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Research Article

Analysis of A-Type and B-Type Highly Polymeric Proanthocyanidins and Their Biological Activities as Nutraceuticals

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Proanthocyanidins have a series of heteroflavan-3-ols, (+)-catechin/(−)-epicatechin units, which are linked through a single B-type linkage and a doubly linked A-type linkage. Recently, we have performed the structural characterization of seed shells of the Japanese horse chestnut and fruits of blueberry and cranberry. The molecular sizes of them were higher in the order of blueberry > cranberry > seed shells of the Japanese horse chestnut between the respective fractions. For the analysis of terminal and extension units in those proanthocyanidins, the isolated fractions were subjected to the thiolytic cleavage of the B-type linkages using 1-dodecanethiol, and the resulting degradation products were identified by ultraperformance liquid chromatography coupled with electrospray-ionization mass spectrometry. These analyses provided fast and good resolution of the degradation products and revealed higher proportions of A-type linkages compared with B-type linkages in both isolated fractions in the order of the seed shells > cranberry > blueberry. Moreover, the isolated fractions with higher molecular sizes and those more abundant in the proportions of A-type linkages were found to be more effective in the inhibition of pancreatic lipase activity. The results suggest that A-type highly polymeric proanthocyanidins are promising for the attenuation of lipid digestion as dietary supplements.

1. Introduction

Proanthocyanidins are well known to be a subclass of polyphenolic compounds that can be detectable in a wide variety of food sources, such as fruits, nuts, beans, apples, and red wine [1]. These compounds are regarded as healthy food supplements due to their activities serving as potent antioxidants to suppress the generation of reactive oxygen species under the oxidative stress in the body [2]. As well, proanthocyanidins have been shown to have other biological activities including antimicrobial, antiallergy, anticancer, and antiobesity actions. To understand the mechanism of specific effects of those compounds on the biological effects, the structural elucidation of polyphenolic polymers is a prerequisite.

Proanthocyanidins comprise a series of heteropolyflavan-3-ols, (+)-catechin/(−)-epicathechin units, which are linked through a single B-type linkage of C4 → C6 or C4 → C8, and a doubly linked A-type linkage including a C4 → C8 bond and an additional ether bond between O7 → C2 (Figure 1). In general, proanthocyanidins are classified as B-type proanthocyanidins mainly consisting of B-type linkages or A-type proanthocyanidins having higher proportion of A-type linkages in addition to the presence of B-type bonds [1, 3, 4]. Recently, we found that seed shells of the Japanese horse chestnut contained the higher levels of A-type highly polymeric proanthocyanidins [2]. Furthermore, in vitro and in vivo studies have revealed that these highly polymeric proanthocyanidins have more healthy effects to

