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Rapid Preparation of Procyanidins B2 and C1 from Granny Smith Apples by Using Low Pressure Column Chromatography and Identification of Their Oligomeric Procyanidins

JUN-SONG XIAO, LIANG LIU, HUA WU, BI-JUN XIE,* ER-NING YANG, AND ZHI-DA SUN

College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, P. R. China

Research in the field of procyanidins is always hindered by the lack of procyanidin standards, and the preparation of procyanidins, especially in large scale, remains difficult and time-consuming. Commercial sources of procyanidin standards are scarce. In this study, a rapid preparation method of procyanidins by using low-pressure column chromatography was developed. Procyanidins in Granny Smith apples were extracted with boiled water and purified on an ADS-17 macroporous resin column to obtain a Granny Smith apple procyanidin extract (GSE). GSE was fractionated according to its degree of polymerization on a Toyopearl TSK HW-40s column. Procyanidins B2 (epicatechin-(4β−8)-epicatechin) and C1 (epicatechin-(4β−8)-epicatechin-(4β−8)-epicatechin) were prepared without HPLC separation. Oligomeric procyanidins from Granny Smith apples were also identified by liquid chromatography−electrospray ionization−mass spectrometry.

KEYWORDS: Granny Smith apple; procyanidin; Toyopearl TSK HW-40s column; preparation

INTRODUCTION

Procyanidins (i.e., condensed tannins) are a class of polyphenols widely present in the plant kingdom and our daily diet, such as grape, cocoa bean, apple, cranberry, litchi, sea buckthorn, peanut, sorghum, hawthorn, almond seed coat, cinnamon, chocolate, red wine, green tea, black tea, and so forth (*1–7*). Procyanidins have also been reported in many traditional and folk medicinal plants (including Chinese traditional medicinal plants), such as *Rheum officinale* (Da-Huang) (*8, 9*), *Melastoma candidum* (*10*), *Davallia mariesii* (*11*), *Davallia divaricata* (*12*), *Nelumbo nucifera* (*13*), *Rosa cymosa*, *Rosa rugosa*, *Cedrela sinensis*, *Eucommia ulmoides* (Du-Zhong), *Sargentodoxa cuneata*, *Zanthoxylum piperitum*, *Cudrania tricuspidata*, *Houttuynia cordata* (*14–18*), *Uncaria tomentosa* (*19*), *Rhamnus lycioides* (*20*), and so forth.

Procyanidins are composed of chains of flavan-3-ol units, that is, (+)-catechin and (−)-epicatechin, linked mainly through C4−C8 or C4−C6 bonds. Some structural variations may occur because of the formation of a second interflavan bond by C−O oxidative coupling to form A-type procyanidins. Some procyanidins may be esterified to gallic acid (**Figure 1**).

The multiple phenolic hydroxyl groups of procyanidins may form complexes with proteins and induce antioxidation properties. The former ability endows procyanidins astringency in taste, antibacterial properties, and the ability to inhibit some enzymes, such as angiotensin I-converting enzyme (*21*), xanthine oxidase, xanthine dehydrogenase, horseradish peroxidase, lipoxygenase (*22*), phosphorylase kinase, protein kinase C, and protein kinase A (*23*). The latter ability endows procyanidins various bioactivities in vivo, including cardiovascular protection (*24*), low-density lipoprotein oxidation inhibition (*25*), anti-inflammation (*26*), antitumor-proliferation (*27*), and so forth. Therefore, procyanidins are believed to play an important role in human health.

Therefore, it is important to acquire detailed information about procyanidins in daily diet and in medicinal plants, including degree of polymerization (DP), composing unit profiles, and interflavan bond types.

Procyanidins DP was determined by acid degradation analysis (including thiolysis and phloroglucinolysis) and gel permeation chromatography (*28–30*). Composing unit profiles can also be obtained from the acid degradation analysis. Enzymatic hydrolysis (*31*) was employed to analyze galloyl esters of procyanidins. Interflavan bond types were determined by NMR. Circular dichroism was used to identify the stereo conformation of C_4 in procyanidins (*32*).

Reverse-phase (RP) HPLC is a common method for the analysis and separation of procyanidins, because it can separate isomers of equivalent molecular mass into different peaks. But it is hard to separate procyanidins with a DP greater than five (*33*). Normal-phase HPLC (or normal-phase low-pressure chromatography) can separate procyanidins of high DP and can be

* To whom correspondence should be addressed. Tel: 86-27-8728-3201. Fax: 86-27-8728-2966. E-mail: bijunxie@sina.com.

