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Profiles and α-Amylase Inhibition Activity of Proanthocyanidins in Unripe *Manilkara zapota* (Chiku)

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Supporting Information

ABSTRACT: Proanthocyanidins in unripe *Manilkara zapota* (chiku) were isolated using solvent extraction followed by Sephadex LH-20 fractionation with a yield of 0.9%. HPLC analysis using a diol column revealed well-resolved oligomers ranging from dimer to hexamer. The majority of the proanthocyanidins are composed of higher-degree oligomers appearing as one large peak in the chromatogram. Analysis of the proanthocyanidins using LC/MS showed that (epi)gallocatechins were the dominant extension unit in the proanthocyanidins. In agreement with this result, thiolysis treatment of the proanthocyanidins using mercaptoacetic acid produced thioether derivatives of (epi)gallocatechins as the major product and (epi)gallocatechin gallate derivatives as the minor product. The mean of the degree of polymerization was estimated to be 9.0. From MALDI-TOF MS, B-type gallocatechin oligomers up to decamer could be detected. The unripe chiku proanthocyanidins are thus good starting material for preparation of (epi)gallocatechin derivatives. The proanthocyanidins was shown to inhibit α-amylase with an IC_{50} value of 4.2 ± 0.2 μg/mL and inhibit α-glucosidase with an IC_{50} of 16.6 ± 0.3 μg/mL.

KEYWORDS: *Manilkara zapota, proanthocyanidins, α-amylase inhibitor, α-glucosidase inhibitor, gallocatechin*

INTRODUCTION

Manilkara zapota, also known as sapodilla or chiku, belongs to the Sapotaceae family. It is an evergreen tree that usually grows up to 10 m high. A native of tropical America, it has been cultivated in tropical areas in Southeast Asia and India. The fruit has a rusty brown skin and a yellowish-brown or red pulp with a pleasant, mild aroma when ripe. Among the commonly consumed tropical fruits in Singapore, chiku was found to possess the highest total phenolic content and water-soluble peroxyl radical scavenging activity as measured by hydrophilic oxygen radical scavenging capacity assay.[1] The polyphenols in unripe chiku were reported as catechin, epicatechin, gallocatechin, 4-hydroxycatechins, chlorogenic acid, and gallic acid.[2−4] Shui and co-workers detected proanthocyanidins in chiku, which might be responsible for its high antioxidant activity.[2] The proanthocyanidins in unripe chiku make it highly astringent; as the fruit matures, the sugar level increases and the proanthocyanidins content decreases. The proanthocyanidins of chiku have not been characterized. Thus, this study was designed to separate and characterize the proanthocyanidins in chiku using a diol HPLC column that has been shown to successfully separate proanthocyanidins in cocoa.[5] In addition, we isolated the proanthocyanidins on a preparative scale and further characterized the distribution of the oligomers by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Proanthocyanidins are known to exhibit a variety of health benefits, including antitumor effects,[6] platelet aggregation inhibition,[7] and modulation of several reactions associated with inflammation.[8] In addition, proanthocyanidins are reported to be an effective inhibitor of α-amylase based on in vitro study.[9] However, as secondary metabolites highly abundant in the plant kingdom and in the agricultural biomass, proanthocyanidins have not yet been widely utilized as renewable resource for industrial applications. Chemically, proanthocyanidins can be good starting materials for synthesis of flavanol derivatives through acid-mediated depolymerization in the presence of nucleophiles. We have demonstrated that proanthocyanidins isolated from mangosteen were excellent starting materials for preparation of epicatechin derivatives.[10] Such application requires the oligomers to have a uniform B-type linkage and one type of flavanol extension unit as well as high mean of degree of polymerization.

MATERIALS AND METHODS

Reagents. α-Amylase (type VI-B, from porcine pancreas), corn starch, acarbose, glucose assay kit (glucose oxidase/peroxidase reagent; *o*-dianisidine reagent), α-glucosidase in the form of rat intestine acetone powder, mercaptoacetic acid, standard gallocatechin, epigallocatechin, catechin, epicatechin, gallocatechin gallate, and epigallocatechin gallate were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO). Standard epicatechin thioether was synthesized according to the reported method.[11] Gallic acid was purchased from Acros Organics (Singapore). Folin−Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Anhydrous sodium carbonate was purchased from Thermo Fisher (Singapore). Unripe *M. zapota* was purchased from local supermarket in Singapore. The proanthocyanidins profiles of chiku from both Vietnam and Indonesia were characterized, and no significant difference between them was found. Therefore, only chiku from Vietnam was used in this study. Liquid chromatography and mass spectra were acquired using a Bruker Amazon ion trap mass spectrometer (Billerica, MA) equipped with a Dionex ultimate 3000RS HPLC system (Bannockburn, IL). The