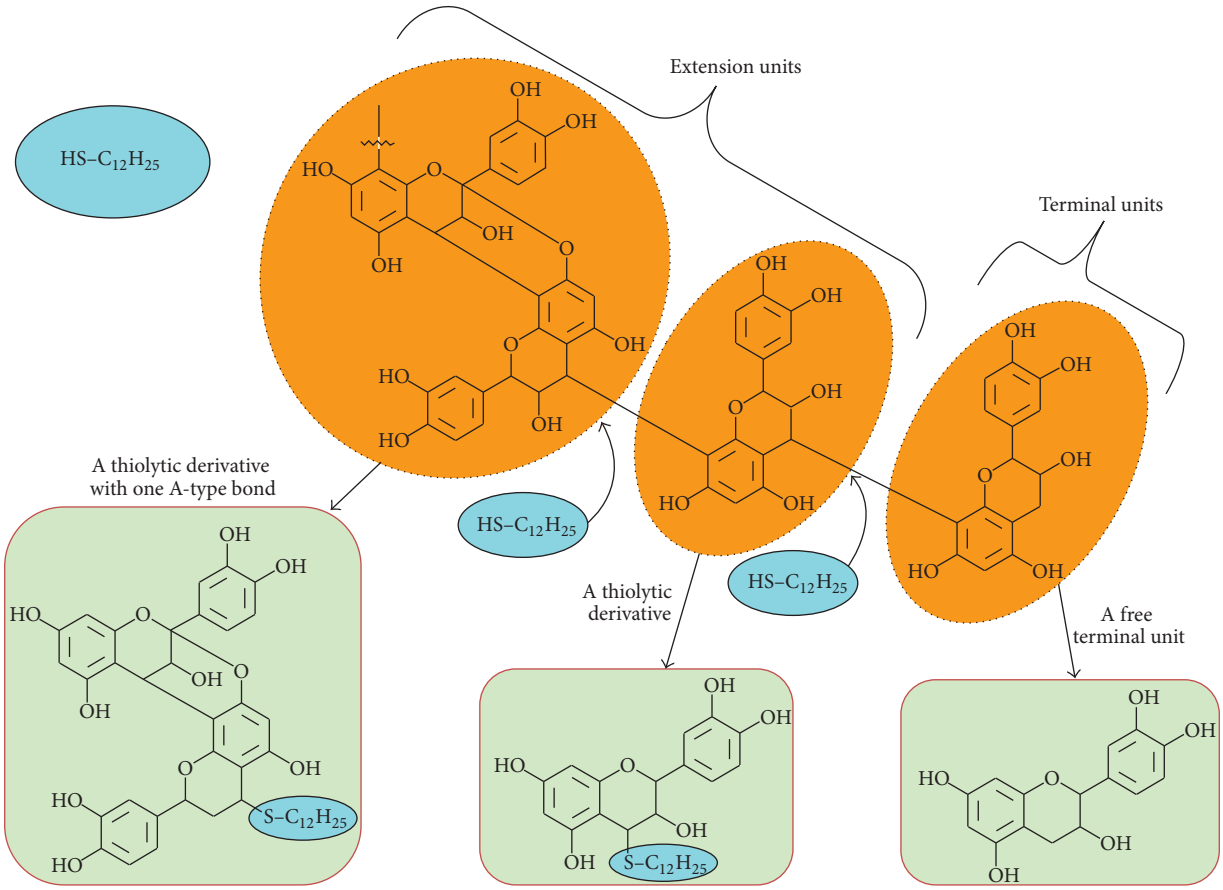

Figure 1: Thiolytic cleavage of highly polymeric proanthocyanidins having a series of heteropolyflavan-3-ols, (+)-catechin/(−)-epicatechins, with doubly linked A-type interflavan linkages and single B-type bonds.

exhibit antidiabetic [5] and antiobesity actions [6, 7] as well as their antioxidant activities [2]. However, the structural characterization of the above highly polymeric proanthocyanidins is limited because of difficulties associated with their highly polymerized forms. Hence, no detailed studies have been described to determine the proportions of terminal and extension units of A-type and B-type highly polymeric proanthocyanidins as well as their polymer sizes.

Recently, we attempted to perform structural characterization of highly polymeric proanthocyanidins from seed shells of the Japanese horse chestnut (*Aesculus turbinata* BLUME) and cranberry (*Vaccinium macrocarpon* Aiton) fruit as rich sources of A-type highly polymeric proanthocyanidins by thiolytic degradation with 1-dodecanethiol. These results were compared with those of the corresponding polymers from blueberry (*Vaccinium angustifolium* Aiton) fruit as a typical source of B-type proanthocyanidins. The thiolytic treatment is expected to cleave only single B-type bond of those proanthocyanidins without affecting the doubly linked A-type linkages, resulting in the formation of thioether derivatives of 1-dodecanethiol from extension units and free terminal units (Figure 1) [8–10]. Using chromatographic and instrumental analyses we have obtained structural information on the proportion of A-type linkages relative to B-type linkages and the molecular sizes of highly polymeric proanthocyanidins isolated differently from three types of food sources. Furthermore, we discuss the implication of different preparations of them as nutraceuticals on the basis of our recent data regarding the inhibitory effects on the digestion of carbohydrates and lipids [6, 7, 11].