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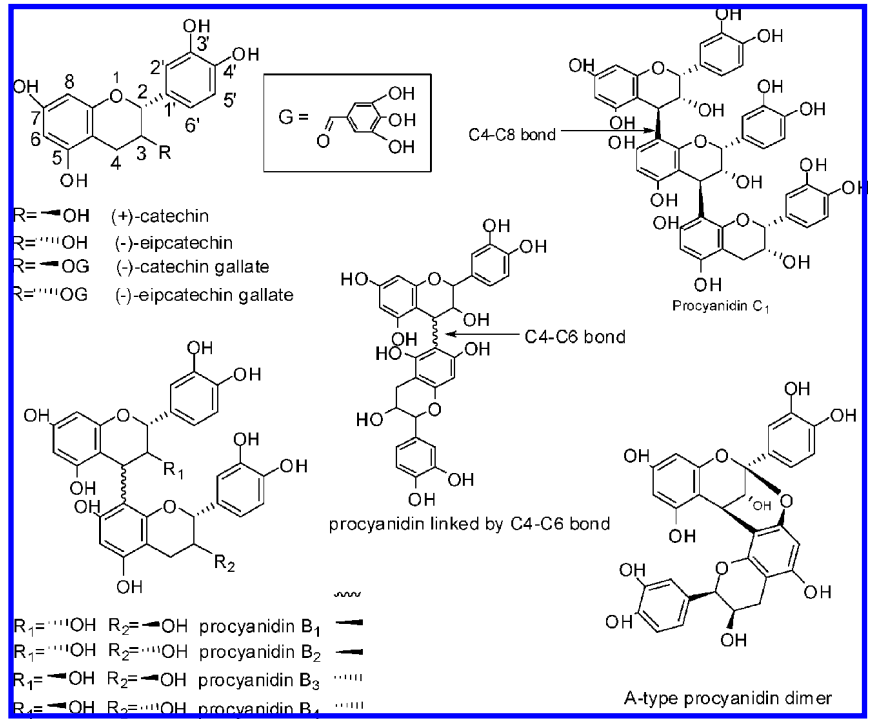

Figure 1. Chemical structure of procyanidins.

used in large-scale preparation. For example, columns packed with silica beads were used to separate procyanidins from cocoa beans, grape seeds and skin, and apple extract (*3, 34, 35*). However, normal-phase chromatography usually uses environment-unfriendly organic solvent (e.g., methylene chloride) as mobile phase. Sephadex LH-20 and Toyopearl TSK HW-40s columns have also been used in procyanidins' fractionation (*31, 36–38*).

To characterize procyanidins in plants and evaluate their biological activities, it is necessary to obtain pure procyanidin compounds. Despite developments in the analysis and preparation of procyanidins, the preparation of oligomeric procyanidins remains time-consuming and cannot be employed in large-scale production. Procyanidin trimers, tetramers, and pentamers are still not commercially available. In the present study, we report a rapid method for the preparation of procyanidins B2 and C1 by using low-pressure column chromatography. The oligomeric procyanidin constituents in Granny Smith apples were also studied.

MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile was HPLC grade and purchased from Fisher Scientific International (Waltham, MA). (+)-Catechin, (−)-epicatechin, and phloroglucinol were from Sigma (St. Louis, MO). Procyanidin B2 standard (97% HPLC) was from Nakahara Science Co., Ltd. (Japan). Procyanidins B1, B3, and C1 prepared from grape seeds (*31*) were donated by Professor Victor A. P. Freitas (Porto University, Portugal). Other chemicals and reagents were of analytical grade.