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heated capillary and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen was operated at 80 psi for sheath gas flow rate and 20 psi for auxiliary gas flow rate. The full scan mass spectra from m/z 50−2000 were acquired in negative ion mode with a scan speed of one scan per second. The MS^n collision gas was helium with collision energy of 30% of the 5 V end-cap maximum tickling voltage. The microplate reader (Synergy HT, Biotek Instruments Inc., Winooski, VT) was used to determine turbidity change and total phenolic content. MALDI-TOF mass spectra were collected on a Bruker microTOF-QII mass spectrometer equipped with delayed extraction and a dinitrogen laser set at 337 nm. The length of one laser pulse was 3 ns. The measurements were carried out using the following conditions: positive polarity, linear flight path with 21 kV acceleration voltage, and 100 pulses per spectrum. The samples were dissolved in methanol (5 mg/mL). Sodium chloride and 2,5-dihydroxybenzoic acid as the matrix were used to enhance ion formation.[12] An aqueous solution of sodium chloride (1.0 μL, 0.1 M) was added to the sample solution (1.0 mL) followed by addition of an equal volume of methanol solution of 2,5-dihydroxybenzoic acid (10 mg/mL). The resulting solution (1.0 μL) was evaporated and introduced into the spectrophotometer.

Extraction of Proanthocyanidins from Chiku. Proanthocyanidins were extracted on the basis of an earlier report with a little modification.[10] Fresh chiku (600 g) was peeled, cut into small pieces, and ground. The resulting paste was extracted by a mixture of acetone/water (7:3, 3 × 5.0 L) with shaking for 2 h at room temperature. The mixture was filtered and the solvent was then evaporated to yield a slurry containing crude proanthocyanidins fraction (20 mL), which was filtered through a PTFE 0.45 μm membrane filter (Epsom) and then loaded on a Sephadex LH-20 column (column i.d., 5 cm) containing 50 g of LH-20 equilibrated with MeOH/water (1:1) for 4 h. The column was washed with MeOH/water (1:1) until the eluent turned colorless. The adsorbed proanthocyanidins were then eluted with aqueous acetone (70%, 500 mL). The acetone was removed on a rotary evaporator at 40 °C and the resulting residue was freeze-dried to give a light brown powder (1.4 g, 0.9% of dry matter).

HPLC and Tandem Mass Spectrometry. Proanthocyanidins (10 mg) from chiku were dissolved in 1 mL of methanol, and 20 μL of the solution was filtered through a PTFE 0.45 μm membrane filter before injection into the LC/MS^n system. The column used was a 250 mm × 4.6 mm i.d., 5 μm, Develosil diol with a 4 mm × 4 mm i.d. guard column of the same materials (Seto). The elution conditions were as follows: flow rate, 1.0 mL/min; column temperature, 35 °C; mobile phase A, 2% acetic acid in acetonitrile; mobile phase B, acidic aqueous methanol (CH_3OH:H_2O:HOAc, 95:3:2 v/v/v). The starting mobile phase condition was 7% B holding isocratic for 3 min before ramping solvent B to 37.6% over 57 min and then to 100% B 3 min thereafter. B was held at 100% for 7 min prior to returning to starting conditions (7% B) in 6 min. The column was equilibrated with 7% B for 5 min prior to the next run.

Thiolysis of Proanthocyanidins for HPLC Analysis. Thiolysis was conducted following a reported method.[13] In a small glass vial, chiku proanthocyanidins solution (50 μL, 2 mg/mL in methanol) was mixed together with methanol acidified with concentrated HCl (50 μL, 3.3%, v/v) and 100 μL of mercaptoacetic acid (5% v/v in methanol). The vial was sealed with an inert Teflon cap, heated in 40 °C water bath for 30 min, kept at room temperature for 10 h, and then kept in the freezer (−20 °C) until the thiolysis media was analyzed using LC/MS. The column used was a 250 mm × 4.6 mm i.d., 3 μm, Atlantis C-18 column (Waters). The binary mobile phases consisted of A (2% acetic acid in water, v/v) and B (methanol), which were delivered in a linear gradient of B from 15 to 80% (v/v) in 45 min. The flow rate was set at 1.0 mL/min.

Determination of α-Glucosidase Activity. The activity of α-glucosidase in the rat intestine acetone powder was determined before use. The rat intestine acetone powder (0.5 g) was dissolved in 20 mL of sodium phosphate buffer (0.1 M, pH 6.9) and stirred in ice bath for 30 min before it was centrifuged at 2000g at 4 °C for 10 min. The supernatant was kept at −20 °C for further use. The unit of α-glucosidase stock solution was determined by glucose assay kit. α-Glucosidase solution (diluted 40×, 150 μL) was preincubated with the same quantity of sodium phosphate buffer (0.1 M, pH 6.9) in a thermoshaker for 5 min at 37 °C. The reaction was started by adding 300 μL of 20 mg/mL maltose solution. Eighty microliters of the mixture was taken out every 2 min, and immediately placed in a boiling water bath to stop the reaction. Forty microliters of inactivated suspension was incubated with 80 μL of glucose assay reagent in a microplate at 37 °C for 30 min. Sulfuric acid (80 μL, 6 M) was added and the solution was mixed by shaking. The absorbance was measured at 540 nm. The glucose calibration curve was prepared at a final concentration range of 5−25 μg/mL and determined in triplicate by glucose assay kits. One unit (U) of enzyme activity was defined as the amount of the enzyme that liberates 1.0 μM glucose from the substrate in 1 min under the test conditions.[14] The optical density (y-axis, OD at 540 nm) was linearly increased over time (x-axis) with $y = 0.0412x + 0.3734$, $R^2 = 0.99$. The α-glucosidase activity in the stock solution was calculated to be 3.08 U/mL.

