which 10 mL of deionized water (w/v, 1:10, testae/water) was added. Beakers were sealed with parafilm and shaken occasionally during 2 h of soaking at 26−28 °C. Then the extracted suspensions were centrifuged (19000*g* at 20 °C) for 15 min and membrane filtered (0.45 μm), and the filtrate was stored at −20 °C for later analyses.

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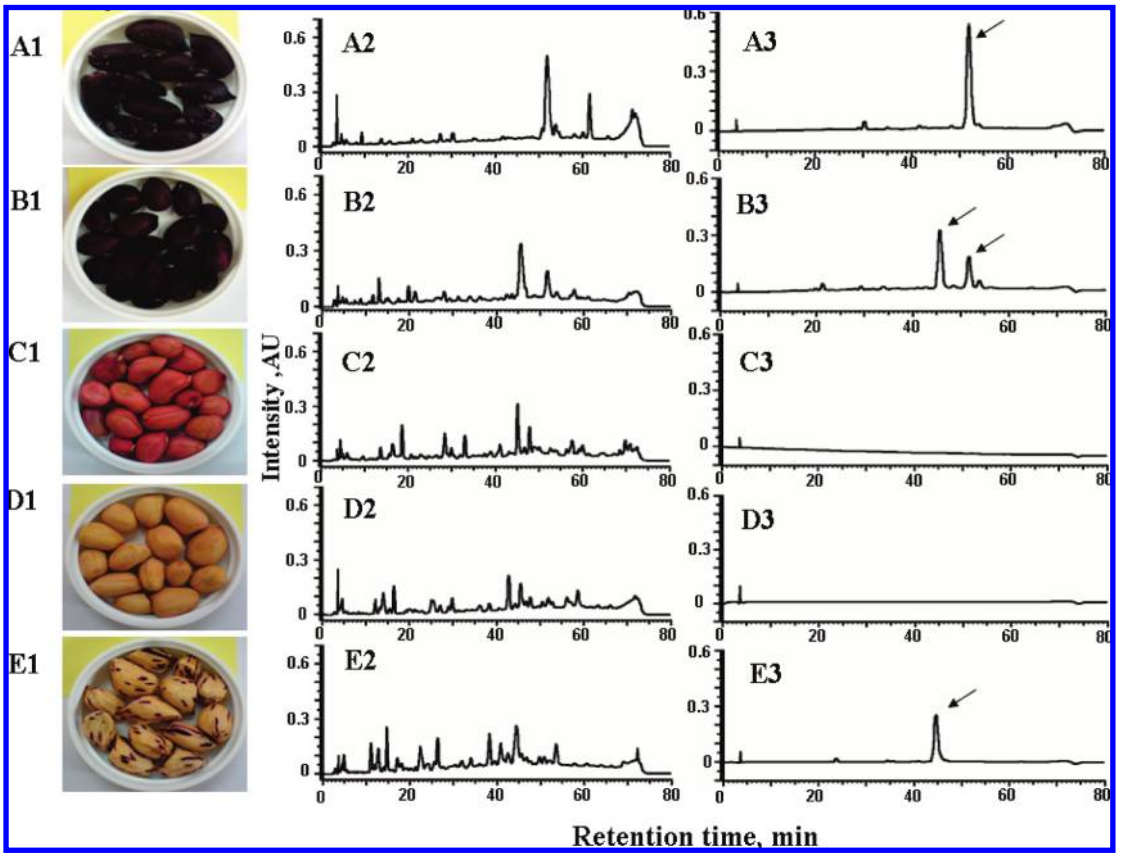

Figure 1. Photographs of five different-colored peanut kernels investigated in this study (A1−E1) and HPLC chromatograms of the water extracts of peanut testae monitored at 280 nm (A2−E2) and 530 nm (A3−E3); A, black kernels (Black Kingkong cultivar); B, dark red kernels (accession VA 10); C, red kernels (accession NI 2); D, pink kernels (accession NI 17); and E, black-pink mix kernels (accession NI 54).

For small-scale extraction and qualitative analysis, 0.1 g of testae was deposited into a test tube and replenished with 2.5 mL of deionized water. The suspended testae were stirred vigorously by a stainless steel spatula for about 5 min. The extract was membrane (0.45 μm) filtered and stored frozen until HPLC analyses.

The membrane-filtered extract of testae was subjected to analysis by HPLC equipped with a dual pump (L-7100) and a photodiode array detector (L-7455) (Hitachi Co., Ltd., Tokyo, Japan) according to a reported procedure (*8*) with modification. The column used was a 250 mm × 4.6 mm i.d., 5 μm, C18 reversed phase column with a guard column of the same material (Thermal Hypersil Ltd., Cheshire, U.K.). The mobile phase contained two components: A (0.05% trifluoroacetic acid in water) and B (acetonitrile containing 0.05% trifluoroacetic acid). The gradient solvent program was set as 0 min, 92% A and 8% B; 60 min, 82% A and 18% B; 65 min, 75% A and 25% B; 66 min, 92% A and 8% B; and 80 min, 92% A and 8% B. The injection volume, flow rate, and two monitoring wavelengths were 20 μL, 1.0 mL/min, and 280 and 530 nm, respectively.