Preparation of Granny Smith Apple Procyanidin Extract (GSE). Granny Smith apples were bought from a local supermarket. A total of 500 g of Granny Smith apples was mashed in 500 mL of distilled water containing 2 g of sodium bisulfate and put into 1500 mL of boiling distilled water. The preparation was boiled for 5 min. The homogenate was cooled to room temperature and filtrated through a 200 mesh filtration cloth. The filtrate was then centrifuged at 4000 rpm for 10 min, and the supernatant was applied to a column (300 mm × 42 mm i.d.) packed with ADS-17 macroporous resin (Nankai Hecheng, Tianjing, China). ADS-17 is a middle-polar resin with hydroxyl groups

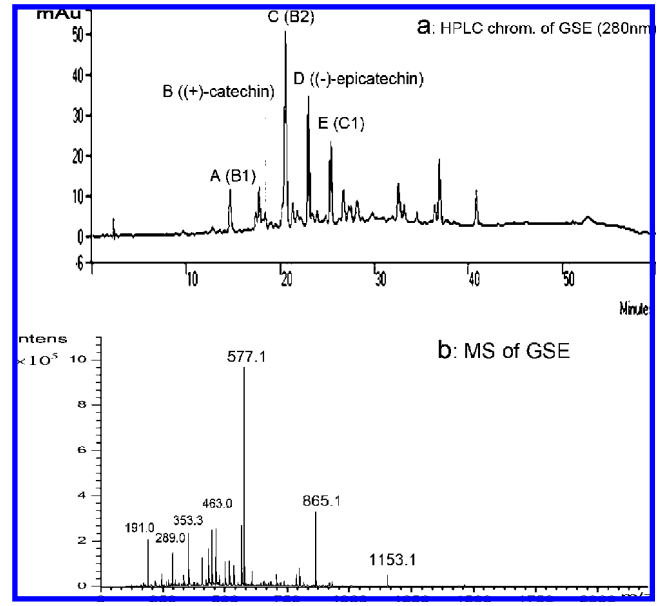

Figure 2. (**a**) HPLC of GSE combined with ESI-MS to identify peaks. (**b**) Direct MS of GSE.

on the surface of beads. The column was washed with 2500 mL of distilled water to remove sugar and low molecular mass materials. Then, the column was eluted with 100 mL of 70% (v/v) aqueous ethanol at a flow rate of 10 mL/min. The eluent was evaporated to about 30 mL under reduced pressure at 35 °C, and the remaining solution was freeze-dried to obtain the GSE.

Fractionation of GSE by Toyopearl TSK HW-40s Column. A Toyopearl TSK HW-40s column (Tosoh, Japan) was packed in a Pharmacia XK16 column (250 × 16 mm i.d.). Methanol was used as mobile phase. A total of 50 mg of GSE was dissolved in 2 mL of methanol and filtered through a 0.22 μm membrane filter to remove the insoluble portion. The filtrate was loaded on the column and eluted at 0.8 mL/min with a LC-1000 pump (Shimadzu, Japan). The eluant was monitored by a HD-3 UV detector at 280 nm and recorded by a XWT-S recorder. Fractions were collected every 5 min, 1 h after the

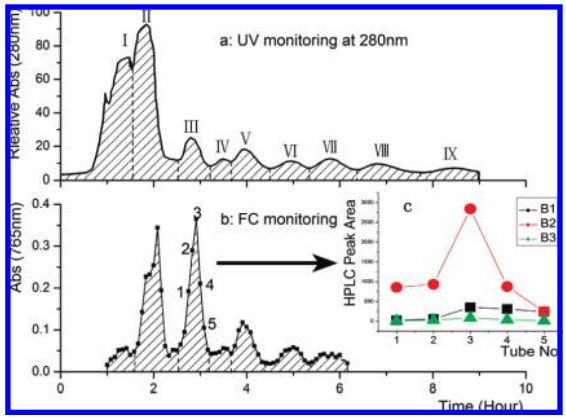

Figure 3. Elution profile of GSE monitored by (**a**) UV detector at 280 nm and (**b**) the FC method . (**c**) Five tubes in peak III were analyzed by HPLC, and procyanidins B1, B2, and B3 contents were determined by peak area. GSE was fractionated into eight fractions by a Toyopearl TSK HW-40s column with methanol as mobile phase at 0.8 mL/min.

elution process began, by using a BSZ-100 fraction collector (the above three instruments are from Huxi Instrumental Co., Shanghai, China).

The total phenolic content in each tube was determined by Folin-Ciocalteu's (FC) method (*39*) with some modifications. The FC method was used to validate whether UV detection alone would be enough for monitoring the fractionation process. A sample of 0.1 mL was mixed with 6 mL of distilled water and 0.5 mL of FC reagent, and then, 1.5 mL of 20% (w/v) sodium carbonate solution was added to the mixture in 8 min. The mixture's volume was increased to 10 mL with distilled water, and the mixture was kept at ambient temperature for 2 h; a deep blue color developed. The absorbance was measured by using a UV-1700 spectrophotometer (Shimadzu, Japan) at 765 nm.