Determination of the Activity of Chiku Proanthocyanidins in Inhibiting α-Amylase and α-Glucosidase. This was done by following a reported method.[15] Inhibition assay solution consisted of enzyme solution (50 μL), inhibitor solution (50 μL), and 1% starch solution (100 μL). The enzyme concentrations used for this assay were 3 U/mL for porcine pancreatic α-amylase and 1×10^{-2} U/mL for rat intestine α-glucosidase. Acarbose was prepared in sodium phosphate buffer (0.1 M, pH 6.9). Chiku proanthocyanidins was first dissolved in methanol and then diluted by sodium phosphate buffer to appropriate concentrations.

In a 96-well microplate, 50 μL of enzyme solution was preincubated with 50 μL of proanthocyanidins solution with series of concentrations in a microplate reader for 15 min at 37 °C. For α-amylase, the final concentrations of the proanthocyanidins were 3, 4, 5, 6, 7, and 8 μg/mL, while for α-glucosidase, the final concentrations of the proanthocyanidins were 8, 12, 16, 20, 24, and 28 μg/mL. The reaction was started by injecting 100 μL of starch solution using a 12-channel multichannel pipet. The turbidity change was immediately monitored at 660 nm for 2 h. The shaking intensity of the microplate reader was set at the highest level to ensure sufficient mixing and avoid starch sedimentation. The percentage of inhibition was defined by eq 1

$$\%\text{inhibition} = \frac{\text{AUC}_{\text{sample}} - \text{AUC}_{\text{control}}}{\text{AUC}_{\text{sample}}} \times 100 \quad (1)$$

In which $\text{AUC}_{\text{sample}}$ is the area under the curve (AUC) of inhibitor; $\text{AUC}_{\text{control}}$ is the area under the curve without inhibitors. The IC_{50} can be defined as the concentration of inhibitor that produces 50% inhibition of enzyme activity under the specified assay condition. It was obtained from interpolation of percentage of inhibition against inhibitor concentration curve.

Determination of Total Phenolic Contents. Total phenolic content for proanthocyanidins from chiku was determined colorimetrically with the Folin−Ciocalteu reagent, using a slightly modified method of Fukumoto and Mazza.[16] The reaction mixture contained 20 μL of proanthocyanidins solution, 160 μL of Folin−Ciocalteu reagent (0.2 M), and 20 μL of sodium carbonate solution; the mixture was kept in the dark under ambient conditions for 30 min to complete the reaction. The absorbance of the resulting solution was measured at 760 nm using the microplate reader. The concentration of total phenolic compounds was expressed as milligrams of gallic acid equivalents per gram of extract.

Statistical Analysis. Statistical analysis was performed using Microsoft Excel. The data were expressed as mean of quintuple runs ± SD with replicate analysis.

RESULTS AND DISCUSSION

Characterization of Proanthocyanidins from Chiku Using $HPLC/MS^n$. Chiku proanthocyanidins were characterized using the diol HPLC column. The diol HPLC column improved oligomeric separation as compared to traditional

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silica normal phase silica column. Other than chiku, diol column has also successfully separated proanthocyanidins from other food. It was first applied to analyze proanthocyanidins from cocoa and then from grape pomace, grape seed, cranberry, blueberry, and strawberry, with good baseline-resolved separation of up to decamers.[5,17,18]

HPLC separation of proanthocyanidins from chiku is shown in Figure 1. To further characterize these peaks, HPLC–ESI-

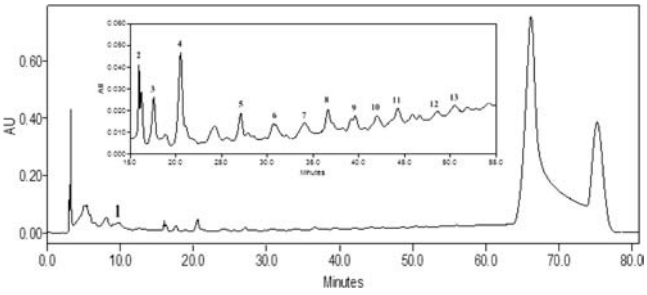

Figure 1. HPLC chromatogram of oligomeric proanthocyanidin in chiku. The identity of the compounds are listed in Table 1.