2. Preparation and Isolation of Highly Polymeric Proanthocyanidins

Seeds of the Japanese horse chestnut were collected from the forest of northern Hyogo Prefecture in Japan, and frozen fruits of blueberry and cranberry were purchased from the commercial sources [2]. These food sources were used for crude extraction of total polyphenolic compounds with 70% acetone as described in our reports [2, 11]. These crude extracts from each food source were fractionated sequentially by column chromatography on Diaion HP-20 and Chromatorex ODS1024T to obtain total fraction of polyphenolic compounds. The resulting fractions were furthermore separated by column chromatography with Sephadex LH-20 into three fractions corresponding to F1, F2, and F3 by eluting with ethanol, methanol, and 70% acetone, respectively. These fractions can be separated on the basis of varied sizes of proanthocyanidins. After further analyses, we found

Table 1: Molecular sizes of two different fractions of highly polymeric proanthocyanidins isolated individually from food sources of seed shells of Japanese horse chestnut, blueberry, and cranberry as determined by gel permeation HPLC.

	Fraction	Retention time (min)	Mp
Seed shells of Japanese horse chestnut	AF2	3.14	14800
	AF3	2.90	30900
Blueberry fruit	BF2	2.96	25500
	BF3	2.81	40800
Cranberry fruit	CF2	3.07	20300
	CF3	2.87	33900

The fractions of F2 and F3 were individually obtained by column chromatography on Sephadex LH-20 from seed shells of Japanese horse chestnut (AF2 and AF3), and fruits of blueberry (BF2 and BF3) and cranberry (CF2 and CF3).

that the fraction of F2 and F3 contained highly polymeric proanthocyanidins from individual food sources while low-molecular-weight phenolic substances are only included in the F1 fraction [2]. The fractions of F2 and F3 from seed shells of the Japanese horse chestnut, blueberry, and cranberry are now referred to as AF2 and AF3, BF2 and BF3, and CF2 and CF3, respectively.

To assess the molecular sizes of highly polymeric proanthocyanidins, the fractions of F2 and F3 from individual food sources were applied to the gel permeation high-performance liquid chromatography (HPLC) on TSK-gel super AW3000 that had been calibrated with standard polystyrenes as described earlier by Bae et al. [12]. The molecular sizes of the samples were estimated by determining individual peak-top molecular weight (Mp) of standard polystyrenes (Table 1). As to the same food source, the molecular sizes of the F3 fractions were found to be always higher than those of the F2 fractions. Moreover, This analysis revealed that the molecular sizes of them were higher in the order of blueberry > cranberry > seed shells of the Japanese horse chestnut by the comparison of them between same fractions from different food sources. We utilized standard polystyrenes with different sizes for the calibration in place of standard polymeric proanthocyanidins that are not available at present. Therefore, the deviations from the estimated molecular sizes of samples could not be excluded. However, the estimation by the gel permeation chromatography is useful enough to compare the relative molecular sizes of highly polymeric proanthocyanidins from different food sources.

3. Thiolytic Cleavage of Highly Polymeric Proanthocyanidins and Their Chromatographic Analysis

Thiolytic cleavage of proanthocyanidins and the related compounds is considered to be a useful method for the analysis of components in the extension and termination units of a series of heteropolyflavan-3-ols and the estimation of the polymerization degree of them. The extension units through the single B-linkages can be cleaved by the nucleophilic attack of benzyl mercaptan [9] or 1-dodecanethiol [10] to produce the respective derivatives, whereas those with the doubly linked A-type bonds are resistant to the thiolysis. The terminal units are released as underivatized (+)-catechin or (−)-epicatechin, or oligomers having other extension units through the A-type bonds. Initially, we compared the thiolytic reactions of highly polymeric proanthocyanidins from seed shells of the Japanese horse chestnut using both benzyl mercaptan and 1-dodecanethiol. The total recovery of the initial compounds was found to be higher with 1-dodecanethiol than with benzyl mercaptan. In addition, we recognized poor resolution of the benzylthioether derivatives with the A-type linkages as the reaction products by reverse-phase HPLC. Considering these issues, we decided to employ 1-dodecanethiol for the thiolytic cleavage of highly polymeric proanthocyanidins. The resulting dodecylsulfide derivatives were separated more efficiently by our conditions of HPLC and ultra-performance liquid chromatography (UPLC).