Liquid Chromatography−Electrospray Ionization−Mass Spectrometry (LC-ESI-MS) Analysis of Procyanidins. GSE and fractions were analyzed by an Agilent 1100 LC-ESI-MS system (Agilent, Foster City, CA). LC conditions were as follows. The column was a Zorbax SB-C18 column (5 μm particle size, 2.1 × 150 mm). The mobile phase consisted of 0.2% aqueous acetic acid as eluent A and acetonitrile as eluent B with the flowing gradient: 5 to 15% B for 10 min, 15 to 20% B for 5 min, 20 to 40% B for 20 min, 40 to 50% B for 10 min, 50 to 5% B for 5 min, re-equilibrated with 5% B for 5 min before the next injection. The flow rate was 0.2 mL/min, and the UV-absorbance of procyanidins was monitored with a diode array detector at 280 nm. Mass spectra were collected in the negative-ion mode with the ESI ion source under the following conditions: fragmenter voltage = 100 V, capillary voltage = 2500 V, nebulizing pressure = 30 psi, dry gas temperature = 300 °C, and mass range = 100−2200 *m/z*.

RESULTS

LC-ESI-MS Analysis of GSE. The extract for the preparation of procyanidins should have simple constituents to avoid interference and have relatively high levels of oligomeric compounds. Apples have simple procyanidin constituents and are rich in oligomeric procyanidins (*31, 38*). Among Granny Smith, Red Delicious, Golden Delicious, and Fuji apples (*2, 3, 40*), Granny Smith apples have more procyanidins and less anthocyanins than the others do; therefore, it was chosen for the preparation of dimeric and trimeric procyanidins in this study.

Granny Smith apples were extracted in boiling water and purified by an ADS-17 macroporous resin column, as described above, to obtain GSE. The GSE was analyzed by LC-ESI-MS.

LC-ESI-MS analysis (**Figure 2**) showed that *m/z* ratios of the peaks at 280 nm were A (577.2), B (289.0), C (577.2), D (289.0), and E (865.5), and the peaks were identified as dimer B1, (+)-catechin, (−)-epicatechin, dimer B2, and trimer C1, respectively, by coelution with standards on RP-HPLC. This suggested that GSE might be a good source for the preparation of procyanidin dimers and trimers.

Fractionation of GSE by Toyopearl TSK HW-40s Column Chromatography. GSE was fractionated on a Toyopearl TSK HW-40s column and monitored by a UV detector at 280 nm, and the polyphenol content in each tube was determined by the FC method. The elution profile obtained by UV detection (**Figure 3a**) matched well with the elution profile obtained by the FC method (**Figure 3b**, in which peaks VIII and IX were not determined), which indicated that UV monitoring alone will be enough for the fractionation monitoring. The nine fractions thus obtained were analyzed by LC-ESI-MS.

Compounds in fractions I and II were combined before analysis. They were identified by comparison with LC, MS, and MS^2 data reported by K. Kahle et al. (*41*) and found to be mainly the glycosides of quercetin and phloretin. They were identified