MS^n at negative mode was performed, and the results are summarized in Table 1. The structures of proanthocyanidins

Table 1. Observed Masses of Chiku Oligomeric Proanthocyanidins by ESI-MS (anionic mode)

peak number	compd identity[a]	m/z $[M - H]^-$	MS^2 fragmentation ions
1	dimer, GC–C (or C–GC)	593	289, 425, 467, 575
2	dimer, C–GCG	745	339, 457, 577
3	A-type trimer, $(C)_3$	861	527, 579, 594, 762
4	A-type trimer, $(C)_2$–GC	879	287, 407, 575, 675, 765
5	trimer, GC_3	913	609, 745
6	trimer, GC_2–GCG	1065	441, 761, 897
7	tetramer, $(GC)_3$–C	1201	897, 1015, 1075
8	tetramer, $(GC)_4$	1217	609, 745, 913
9	tetramer, $(GC)_3$–GCG	1369	761, 939, 1065, 1183
10	pentamer, $(GC)_4$–C	1505	725, 1201, 1337
11	pentamer, $(GC)_5$	1521	609, 913, 1217, 1353
12	hexamer, $(GC)_5$–C	1809	1201, 1351, 1453, 1505
13	hexamer, $(GC)_6$	1825	913, 1217, 1521, 1657

[a]C, GC, and GCG are the abbreviation for (epi)catechin,(epi)-gallocatechin, and (epi)gallocatechin gallate, respectively. The stereochemistry of the chiral carbons on the C ring of flavanols units is not defined.

were assigned on the basis of the m/z of their parent ions and their corresponding fragment ions from MS^2. Because mass spectrometry could not differentiate the chirality of C3 on the flavan-3-ols, we used (epi)catechin to indicate that the product could be either catechin or epicatechin.

Peak 1 gave the m/z value of 593 [306 + 290 − (2 − 1) × 2 − 1]. Its anionic MS^2 gave main daughter ion peaks at m/z 289, 425, 467, and 575. The main fragment ion at m/z 289 $[M - H - 304]^-$ was assigned to be (epi)catechin and might arise from quinone–methide cleavage of the interflavanoid bond; m/z 425 $[M - H - 168]^-$ might arise from retro-Diels–Alder fission of the heterocyclic rings, and m/z 467 $[M - H - 126]^-$ might arise from heterocyclic ring (HR) fission of the heterocyclic rings. The fragmentation pathway of this dimer is shown in Figure 2. Thus, peak 1 was assigned as a dimer of (epi)gallocatechin (G) and (epi)catechin (C). However, from the MS^2 fragmentation pattern it is not possible to distinguish the linkage sequence (i.e., GC or CG). Peak 3 gave the m/z value of 861 [290 × 3 − (3−1) × 2 − 2 × 2 − 1] and thus was assigned to be double A-type (epi)catechins trimer. Peak **4** had the m/z of 779 [290 × 2 + 306 − (3−1) × 2 − 1 × 2 − 1]. Its main fragment ions at m/z 287 $[M - H - 288 - 304]^-$ and 575 $[M - H - 304]^-$ are common fragments from interflavanoid bond cleavage. m/z 287 was a quinone methide (epi)catechin, which indicated that (epi)catechin was the extension unit. Thus peak 4 was assigned as an A-type trimer of two (epi)catechins and one (epi)gallocatechin. Peak 7 had the m/z of 1201 [306 × 3 + 290 − (4 − 1) × 2 − 1]. Its main daughter ion at m/z 897 $[M - H - 304]^-$ might arise from the interflavanoid bond cleavage by losing a neutral (epi)-gallocatechin quinone methide fragment; this indicated that the terminal unit was a (epi)gallocatechin. The peak at m/z 1015 $[M - H - 168 - 18]^-$ might arise from retro-Diels–Alder fission of the heterocyclic ring and further loss of water, while m/z 1075 $[M - H - 126]^-$ might arise from fission of the heterocyclic rings. Peak 7 was therefore assigned to be a tetramer of three (epi)gallocatechins and one (epi)catechin unit. Peak 9 had the m/z of 1369 [306 × 3 + 458 − (4 − 1) × 2]. The three fragment ions at m/z 1065 $[M - H - 304]^-$ and 761 $[M - H - 304 \times 2]^-$ might arise from interflavanoid bond cleavage; m/z 1183 $[M - H - 168 - 18]^-$ might arise from retro-Diels–Alder fission of the heterocyclic rings and loss of water. Therefore, peak 9 was assigned to be a tetramer of three (epi)gallocatechins and one (epi)gallocatechin gallate. Peak 10 gave the m/z of 1505 [306 × 4 + 290 − (5 − 1) × 2 − 1]. Its main fragment ion at m/z 1201 $[M - H - 304]^-$ was from interflavanoid bond fragmentation losing a neutral gallocatechin quinone methide. Therefore, peak 10 was assigned to be a B-type pentamer of four (epi)gallocatechins and one (epi)-catechin. Peak 12 had the m/z of 1809 [306 × 5 + 290 − (6 − 1) × 2 − 1]. Its main fragment ions at m/z 1505 $[M - H - 304]^-$ and 1201 $[M - H - 304 \times 2]^-$ were derived from interflavanoid bond cleavage. Therefore, peak 12 was assigned to be a hexamer of five (epi)gallocatechins and one (epi)catechin.