Acid Hydrolysis and HPLC Analysis. Acid hydrolysis of the water extracts was carried out to release the anthocyanin aglycones. A reported procedure (*9*) was followed with modification. As a preliminary experiment, aliquots of 0.1 mL extract were deposited into a series of screw-capped test tubes, and each was mixed with 0.9 mL of 2 N HCl (prepared in methanol). Then, the tubes were screw-capped and heated with a thermal module set at 90 °C for 10, 20, 30, 40, 50, and 60 min, respectively. The solution in each tube was rapidly cooled to ambient temperature in an ice bath and membrane (0.45 μm) filtered, followed by HPLC analysis to determine the change of chromatogram. It was observed that a 40 min heat treatment was sufficient for hydrolysis and was used in the later experiments.

For HPLC analysis of the acid hydrolysates, the same chromatographic instruments, C18 reversed phase column, and mobile solvents described above were used. A gradient solvent program was set as 0 min, 90% A and 10% B; 10 min, 60% A and 40% B; 30 min, 40% A and 60% B; 31 min, 90% A and 10% B; and 35 min, 90% A and 10% B. The injection volume, flow rate, and two monitoring wavelengths were 20 μL, 1.0 mL/min, and 280 and 530 nm, respectively. An authentic sample of cyanidin chloride (Extrasynthese, Genay, France) was run concurrently as a reference standard. The referenced cyanidin was also spiked into the acid hydrolysates of testa extracts, followed by HPLC analysis to differentiate their retention times and photodiode UV−visible spectra (220−550 nm).

Roasting of Kernels of Black Kingkong and Extraction of Soluble Testa Pigments. For roasting, kernels of Black Kingkong in PE bags removed from a freezer were kept at 26 ± 2 °C overnight and subjected to roasting at 175 °C for 20 min (300 g per batch) in a drum oven (Sanyo Co., Tokyo, Japan) (simulating conventional roasting). Testae of the raw and roasted peanut kernels were manually removed and weighed to determine the percentage contribution to intact kernels and crushed into flour. A 1 g sample of each testa was weighed and deposited into a 50 mL beaker and extracted with 10 mL of deionized water (w/v, 1:10, testae/water) following the procedure described above.

Determination of Total Phenolic and Flavonoid Contents. The procedures reported previously (*10*, *11*) were followed for determining the total phenolic contents with minor modification. Briefly, 1 mL of testa extract was subjected to a 20-fold dilution in deionized water; 0.1 mL of the diluted solution was mixed thoroughly with 0.5 mL of Folin−Ciocalteu reagent. After 3 min, 0.4 mL of 7.5% Na_2CO_3 aqueous solution was added and mixed. Absorbance at 750 nm was measured after a 30 min reaction time. As a reference, a series of gallic acid solutions (0, 10, 25, 50, 75, 100, and 150 μg/mL) was subjected to reaction and absorbance determination to construct a standard curve for estimating the amount of total phenolic compounds in the testa extracts.

For determination of flavonoid content, a previously reported procedure (*12*) was followed with modification. Briefly, a 1 mL extract of testae was subjected to a 5-fold dilution in deionized water. The diluted solution (0.5 mL) was mixed thoroughly with 1.5 mL of deionized water, 0.1 mL of 10% aluminum nitrate, and 0.1 mL of 1.0 M potassium acetate and reacted for 40 min. Absorbance at 415 nm was measured. As a reference, a series of quercetin solutions, namely, 0, 10, 25, 50, 75, 100, 150, and 200 μg/mL, was prepared and subjected to reaction and absorbance determination

to construct a standard curve for estimating the amount of flavonoid compounds in the extracts of testae.

Peanut Testa Pigment Isolation. The membrane-filtered extract of testae of Black Kingkong was subjected to HPLC analysis as described above except that a gradient solvent program was substituted to save running time. The mobile phase contained two components: A (0.05% trifluoroacetic acid in water) and B (acetonitrile containing 0.05% trifluoroacetic acid). The gradient solvent program was set as 0 min, 95% A and 5% B; 10 min, 40% A and 60% B; 18 min, 95% A and 5% B; and 20 min, 95% A and 5% B. The injection volume, flow rate, and monitoring wavelength were 20 μL, 1.0 mL/min, and 280 nm, respectively. Because the chromatograms obtained from the raw and roasted kernel testae were essentially identical, the extracts of raw kernel testae were subjected to further fractionation for structure identification.