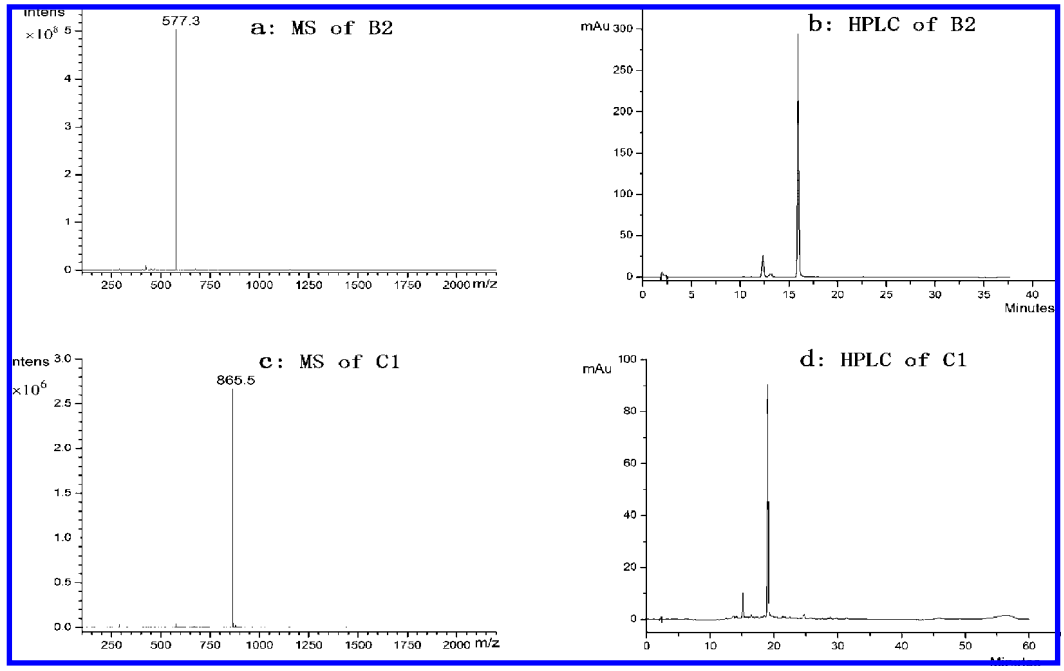

Figure 4. MS and HPLC spectra of procyanidin B2 (**a**, **b**) and C1 (**c**, **d**).

Table 1. Retention Time, λ_{max}, MS and MS^2 Data, and Tentative Compounds in Different Fractions from GSE

t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*, relative abundance (%))[a]	tentative compounds
			Fractions I+II	
16.4	280	577.0	424.9(100), 406.9(41), 288.9(23), 451.0(22), 559.0(20)	dimer B_2
17.5	280	289.1	244.9(100), 204.9(34)	(−)-epicatechin
22.9	355	463.1	300.8(100)	quercetin-3-*O*-galactoside
23.1	355	463.0	300.8(100)	quercetin-3-*O*-glucoside
24.4	354	433.4	300.7(100)	quercetin-3-*O*-arabinoside or Quercetin-3-*O*-xyloside
25.0	284	567.3	273.0(100)	phloretin-2′-*O*-xyloglucoside[b]
25.4	350	447.1	300.8(100)	quercetin-3-*O*-rhamnoside[b]
27.3	284	435.2	272.9(100)	phloridzin
27.5	286	273.1	166.8(100)	phloretin
			Fraction III	
13.1	280	577.2	425.1(100), 407.1(93), 289.0(59), 451.1(52), 559.1(21)	dimer B1
16.5	280	577.2	425.0(100), 407.0(49), 451.1(39), 289.0(24), 559.0(12)	dimer B2[b]
			Fraction IV	
18.6	280	865.1	695.0(100), 576.9(91), 739.2(74), 713.0(44), 287.2(11)	trimer Y1
19.1	280	865.0	ND	trimer C1
22.0	280	577.1	406.9(100), 424.9(99), 451.0(72), 288.9(64), 559.0(21)	dimer X[b]
			Fraction V	
15.1	280	865.5	695.2(100), 577.0(87), 739.2(81), 575.3(56), 713.2(42), 407.1(20), 287.1(24), 847.3(23), 451.1(23)	trimer Y2
19.1	280	865.5	695.2(100), 577.1(77), 739.3(50), 575.3(44), 713.2(34), 451.1(23), 587.0(21), 407.1(21), 287.1(21), 847.3(19), 425.1(15)	trimer C1[b]
			Fraction VI	
15.2	280	865.5	695.2(100), 577.0(77), 739.3(63), 713.2(36), 575.3(33), 847.3(29), 587.0(21), 287.1(13), 451.1(13)	trimer X2
16.2	280	865.2	577.0(100), 739.3(93), 695.1(91), 713.2(51), 575.3(58), 847.3(28), 587.1(34), 286.8(30), 451.1(30)	trimer Y3[b]
18.6	280	865.5	577.1(100), 739.2(72), 695.2(97), 713.2(72), 847.3(23)	trimer Y1
19.8	280	865.1	713.1(100), 575.3(100), 577.1(87), 739.3(91), 695.1(97), 847.1(25), 286.9(62), 450.9(27), 424.8(56), 407.0(41)	trimer Y4
20.5	280	865.5	ND	trimer Y5
			Fraction VII	
15.4	280	1153.4	864.9(100), 1027.2(58), 983.1(53), 1001.1(35), 1135.2(46), 575.2(27), 739.0(19)	tetramer Z1
16.9	280	865.1	695.0(100), 577.0(88), 739.1(71), 713.1(48), 450.9(39), 406.9(33), 286.9(28), 424.9(23), 847.1(21)	trimer Y6
17.8	280	1153.2	ND	tetramer Z2
19.7	280	1153.3	694.9(100), 983.1(83), 575.1(71), 577.0(41), 1027.2(57), 739.0(34), 1001.1(34), 1135.2(28), 424.9(23), 407.0(20)	tetramer Z3[b]
24.5	280	1153.1	ND	tetramer Z4
			Fraction VIII	
13.2	280	1153.2	1027.2(100), 865.0(97), 574.9(95), 863.2(89), 576.9(83), 983.2(78), 1135.3(62), 738.9(47), 1001.1(42), 406.8(40), 448.9(31)	tetramer Z5
16.9	280	1153.3	865.0(100), 863.3(70), 983.2(70), 575.0(68), 576.9(40), 1027.2(48), 1135.3(40), 1001.1(34), 738.9(22), 448.9(20)	tetramer Z6[b]
17.3	280	1153.5	983.1(100), 863.1(95), 1027.2(64), 865.1(60), 575.0(51), 1135.1(36), 449.1(34), 577.3(26), 739.1(26), 1001.0(22)	tetramer Z7[b]
20.5	280	1153.2	863.2(100), 576.9(57), 575(57), 864.9(56), 983.1(34), 1027.2(27), 1001.1(24), 739(22), 1135.1(16), 448.9(15)	tetramer Z8
			Fraction IX	
14.8	280	1153.3	863.4(100), 864.7(36), 1027.2(35), 1001(24), 1135.2(24), 577.3(14)	tetramer Z9
17.1	280	865.1	ND	trimers Y7
20.8	280	1441.5	865.0(100), 864.3(70), 1153.1(64), 863.4(55), 1271.3(38), 1152.3(32), 1027.0(26), 575.0(18), 1423.0(16), 1315.3(17), 577.3(14), 1289.2(14)	pentamer[b]