Parent ions at m/z $[M - H]^-$ for peaks 2, 5, 6, 8, 11, and 13 were 745, 913, 1065, 1217, 1521, and 1825, respectively. These peaks have been identified by a previous report,[2] and our assignments were consistent with their results. Peak 2 gave m/z 745 [290 + 458 − (2 − 1) × 2 − 1] Da. Its main fragment ions at m/z 457 $[M - H - 288]^-$ might arise from interflavanoid bond cleavage. Therefore, peak 2 was assigned as a dimer of one (epi)catechin and one (epi)gallocatechin gallate. Peak 5 gave the m/z of 913 [306 × 3 − (3 − 1) × 2 − 1]. Its main fragment ions at m/z 609 $[M - H - 304]^-$ might arise from interflavanoid bond cleavage; m/z 745 $[M - H - 168]^-$ might arise from retro-Diels–Alder fission of the heterocyclic rings. Therefore, peak 5 was identified to be a trimer of (epi)-gallocatechins. Peak 6 gave the m/z of 1065 [306 × 2 + 458 − (3 − 1) × 2 − 1]. Its main fragment ions at m/z 761 $[M - H - 304]^-$ might arise from interflavanoid bond cleavage; m/z 897 $[M - H - 168]^-$ might arise from retro-Diels–Alder fission of the heterocyclic rings. Therefore, peak 6 was assigned to be a timer of two (epi)gallocatechins and one (epi)gallocatechin gallate. Peaks 8, 11, and 13 gave m/z values of 1217 [306 × 4 −

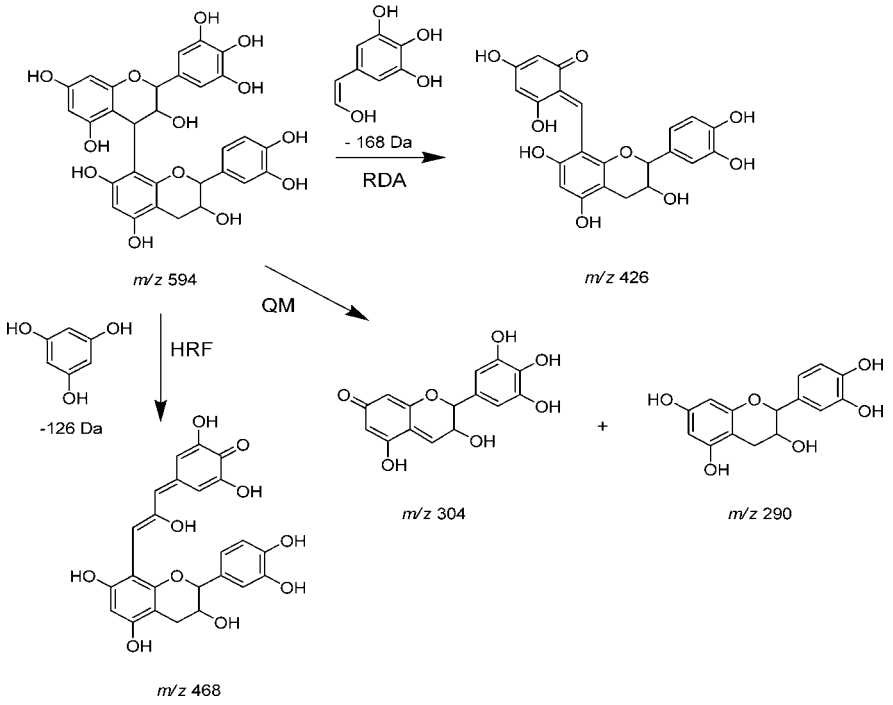

Figure 2. Fragmentation pathway of a GC−C dimer detected in proanthocyanidins from chiku.

$(4-1)\times 2-1]$, 1521 $[306\times 5-(5-1)\times 2-1]$, and 1825 $[306\times 6-(6-1)\times 2-1]$, respectively. Similar fragmentation patterns of these peaks to peak 5 have been tabulated in Table 1. Therefore, peak 8, 11, and 13 were assigned to be a tetramer, pentamer, and hexamer of (epi)gallocatechins, respectively. The commonly seen pattern of the fragmentations of these oligomers is the release of a neutral fragment of 304, which suggests that the oligomers have the same extension unit of (epi)gallocatechin. For the identity of the terminal unit, it is likely to be (epi)catechin, as shown from the thiolyzed product that produces catechin thioether derivative.

The proanthocyanidins with one A-type linkage were identified readily on MS by their m/z of $[M-H]^-$ being two units less than those of the B-type proanthocyanidins. Therefore, our LC−MS^2 results showed that, among the 13 compounds, only peaks 3 and 4 had the A-type linkages; the rest were all B-type linkages. In addition, in the presence of B-type interflavanoid bond, A-type interflavanoid bonds in the same molecule do not undergo quinine methide cleavage. Therefore, the position of an A-type linkage in the proanthocyanidins could be identified according to their product ion spectra. Therefore, the A-type trimer of peak 4 had an (epi)catechin→A→(epi)catechin→(epi)gallocatechin connection.