For semipreparative HPLC purification, the column was replaced by a semipreparative 250 mm × 10 mm i.d., 8 μm, C18 reversed phase column (Thermo Hypersil Ltd.), and the same solvent system described above was used. The injection volume, flow rate, and monitoring wavelength were 0.1 mL, 3.0 mL/min, and 280 nm, respectively. The major peak fractions were collected, pooled, lyophilized, designated as compound **1**, and stored at −20 °C for structure identification.

Structure Identification. The NMR sample was prepared by dissolving the purified compound **1** (ca. 5 mg) in 500 μL of methanol-d_4. All NMR spectra were obtained using Bruker Avance 600, 500, and DRX 500 MHz NMR spectrometers equipped with Bruker TXI and QNP probes (Bruker BioSpin AG, Fallalden, Switzerland). For molecular weight determination, the sample dissolved in methanol was subjected to ESI-MS analysis by a mass spectrometer (Thermo Finnigan, San Jose, CA).

Compound **1** is a dark violet amorphous powder: ESI (+)-MS, m/z 581 [M^+]; ^{1}H NMR (500 MHz, methanol-d_4) δ 8.96 (1H, s, H-4), 6.65 (1H, s, H-6), 6.89 (1H, s, H-8), 8.04 (1H, d, J = 2.2 Hz, H-2′), 7.02 (1H, dd, J = 8.9 Hz, H-5′), 8.30 (1H, dd, J = 2.2, 8.9 Hz, H-6′), 5.45 (1H, d, J = 7.8 Hz, H-1″), 3.98 (1H, t, J = 7.8 Hz, H-2″), 3.80 (1H, t, J = 9.0 Hz, H-3″), 3.51 (1H, t, J = 9.0 Hz, H-4″), 3.62 (1H, m, H-5″), 3.74 (1H, m, H-6″), 3.94 (1H, m, H-6″), 4.75 (1H, d, J = 7.6 Hz, H-1‴), 3.18 (1H, t, J = 9.0 Hz, H-2‴), 3.33 (1H, m, H-3‴), 3.42 (1H, m, H-4‴), 3.09 (1H, m, H-5‴), 3.71 (1H, m, H-5‴); ^{13}C NMR (125 MHz, methanol-d_4) δ 162.7 (C-2), 143.8 (C-3), 134.8 (C-4), 157.8 (C-5), 101.9 (C-6), 169.0 (C-7), 93.6 (C-8), 156.1 (C-9), 111.7 (C-10), 119.8 (C-1′), 117.0 (C-2′), 146.0 (C-3′), 154.4 (C-4′), 115.9 (C-5′), 127.2 (C-6′), 100.1 (C-1″), 80.3 (C-2″), 76.7 (C-3″), 69.3 (C-4″), 77.2 (C-5″), 60.8 (C-6″), 104.3 (C-1‴), 74.2 (C-2‴), 76.4 (C-3‴), 69.5 (C-4‴), 65.7 (C-5‴). The structure was unambiguously identified as cyanidin 3-sambubioside by comparing those data with the literature (*19*−*21*).

Assessments of Antioxidant Activities. For determination of α,α-diphenyl-β-picrylhydrazyl (DPPH) scavenging activity, a previously reported procedure (*13*) was followed. For each measurement, a 1 mL extract of testae was diluted 1000-fold in deionized water. The diluted solution (2 mL) was mixed thoroughly, in the dark, with 0.1 mL of DPPH solution (2 mM) at 26 °C for 30 min, and then the absorbance at 517 nm was measured. Butylated hydroxytoluene (BHT) solutions at concentrations of 0.1, 1, 5, and 25 μg/mL in methanol served as references.

For antioxidative potency (AOP) determination, a previously reported procedure (*14*) was followed. Testa extract (1.0 mL) after freeze-drying was dissolved in 1.0 mL of methanol. The same BHT solutions above also served here as references.

To determine the reducing power, the procedure of ref *15* was followed with modification. Briefly, a 1.0 mL extract of testae was diluted 15-fold in deionized water. The diluted solution (0.5 mL) was mixed thoroughly with 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then, 0.5 mL of 1% trichloroacetic acid was added, and the mixture was centrifuged (1000g at 20 °C, 10 min). The upper layer of the solution was mixed with deionized water and 0.1% $FeCl_3$ at a ratio of 1:1:2, and then the absorbance at 700 nm was measured. An increase in absorbance of the reaction mixture indicates increased reducing power. A series of ascorbic acid solutions at concentrations of 0, 5, 10, 25, 50, and 75 μg/mL in deionized water were prepared for construction of a reference curve for equivalency estimation.

Cultivation of RAW 264.7 Macrophage Cells, Viability, and Nitric Oxide (NO) and Prostaglandin E_2 (PGE_2) Determination. RAW 264.7 cells were cultured with freshly prepared Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 75 mm^2 tissue culture flask. Flasks were incubated 16−18 h at 37 °C with 95% atmospheric relative humidity under a 5% CO_2 atmosphere. The cells were harvested, and populations were adjusted to 5×10^5 cells/mL.