Until now, the thiolytic products of proanthocyanidins have been analyzed by the injection to the HPLC column. Although this method can simultaneously detect both extension units as the dodecylsulfide derivatives and released terminal units, the accurate determination of the amount of the terminal units became difficult due to much higher levels of the extension units compared with the terminal units especially in highly polymerized forms. To overcome this issue, we introduced a revised method to determine the amounts of both units by chromatographic analysis after the separation of the derivatives of extension units from terminal units. For this, the phase separation with water and chloroform was effective because the dodecylsulfide derivatives of the extension units are soluble in organic phase while the terminal units are only soluble in aqueous phase [11].

4. Identification of Terminal and Extension Units in A-Type and B-Type Highly Polymeric Proanthocyanidins by Thiolytic Analysis

To obtain the structural information on the terminal and extension units in highly polymeric proanthocyanidins prepared from three types of food sources, the isolated individual fractions of F2 and F3 from different foods were used for the thiolytic cleavage. The resulting thiolytic products were subjected to the analysis by ultra-performance liquid chromatography coupled with electrospray-ionization mass spectrometry (UPLC-ESI/MS) using a Waters Synapt G2 High Definition Mass Spectrometry System (Nihon Waters,

Table 2: Proportions of terminal units in highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut, blueberry, and cranberry as determined by UPLC-ESI/MS of the thiolytic products.

	Cleaved terminal unit	Proportion (mol%)					
		AF2	AF3	BF2	BF3	CF2	CF3
1	(+)-Catechin	10.2	8.9	51.8	35.8	7.8	7.8
2	(−)-Epicatechin	70.7	74.6	48.2	64.2	56.7	52.2
3	Procyanidin A2	19.1	16.5	N.D.	N.D.	35.5	40.0

The terminal units were quantified by monitoring the absorbance at 280 nm. Data represent the mean of three independent experiments. N.D.: not detectable.