[a] ND means that MS^2 was not detected. [b] Main compounds of one fraction according to UV and MS.

as dimer B2 (*m*/*z* = 577.0), (−)-epicatechin (*m*/*z* = 289.1), quercetin-3-*O*-glucoside (*m*/*z* = 463.1), quercetin-3-*O*-galactoside (*m*/*z* = 463.0), quercetin-3-*O*-arabinoside (*m*/*z* = 433.4) or quercetin-3-*O*-xyloside (*m*/*z* = 433.4), phloridzin (*m*/*z* = 435.2), phloretin (*m*/*z* = 273.1) and phloretin-2′-*O*-xyloglucoside (*m*/*z* = 567.3), and quercetin-3-*O*-rhamnoside (*m*/*z* = 447.1). Strangely, chlorogenic (*m*/*z* = 353.0), which is present in **Figure 2b**, was not present in fractions I and II. One reasonable explanation is that it was eluted out before fraction I was collected.

Fraction III contained mainly dimers, including procyanidins B1 (epi-(4β−8)-cat, *m*/*z* = 577.2), B2 (epi-(4β−8)-epi, *m*/*z* = 577.2), and traces of B3 (cat-(4β−8)-cat, *m*/*z* = 577.1), which were identified by their MS and MS^2 data and by coelution with dimer standards by HPLC. Five tubes (tubes no. 1, 2, 3, 4, and 5, seen in **Figure 3c**) that belonged to fraction III were analyzed by HPLC, and the content of dimers B1, B2, and B3 in the five tubes was estimated from their HPLC peak areas, given that they have the same molar absorptivity. According to **Figure 3c**, the procyanidin B2 content was higher in eluent from 160 to 175 min (i.e., tubes 1−3) than in tubes 4 and 5. So, tubes 1, 2, and 3 were collected and freeze-dried, and a slightly yellow amorphous powder was obtained. HPLC and MS (**Figure 4a**, **b**) analyses showed that the powder contained 6.4% of B1 and 92.5% of B2. If the whole peak III

(i.e., tubes 1–5) were collected, it would be composed of 22.0% of B1 and 75.5% of B2.