Cell viability was determined by the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) test (*16*, *17*). Aliquots (200 μL) were dispensed into the wells of a 96-well ELISA plate. The plate was incubated at 37 °C with 95% atmospheric relative humidity under a 5% CO_2 atmosphere for 15 h. The medium was removed and replaced with a medium (200 μL) containing various concentrations of cyanidin 3-sambubioside (0, 50, 100, 200, and 400 μM), and the plates were further incubated at 37 °C for 24 h. Later, 10 μL of MTT working solution (0.5 mg/mL) was added to each well, followed by further incubation at 37 °C for 4 h and the addition of 100 μL of color development solution (0.04 N HCl in isopropanol). After 15 min of shaking, absorbance at 570 nm of the mixture in each well was determined with an ELISA reader, and values were used for estimation of cell viability.

As a prerequisite in the assessment of the likelihood of cyanidin 3-sambubioside to cause inflammation, RAW 264.7 cells were cultured as described above to give a population of 5.0×10^5 cells/mL. Aliquots (200 μL) were dispensed into the wells of a 96-well ELISA plate. The plate was incubated at 37 °C with 95% atmospheric relative humidity under a 5% CO_2 atmosphere for 15 h. The medium was removed, replenished with fresh medium, and incubated at 37 °C for 0, 2, 14, 18, and 24 h. The cultured medium in specified wells was replaced with medium containing 400 μM cyanidin 3-sambubioside and further incubated for 24, 22, 10, 6, and 0 h. Finally, media were withdrawn from wells and subjected to NO quantification.

To determine the NO concentration, aliquots (100 μL) of cultured fluids were dispensed into each well of a 96-well plate to which 50 μL of sulfanilamide solution was added. After incubation of the plates at room temperature (26 °C) without exposure to light, 50 μL of *N*-(1-naphthyl)ethylenediamine dihydrochloride solution was added to each well, thoroughly mixed, and incubated for 15 min. NO production was determined by measurement of absorbance at 550 nm. $NaNO_2$ was used to generate a standard curve for estimation of NO concentration (*18*). The concentration of NO_2^- in the extracellular medium was determined with a Griess Reagent System (Promega, Madison, WI). Briefly, supernatant was combined with an equal volume of Griess reagent and incubated at room temperature (26−28 °C) for 10 min. NO production was determined by measurement of absorbance at 540 nm.

In the determination of lipopolysaccharide (LPS)-induced NO and PGE_2 production as affected by cyanidin 3-sambubioside, mouse macrophage RAW 264.7 cells were cultured as described above to give a population of 5.0×10^5 cells/mL. Aliquots (200 μL) of the suspension were dispensed into the wells of a 96-well ELISA plate. The plate was incubated at 37 °C with 95% atmospheric relative humidity under a 5% CO_2 atmosphere for 15 h. The medium was removed and replaced with a medium containing various concentrations of cyanidin 3-sambubioside (0, 25, 50, 100, 200, and 400 μM) and 10 μL of LPS (5 μg/mL) for inflammation mediation. Plates were incubated for an additional 24 h prior to NO quantification according to the procedure described above.

For PGE_2 quantification by a PGE_2 ELISA kit (Cayman, Ann Arbor, MI), mouse macrophage RAW 264.7 cells were cultured and treated with cyanidin 3-sambubioside (0, 50, 100, 200, and 400 μM) and 10 μL of LPS (5 μg/mL) for inflammation mediation. Plates were incubated for an additional 24 h, and aliquots (50 μL) of the cell suspension were dispensed into the wells of a 96-well ELISA plate. After incubation at 4 °C overnight, the cultivation fluid was removed and washed with wash buffer four times. Each well was replenished with 200 μL of Ellman's reagent and incubated for an additional 1 h under ambient temperature. Then, the incubated fluid was removed and washed with wash buffer six times, and each well was replenished with 100 μL of avidin−peroxidase and incubated under ambient temperature for 1.5 h without exposure to light. Then, absorbance at 405 nm of each well was determined with an ELISA reader.