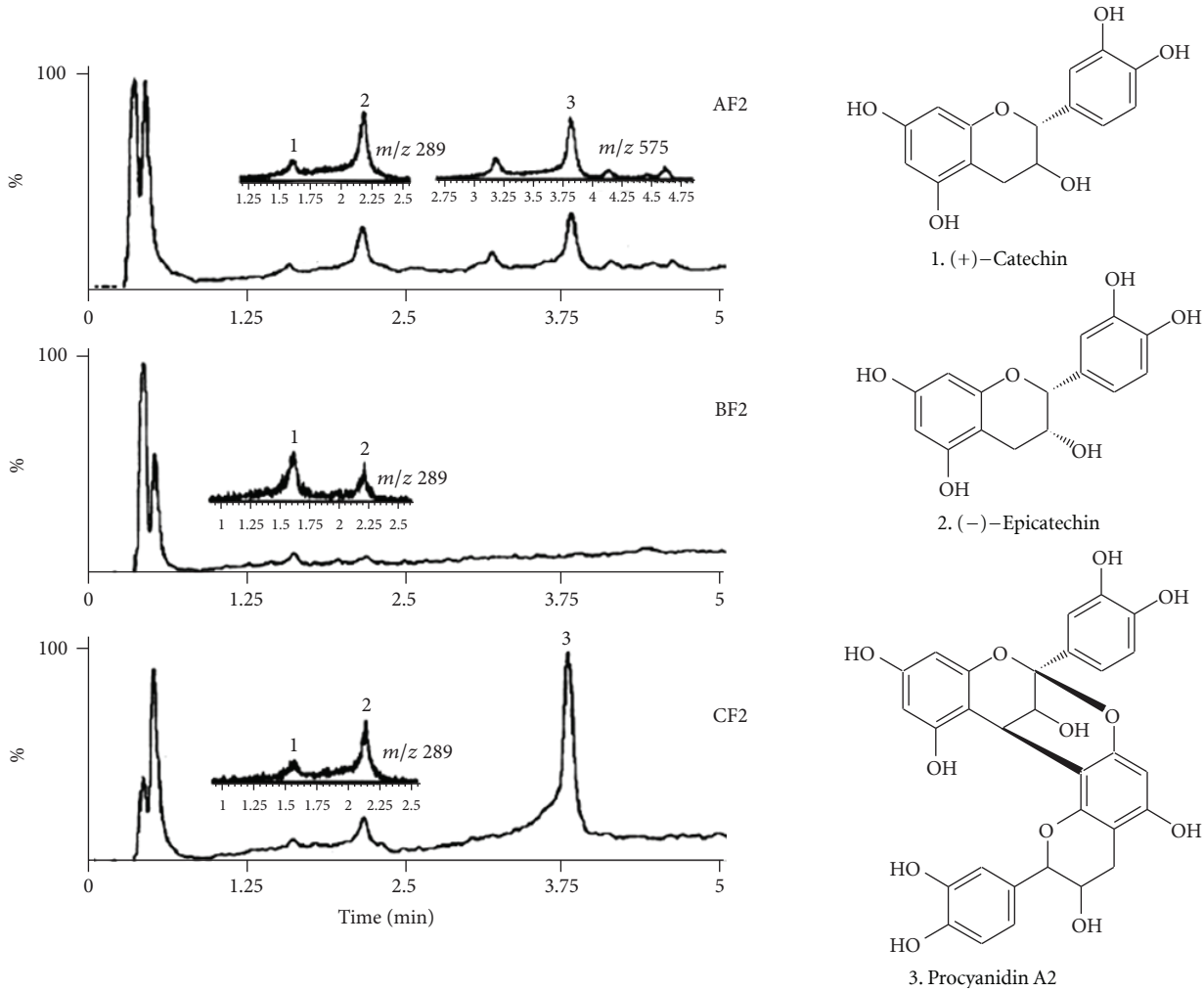

Figure 2: Analysis of terminal units of highly polymeric proanthocyanidins from three food sources using the individual fractions of F2 after thiolytic cleavage by UPLC-ESI/MS.

Tokyo, Japan) connected with an analytical UPLC column of Cosmosil 2.5C18-MS-II (75 mm × 2 mm i.d., 2.5 μm particle, Nacalai, Japan) as described [11]. Based on the mass spectral data of negative ions $[M–H]^-$ reflecting molecular ions and the product ions characteristic of individual compounds, we identified the thiolytic products of the terminal and extension units and quantified the proportion of the thiolytic products. The analysis of the terminal units revealed that all of the food sources contained the monomeric (+)-catechin (**1**) and (−)-epicatechin (**2**) (Figure 2, Table 2). In addition, procyanidin A2 (**3**) with one A-type bond was recognized in the fractions F2 and F3 from seed shells of the Japanese horse chestnut (AF2 and AF3) and cranberry fruit (CF2 and CF3), reflecting characteristic A-type proanthocyanidins. In sharp contrast, the peak of **3** was not detectable in the terminal units of both BF2 and BF3 fractions from blueberry fruit, demonstrating the typical source of B-type proanthocyanidins.

The extension units of highly polymeric proanthocyanidins can be analyzed as the dodecylsulfide derivatives after their thiolytic degradation with 1-dodecanethiol and the

Table 3: Proportions of extension units in highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut, blueberry, and cranberry as determined by UPLC-ESI/MS of the dodecylsulfide derivatives after thiolytic cleavage.

	Cleaved extension unit	Proportion (mol%)					
		AF2	AF3	BF2	BF3	CF2	CF3
4	(Epi)gallocatechin dodecylsulfide	1.9	1.7	1.5	3.1	7.6	6.3
5	(Epi)gallocatechin-(epi)catechin A-type dimer dodecylsulfide	3.6	2.8	N.D.	N.D.	N.D.	N.D.
6	(Epi)catechin A-type trimer dodecylsulfide	0.9	0.8	N.D.	N.D.	N.D.	N.D.
7	(Epi)catechin A-type dimer dodecylsulfide	32.2	25.3	6.8	6.4	25.4	20.3
8	(Epi)catechin dodecylsulfide	61.3	69.4	91.6	90.6	67.0	73.3

The dodecylsulfide derivatives of extension units were quantified by monitoring the absorbance at 280 nm. Data represent the mean of three independent experiments. N.D.: not detectable.