Thiolysis of Proanthocyanidins from Chiku. To investigate the main components of proanthocyanidins and the sequence of the monomeric units in chiku, depolymerization through a thiolysis reaction was carried out by following standard conditions using mercaptoacetic acid.[11] The advantage of using mercaptoacetic acid over commonly used mercaptotoluene is that the former has no UV/vis absorbance at the detection wavelengths (280 nm) of the (epi)catechins and gives a better HPLC chromatogram for thiolysis products in the reaction mixture (Figure 3). We have compared the conversion of mangosteen proanthocyanidins by different thiol compounds

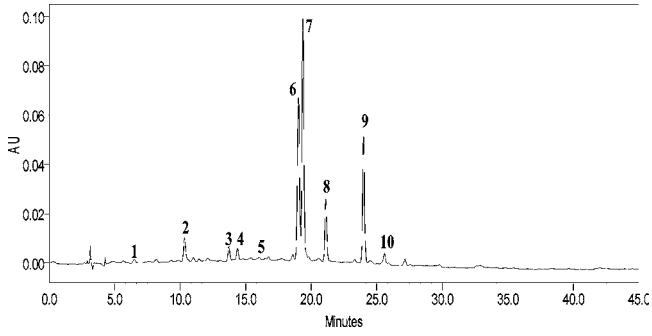

Figure 3. HPLC chromatogram of thiolytic products of proanthocyanidins by mercaptoacetic acid: gallocatechin, 1; epigallocatechin, 2; catechin, 3; epigallocatechin gallate, 4; epicatechin, 5; (epi)-gallocatechin thioether, 6; (epi)gallocatechin thioether, 7; (epi)-gallocatechin gallate thioether, 8; epicatechin thioether, 9; (epi)-catechin gallate thioether, 10.

and found that mercaptoacetic acid gave the highest conversion (55% yield) to epicatechin thioether.[19] In this case, the peaks of thiolyzed derivatives were identified using the LC−MS and standard compounds. The major product observed was the 4β-(carboxymethyl)sulfanyl-(−)-(epi)gallocatechin methyl ester along with significant amounts of 4β-(carboxymethyl)sulfanyl-(−)-epicatechin methyl ester and 4β-(carboxymethyl)sulfanyl-(−)-(epi)gallocatechin gallate methyl ester and much smaller peaks for gallocatechin, epigallocatechin, catechin, epigallocatechin gallate, epicatechin, and 4β-(carboxymethyl)sulphanyl-(-)-(epi)catechin gallate methyl ester. This result suggested that there were significant amounts of (epi)gallocatechin extension units in chiku proanthocyanidins. In addition, the major terminal unit in chiku proanthocyanidins was epigallocatechin judging from the HPLC peak intensity. The mean degree of polymerization (mDP) of chiku proanthocyanidins was estimated to be 9.0 by comparing the peak areas from the area under the curves of the HPLC chromatogram based on eq

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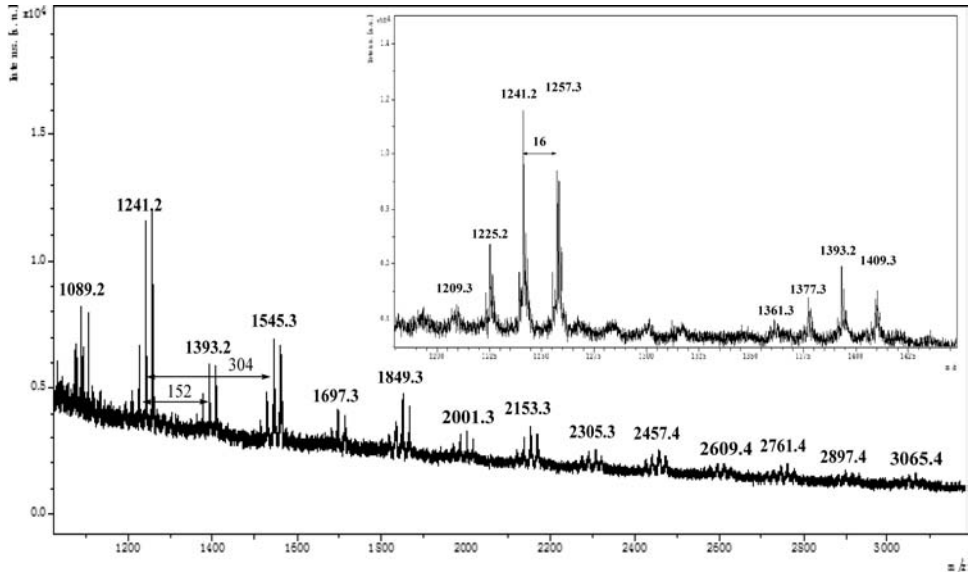

Figure 4. MALDI-TOF mass spectrum of chiku proanthocyanidins.