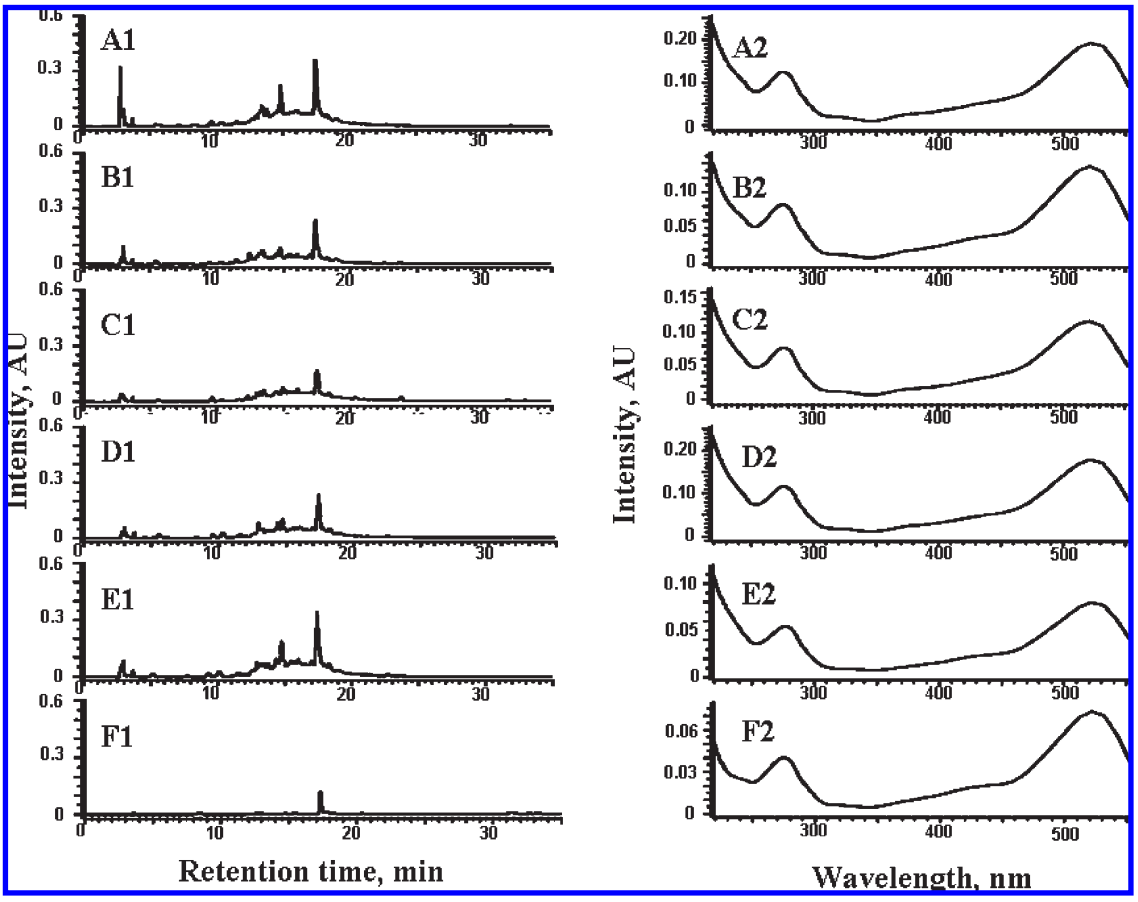

Figure 2. HPLC chromatograms monitored at 280 nm of the acid hydrolysates of extracts of testae of five different-colored peanut kernels (A1−E1) and authentic cyanidin (F1) and photodiode UV−vis spectra (220−550 nm) of the major peaks with 17.5 min of retention time (A2−E2) and authentic cyanidin (F2); A, black kernels (Black Kingkong cultivar); B, dark red kernels (accession VA 10); C:, red kernels (accession NI 2); D, pink kernels (accession NI 17); and E, black-pink mix kernels (accession NI 54); and F: authentic cyanidin.

Statistics. At least three replicate experiments for each treatment were conducted. Means of values with standard deviations are expressed. Analysis of variance ($P = 0.05$) among the test groups were analyzed by SAS (Statistical Analysis System, Cary, NC).

RESULTS AND DISCUSSION

In this study, when the testae of five different-colored peanut kernels (**Figure 1**A1−E1) were subjected to water extraction and HPLC analysis, their HPLC chromatograms monitored at 280 nm (**Figure 1**A2−E2), with an attempt to indicate flavonoids, varied remarkably. At 530 nm (**Figure 1**A3−E3), the chromatograms indicated peaks with red color based on chromatographic responses of anthocyanins (*19*), two noticeable peaks between 45 and 55 min of retention time (indicated by arrows) (**Figure 1**A3, B3, and E3) were detected for the A1, B1, and E1 kernels. These kernels were observed to be completely or partially black color.

When the extracts of testae were subjected to acid hydrolysis and subjected to HPLC photodiode array analyses, their HPLC chromatograms and UV−vis spectra (220−550 nm) of the major peaks with 17.5 min of retention time are shown in **Figure 2**. An authentic cyanidin was also run concurrently for reference (**Figure 2**F1). On the basis of the identical retention times and spectra for the specified peaks, it is obvious that cyanidin was detected in each of the acid hydrolysates of extracts of testae of different-colored kernels. For further confirmation of this finding, all acid hydrolysates were mixed in equal volumes and/or further spiked with authentic cyanidin and subjected to HPLC photodiode array analyses (**Figure 3**). No additional peak was detected (**Figure 3**A1 and B1).