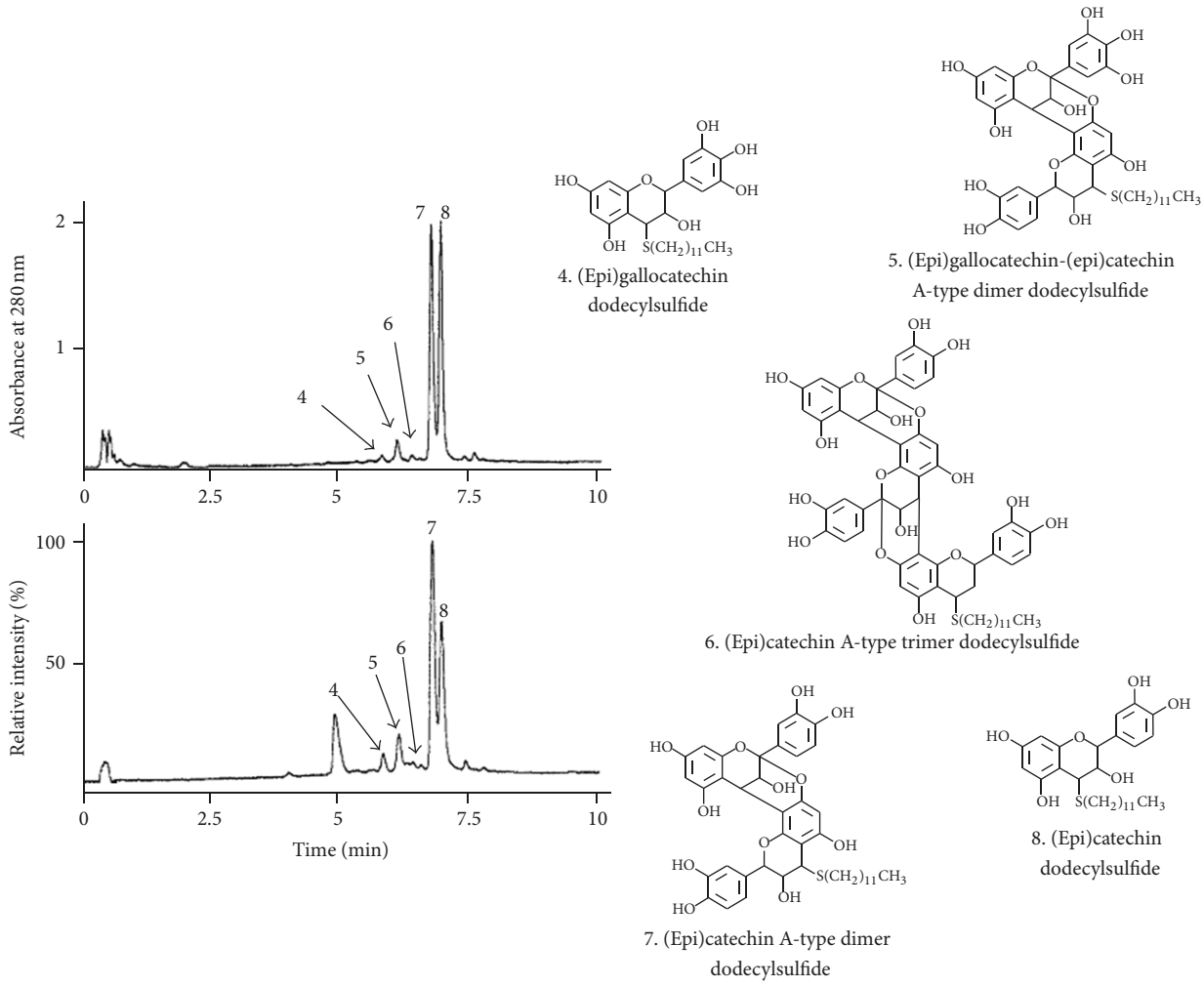

Figure 3: Analysis of extension units of the AF2 fraction of highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut as the dodecylsulfide derivatives after thiolytic cleavage by UPLC-ESI/MS.