2. It should be pointed out that, due to the lower absorbance coefficient of the gallocatechin in comparison with that of epicatechin, the actual mean degree of polymerization is likely to be a little higher.

$$\mathrm{mDP} = \frac{\sum[\text{extension units}] + \sum[\text{terminal units}]}{\sum[\text{terminal units}]} \quad (2)$$

Proanthocyanidins isolated from different plants show various mDPs and characteristics of monomeric units. Prodelphinidins are less common in nature compared with the procyanidins that exist most widely in plants. They are most heterogeneous in their constituent units and coexist with procyanidins.[13] Other than chiku proanthocyanidins, blackcurrants,[13] fruits of the poisonous plant *Iris pseudacorus*,[20] and sea buckthorn pomace[21] were all observed to have high distributions of (epi)gallocatechin subunits within the mixture of proanthocyanidins.

The A-type linkage in proanthocyanidins remains stable during the thiolytic degradation.[22] The A-type linkage in terminal units is released as an A-type dimer, whereas the A-type linkage between the extension units yields an A-type dimer thioether. However, in our thiolysis result, no A-type dimer derivatives were observed, although they were detected in small amounts (peaks 3 and 4 in Figure 1). The thiolyzed product concentration could be below the detection limit.

MALDI-TOF MS Analysis of Chiku Proanthocyanidins. A series of peaks arising from proanthocyanidins at m/z $[M + Na]^+$ as well as $[M + K]^+$ were obtained from trimer to decamer (Figure 4). The major peak assignments are listed in Table 2. The difference between the major peaks was at m/z 304, which coincides with the mass of (epi)gallocatechin. The spectra revealed a second m/z increment of 152, coinciding with the mass of the galloyl group, which indicated the existence of (epi)gallocatechin gallate. The major peaks were accompanied by peaks that were m/z 16 smaller or larger (Figure 4). The peak that was 16 m/z smaller is likely due to one less −OH group in one of the monomeric units, while the peak that was 16 m/z larger can arise from two possibilities: K^+ instead of Na^+ ion (the mass difference is also 16) or one more −OH group on one of the monomeric unit. However, for oligomers with gallocatechin as the monomeric unit, it is unlikely to have one more −OH group on the B ring. The thiolyzed product profile does not support the presence of such product either. On the basis of this analysis, peak 1241.2 could be assigned as the tetramer of (epi)gallocatechins ($+Na^+$). Peak 1225.2 might be the tetramer of three (epi)gallocatechins and one (epi)catechin. Peak 1209.3 might be the tetramer of two (epi)gallocatechins and two (epi)catechins. Peak 1257.3 could also be assigned to the tetramer of (epi)gallocatechins, but with K^+ ion instead of Na^+ attached.

Table 2. Observed Masses of Chiku Oligomeric Proanthocyanidins by MALDI-TOF

m/z $[M + Na]^+$	polymer	basic unit[a]	interflavan bond
1089.2	trimer	$(GC)_2$−GCG	B type
1241.2	tetramer	$(GC)_4$	B type
1393.2	tetramer	$(GC)_3$−GCG	B type
1545.3	pentamer	$(GC)_5$	B type
1697.3	pentamer	$(GC)_4$−GCG	B type
1849.3	hexamer	$(GC)_6$	B type
2001.3	hexamer	$(GC)_5$−GCG	B type
2153.3	heptamer	$(GC)_7$	B type
2305.3	heptamer	$(GC)_6$−GCG	B type
2457.4	octamer	$(GC)_8$	B type
2609.4	octamer	$(GC)_7$−GCG	B type
2761.4	nonamer	$(GC)_9$	B type
2897.4	nonamer	$(GC)_8$−CG/$(GC)_7$−C−GCG	B type
3065.4	decamer	$(GC)_{10}$	B type

[a]C, GC, CG, and GCG are the abbreviation for (epi)catechin, (epi)gallocatechin, (epi)catechin gallate, and (epi)gallocatechin gallate, respectively; the stereochemistry of the chiral carbons on the C ring of flavanol units is not defined.

Inhibition Activity of Chiku Proanthocyanidins on α-Amylase and α-Glucosidase. We applied a recently reported high-throughput method for rapid determination of starch hydrolase inhibition of chiku proanthocyanidins.[15] Figure 5 summarized the inhibitory effects of different concentrations of proanthocyanidins against α-amylase and α-glucosidase. A dose-dependent effect can be observed, and the result showed