Among the test peanuts in this study, the black-colored kernels of Black Kingkong (**Figure 1**A1) are unique and popularly accepted by consumers. This raised our research attention and, thus, kernels of Black Kingkong were used in the following studies. As estimated, weight percentages of the peanut testae collected from the raw and roasted kernels were 3.4 and 3.5% (w/w), respectively. This was in agreement with a previous report (*2*) that whole peanut kernels constitute 3.3% of testae. When the testae from raw and roasted kernels were subjected to extraction with water and lyophilized, the solid yields were 162.6 ± 0.8 and 157.8 ± 11.6 mg/g of testae, respectively. As reported previously (*6*), when the *n*-hexane-defatted peanut skins of an unspecified cultivar were directly extracted with 50% ethanol, the yield of solids was 107 mg/g of testae.

As determined on the basis of the initial kernel testa weights before water extraction, the total phenolic and flavonoid compounds in the testae of raw and roasted kernels were 35.4 and 36.9 mg/g of testae, respectively. Flavonoid contents were 21.3 and 25.5 mg/g of testae (quercetin equivalency) of raw and roasted kernels, respectively. Total phenolic and flavonoid contents in the roasted testae were slightly higher than those in the raw testae. In a previously published paper (*2*), in which raw peanut testae were extracted with water, 80% methanol, and 100% methanol, the contents of the total phenolic compounds were 56.7, 89.9, and 90.1 mg/g of testae, respectively. When roasted (175 °C, 5 min) peanut testae were extracted with the same series of solvents, phenolic contents were 79.0, 125, and 96.7 mg/g of testae, respectively. As compared quantitatively, contents of total phenolics in the peanut testae of unspecified cultivars were 118 and

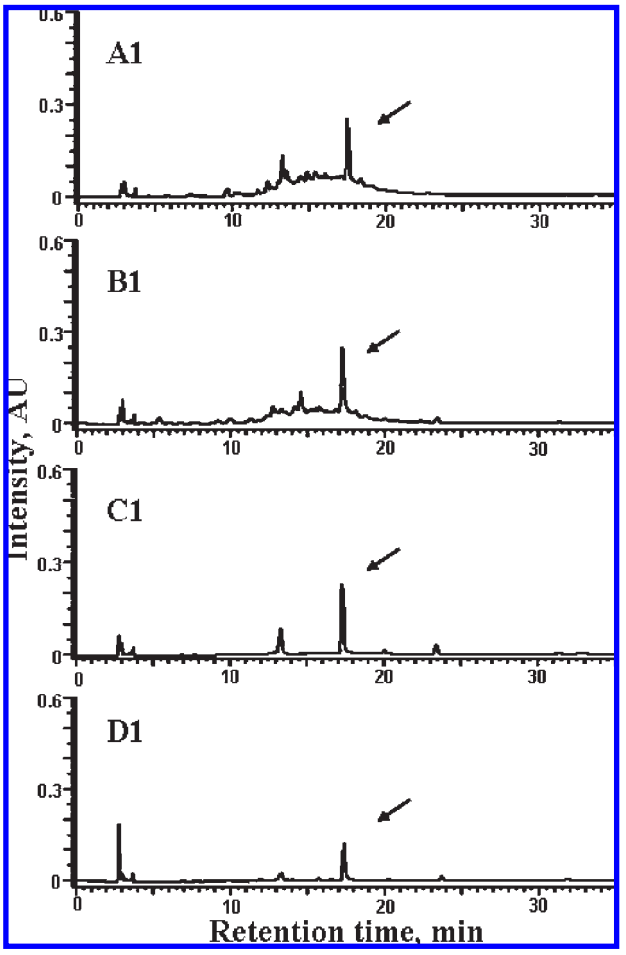

Figure 3. HPLC chromatograms monitored at 280 nm of the equally mixed hydrolysates prepared from the five different-colored peanut tesate (A1), the mixture spiked with an equal volume of authentic cyanidin (B1), acid hydrolysate of cyanidin 3-sambubioside (C1), and hydrolysate of cyanidin 3-sambubioside spiked with an equal volume of authentic cyanidin (D1).

97 mg/g of testae, respectively (*3*, *4*). It is apparent that peanut testae contain substantial amounts of phenolics and that the extracted phenolic contents vary depending upon peanut cultivar, extraction solvent, and procedures used for extraction.

When the water extracts of raw and roasted peanut testae were subjected to reversed phase HPLC analyses, a major peak (assigned as compound **1**) was detected at retention times of 11.73 and 11.79 min, respectively. Their photodiode UV−vis spectra (220−550 nm) were identical (refer to the Supporting Information). This reveals that the effect of roasting kernels on chemical composition was inconsequential based on the fact that the chromatograms and major peak spectra were essentially identical. Then, the major peak fractions from raw kernels were repeatedly collected by semipreparative HPLC, pooled, freeze-dried, and weighed, and the recovered yield was 11.0 mg of compound **1**/g of testae.