Fraction IV contained mainly dimer X (m/z = 577.1), but the isomer type was unknown. Fraction V contained mainly trimer C1, which was identified by MS, MS^2, and coelution with standard. Fraction V (eluent from 225 min to 255 min) was collected, methanol was removed, and the sample was freeze-dried. A slightly brown amorphous powder was obtained. HPLC and MS analyses (**Figure 4c**, **d**) indicated that fraction V contained 90.3% of trimer C1 (epi-(4β−8)-epi-(4β−8)-epi).

Fraction VI contained five trimer isomers, in which trimer Y3 dominated. Fraction VII contained three tetramer isomers (m/z = 1153.5). Tetramer Z3 was the main compound in fraction VII. Fraction VIII contained four tetramer isomers, in which tetramers Z6 and Z7 were the main compounds. Fraction IX contained mainly pentamer (m/z = 1441.0).

Retention time (t_R), λ_{max}, MS and MS^2 data, and related analysis of each fraction are summarized in **Table 1**. A total of one monomer, three dimers, eight trimers, nine tetramers, and one pentamer were found among all the fractions via HPLC chromatography.

Procyanidin oligomers in GSE were eluted from a Toyopearl TSK HW-40s column according to their DP. Furthermore, they were well separated from each other (see **Figure 3a**) and could be collected easily.

Reproducibility of the Fractionation. In the current study, the yield of GSE from fresh apples (Granny Smith) was 0.135%, and the yields of procyanidin B2, trimer C1, and tetramers (i.e., peaks VII and VIII) from GSE were 16.7, 22.0, and 5.4%, respectively. GSE was fractionated by a Toyopearl TSK HW-40s column 12 times, and no obvious separation-efficiency decrease was observed (data not shown).

In an effort to decrease fractions I and II in GSE, the extraction was performed with methyl acetate, and the methyl acetate phase was fractionated by a Toyopearl TSK HW-40s column. Finally, the peak height of peaks I, VII, VIII, and IX decreased, but that of peak II did not (data not shown). So, the extraction of GSE was not favorable. In another experiment, adding 1% of acetic acid in methanol as mobile phase had no observed effect in the fractionation process (data not shown).

DISCUSSION

Both normal- and reverse-phase chromatographies have been used to separate and prepare procyanidins. Each chromatography has its advantages. Procyanidins were usually eluted according to their DP in normal-phase chromatography, and silica beads were often used as column material. However, in reverse-phase chromatography, procyanidins were eluted according to their polarity, and procyanidin isomers with the same DP may have different retention times.

In recent years, Toyopearl TSK HW-40s columns were widely used to fractionate polyphenols, such as proanthocyanidins from grape seeds (*31, 38*) and hawthorn barks (*42*). A toyopearl TSK HW-40s column is made from a vinyl polymer, and the surface of the beads is covered by hydroxyl groups. The fractionation depends on the interactions between the Toyopearl TSK HW-40s column's surface and the procyanidins, including hydrogen-bond formation between the hydroxyl groups and hydrophobic interactions between the vinyl groups of the resin beads and the aromatic rings of procyanidins. Recently, Yanagida et al. (*36*) developed a method to separate apple extract by size-exclusive chromatography without needing derivatization, in which an acetone−8 M aqueous urea (6:4; adjusted to pH 2 by HCl) mobile phase was employed.

In the current study, oligomeric procyanidins were prepared by the combination of ADS-17 column chromatography and Toyopearl TSK HW-40s column chromatography. Both chromatography systems operated at low pressure. This made it easy to handle the systems and made it possible to use a common apparatus. The preparation of procyanidins is rapid and simple and gives high yields.

After elution of the oligomeric procyanidin fraction on a Toyopearl TSK HW-40s column with methanol, no re-equilibration or regeneration of the column is needed, and the column is ready for the next separation. It is thus suitable for the preparation of procyanidin oligomers (from dimers through tetramers in one run) for laboratory research and potentially applicable in industry for large-scale production.

Furthermore, this method could be used to characterize oligomeric procyanidins in food and medicinal plants. Procyanidins in these materials could be extracted and purified by an ADS-17 resin column and then fractionated by a Toyopearl TSK HW-40s column to obtain their oligomeric features.

ABBREVIATIONS USED

GSE, Granny Smith apple procyanidin extract; DP, degree of polymerization; FC, Folin-Ciocalteu's; UV, ultraviolet; and LC-ESI-MS, liquid chromatography−electrospray ionization−mass spectrography.

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Supporting Information Available: HPLC spectra of nine fractions from GSE. This material is available free of charge via the Internet at http://pubs.acs.org.

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