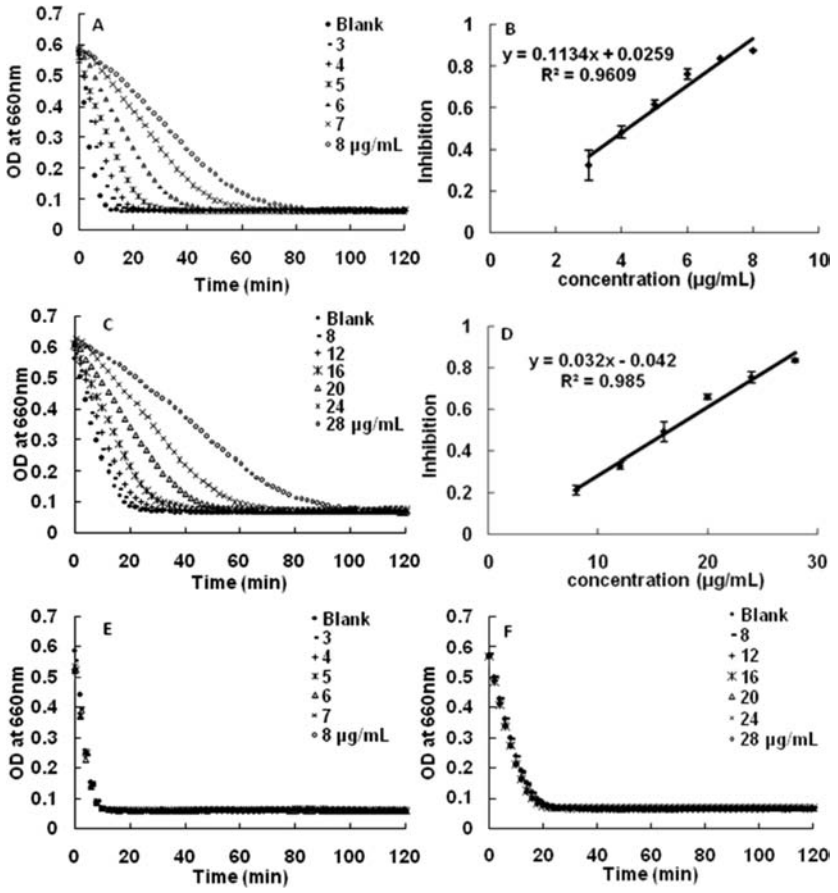

Figure 5. (A) Turbidity change at different concentrations of chiku extract that reflects chiku proanthocyanidins' inhibitory effect on starch hydrolysis by α-amylase. (B) The relationship between AUC and the chiku proanthocyanidins concentration. (C) The representative kinetic curve of starch hydrolysis by α-glucosidase in the presence of chiku extract. (D) The net AUC and percent of inhibition change by chiku extract. (E) The representative kinetic curve of starch hydrolysis by α-amylase in the presence of thiolyzed proanthocyanidins. (F) The representative kinetic curve of starch hydrolysis by α-glucosidase in the presence of thiolyzed chiku proanthocyanidins.

that the proanthocyanidins caused significant α-amylase inhibition with a concentration as low as 3 μg/mL. The inhibition activity increased linearly with the increased concentrations of proanthocyanidins. When the extract concentration reached 8 μg/mL, this inhibition activity could reach about 90%. From the dose response curve, we calculated the IC_{50} of 4.2 ± 0.2 μg/mL for α-amylase and IC_{50} of 16.6 ± 0.3 μg/mL for α-glucosidase. To investigate that chiku proanthocyanidins are responsible for this enzyme inhibition activity, we tested the inhibiton activity of the thiolyzed product mixtures and found that there is no detectable inhibitory activity for both α-amylase and α-glucosidase (Figure 5E,F). Therefore, we concluded that it might be the proanthocyanidins, not other compounds or the proanthocyanidins monomers in chiku proanthocyanidins, that cause this inhibition activity. Although our results are in agreement with the fact that proanthocyanidins are known amylase inhibitors, it is also possible that the reaction conditions (acid + mild heating) may inflict changes also on other active compounds in the mixture and inhibitory activity may be lost. Other proanthocyanidins sources include berry,[9] chestnut skins,[23] and sorghum bran.[24]

Total Phenolic Content. The total phenolic content of the proanthocyanidins from chiku was determined to be 295 ± 14 mg GAE/g extract. We recently found that the total phenolic content of grape seed extract had a value of 734 ± 27 mg GAE/g extract.[15] The total phenolic content of proanthocyanidins from chiku is much lower than that of grape seed extract. However, the α-amylase inhibition activity is even stronger than that of grape seed extract. This indicates that the total phenolic content is not directly responsible for the relevant inhibition effects between total phenolic contents and starch hydrolase inhibition activity. This inhibition activity mainly depends on the specific structure of phenolic compounds.

In summary, we have shown that chiku is a good source of oligomeric proanthocyanidins dominated by B-type linkages. Characterization of the components of proanthocyanidins in chiku was done by LC−ESI-MS^n. Results showed that proanthocyanidins in chiku were mainly composed of gallocatechin and/or epigallocatechin units. The chiku proanthocyanidins exhibited good activity toward inhibition of α-amylase and α-glucosidase.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Selected ESI-MS/MS mass spectra of chiku proanthocyanidins in negative mode. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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