Compound **1** was obtained as a dark violet amorphous powder and showed a [M^+] ion peak at *m*/*z* 581 in the ESI(+)-MS. The maximum absorbance wavelengths at 280 and 530 nm displayed by compound **1** belong to anthocyanin. The cyanidin detected by HPLC after acid hydrolysis of compound **1** indicated the cyanidin as its aglycone. The presence of cyanidin was also verified by ^{1}H NMR with three 1H singlets at δ 6.65, 6.89, and 8.96 and ABX-type signals at δ 8.04, 7.02, and 8.30. The presence of glucose and xylose in compound **1** was confirmed by ^{1}H−^{1}H COSY, and the sugar linkage of compound **1** was determined by NOESY,

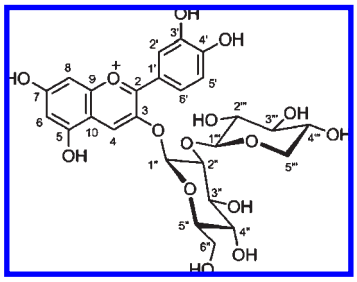

Figure 4. Chemical structure of cyanidin 3-sambubioside (cyanidin 3-*O*-(2-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside).

HMQC, and HMBC (refer to the Supporting Information). By comparing those NMR spectra data with the literature (*19*−*21*), compound **1** was identified as cyanidin 3-*O*-(2-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside, cyanidin 3-sambubioside, or cyanidin-3-glucosyl xyloside (**Figure 4**).

Cyanidin 3-sambubioside has been identified to be present in *Sambucus nigra* (*19*), *Vaccinium padifolium* (*20*), and *Viburnum dilatatum* Thunb. (*21*). We report here for the first time its presence in peanut testae.

For further characterization of the anthocyanidin (aglycones) composition, cyanidin 3-sambubioside was subjected to acid hydrolysis and followed by HPLC photodiode array analyses. Its retention time (**Figure 3**C1) was identical to that of authentic cyanidin (**Figure 2**F1). When the acid hydrolysate of cyanidin 3-sambubioside was spiked with an equal volume of authentic cyanidin and subjected to HPLC analysis (**Figure 3**D1), a complete match of retention times was observed.

When various concentrations of cyanidin 3-sambubioside were subjected to DPPH scavenging activity, AOP, and reducing power assessments, a general dose-dependent antioxidant activity was observed (**Figure 5**). This was in agreement with reports of cyanidin 3-sambubioside purified *V. dilatatum* Thunb. (*21*) and cyanidin-based anthocyanins (cyanidin 3-sambubioside was not included) extracted from elderberry, evergreen blackberry, black carrot, red cabbage, and purple sweet potato (*22*). We observed an increased DPPH scavenging activity with increasing cyanidin 3-sambubioside concentration (**Figure 5A**). For its concentrations of 5 and 25 μg/mL, the scavenging activities were equivalent to 5.5 and 27.7 μg/mL BHT, respectively, giving an equivalent of 1.1 μg of BHT/μg of cyanidin 3-sambubioside. For concentrations of 25, 50, and 100 μg/mL for AOP assessment (**Figure 5B**), these activities were equivalent to 0.5, 1.2, and 2.0 μg/mL of BHT, equivalent to ca. 0.02 μg of BHT/μg of cyanidin 3-sambubioside. The low detected AOP might be caused, in part, by hindrance of reaction of the hydrophilic cyanidin 3-sambubioside by an emulsified substrate of linoleic acid. As estimated by using concentrations of 25, 50, and 100 μg/mL in reducing power assessment (**Figure 5C**), the reducing powers were equivalent to 13.4, 24.4, and 44.4 μg/mL of ascorbic acid (vitamin C), equivalent to ca. 0.48 μg of ascorbic acid/μg of cyanidin 3-sambubioside.

As a prerequisite test for assessment of anti-inflammatory activities of cyanidin 3-sambubioside, RAW 264.7 cells were incubated in solutions containing cyanidin 3-sambubioside concentrations of 0, 50, 100, 200, and 400 μM for 24 h. Viability of cells as determined by the MTT test showed that no obvious cytotoxicity was detected. As a preliminary counterpart experiment to assess NO production induced by cyanidin 3-sambubioside, media were supplemented with 100 μg/mL (172 μM) cyanidin 3-sambubioside at specified time intervals of 0, 2, 14, 18, and 24 h and also incubated for 24, 22, 10, 6, and 0 h individually. The resulting NO contents ranged from 6.4 to

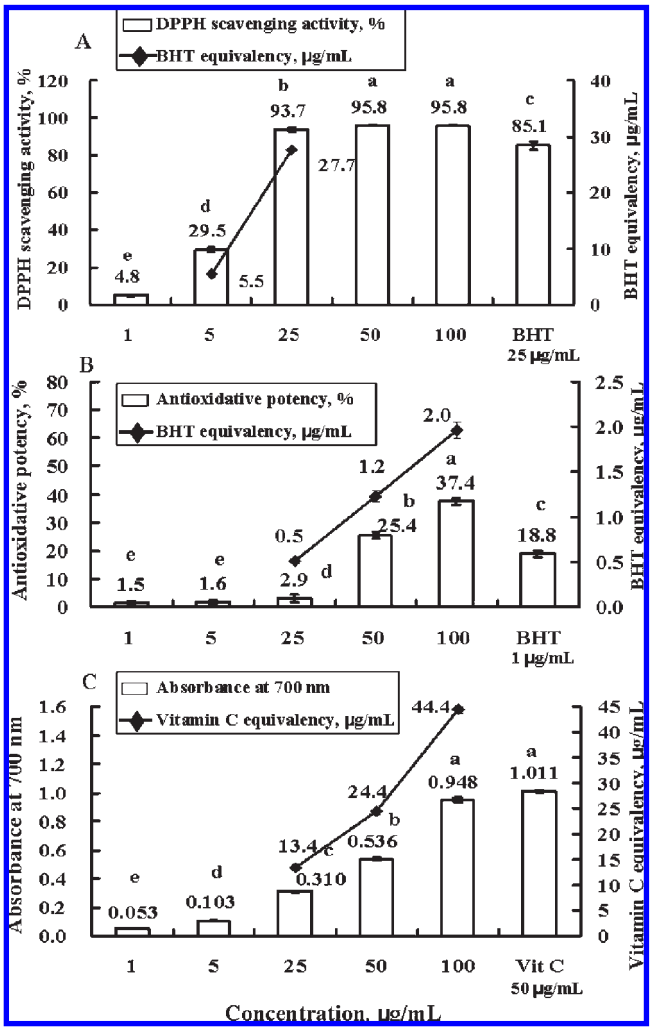

Figure 5. Bioactivities of the extracted cyanidin 3-sambubioside at various concentrations: (**A**) α,α-diphenyl-β-picrylhydrazyl (DPPH) scavenging activities; (**B**) antioxidative potencies; (**C**) reducing powers. Each value represents mean ± SD (n = 3). Bars in each panel marked with different letters are significantly different (P < 0.05).

6.7 μM (detailed data not shown). This indicates that a cyanidin 3-sambubioside concentration of up to 172 μM is unlikely to cause an increase of NO biosynthesis.

When the RAW 264.7 macrophage cells were exposed to cyanidin 3-sambubioside at concentrations of 0, 50, 100, 200, and 400 μM and mediated with LPS at 5 μg/mL, a dose-dependent inhibition of NO production was observed (**Figure 6A**). However, under these doses PGE_2 production was affected insignificantly (**Figure 6B**). Nevertheless, it is of great interest to find that cyanidin 3-sambubioside is a potent natural NO inhibitor. On the basis of the fact that anthocyanins are substantial sources of dietary flavonoids and natural colorant, their contributions to human health are notable and deserve further investigation.

In conclusion, cyanidin was detected in the testa extracts of the five different-colored peanut kernels after acid hydrolysis. When black testae from kernels of Black Kingkong were further investigated, cyanidin 3-sambubioside, a major anthocyanin, was purified and identified in peanut testae for the first time. Cyanidin 3-sambubioside was stable against roasting at 175 °C for 20 min. In addition to cyanidin 3-sambubioside, water extracts of testae either from raw or roasted kernels also contained substantial amounts of flavonoids and exhibited potent

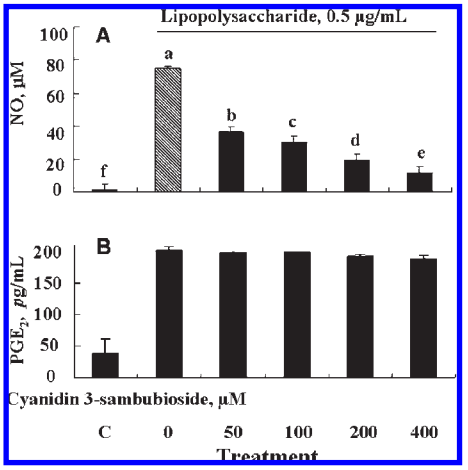

Figure 6. Nitric oxide (NO) and prostaglandin E_2 (PGE_2) inhibitory activities of cyanidin 3-sambubioside on lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells. Each value represents mean ± SD (n = 3). Bars marked with different letters are significantly different (P < 0.05).

antioxidant activities. Cyanidin 3-sambubioside was further identified as a potent natural inhibitor of NO biosynthesis. The high recovery yield (11.0 mg of cyanidin 3-sambubioside/g of testae) and potent bioactivities render peanut cyanidin 3-sambubioside a valuable candidate as a natural colorant, antioxidant, and bioactive ingredient for the development of health-related food products.

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Supporting Information Available: HMBC correlations for compound **1** and HPLC chromatograms and photodiode UV−vis spectra of the water extracts of peanut testae. This material is available free of charge via the Internet at http://pubs.acs.org.

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