identification by UPLC-ESI/MS (Figure 3, Table 3). The analysis of the fractions of F2 and F3 from all the food sources revealed that (epi)catechin dodecylsulfide (**8**) are present as the most abundant thiolytic product. Higher proportions of it were found in the fractions of blueberry fruit, reflecting typical B-type proanthocyanidins. In addition, (epi)catechin A-type dimer dodecylsulfide (**7**) were commonly detected as the secondly predominant cleaved products from all the fractions. The higher proportions of it was detectable in the order of the seed shells of the Japanese horse chestnut > cranberry fruit > blueberry fruit. These findings indicate much more abundant proportions of A-type linkages in the fractions from the seed shells of the Japanese horse chestnut (AF2 and AF3) and cranberry frit (CF2 and CF3)

than those from blueberry fruit (BF2 and BF3). As well, the fractions of AF2 and AF3 contained other thiolytic products of (epi)gallocatechin-(epi)catechin A-type dimer dodecylsulfide (**5**) and (epi)catechin A-type trimer dodecylsulfide (**6**) as typical A-type proanthocyanidins. Interestingly, (epi)gallocatechin dodecylsulfide (**4**) was detectable in the fractions of F2 and F3 from all the food sources and more abundant in the fractions from cranberry fruit (CF2 and CF3). With respect to the comparisons between the F2 and F3 fractions from individual food sources, we found no marked differences between them. Taken together, our analysis provided the evidence that seed shells of the Japanese horse chestnut contained apparently higher proportions of doubly liked A-type linkages with variations in the binding mode than fruits of blueberry and cranberry.

Earlier, the thiolytic cleavage of oligomeric procyanidins with only B-type linkages have been accomplished using benzylmercaptan to calculate the mean degree of polymerization after the quantification of those derivatives and monomeric flavan-3-ols [9]. On the other hand, highly polymeric proanthocyanidins with both A-type and B-type linkages are more complicated to analyze it due to the lack of the authentic compounds corresponding to thiolytic products and much higher proportion of the extension units compared with the terminal units. We tried to estimate the average degree of polymerization by the determination of the thiolysis of our highly polymeric proanthocyanidins. However, the calculated values did not match the molecular sizes estimated by the gel permeation chromatography. These erroneous results could be partly explained by the more preferential degradation of terminal units during the extended reaction of thiolytic cleavage. Hence, more extensive efforts to overcome these issues associated with the reaction conditions should be done for the accurate estimation of the polymerization degree of highly polymeric proanthocyanidins by thiolytic cleavages.

5. Biological Activities of A-Type and B-Type Highly Polymeric Proanthocyanidins as Neutraceuticals

Excess consumption of high-fat diets is well known to lead to the development of obesity, which is a major risk factor for the onset of insulin resistance and the increased morbidity of diabetes mellitus and cardiovascular disease. Therefore, much attention has been paid to the neutraceuticals that have antiobesity effects. Recently, we have described that highly polymeric pronanthocyanidins from seed shells of the Japanese horse chestnut can inhibit pancreatic lipase in a dose-dependent manner in vitro assay system [6]. The effectiveness of these isolated fractions in suppressing the digestion and absorption of lipids was furthermore confirmed by in vivo oral fat tolerance test in mice [6] and long-term antiobesity effects in obese mice fed a high-fat diet supplemented with the total fraction as a drink [7].

More recently, we have isolated the fractions of F2 and F3 from highly polymeric proanthocyanidins of three types of food sources and tested the individual fractions for their inhibitory effects on the enzymatic activity of pancreatic lipase [11]. By analyzing the dose-response curves of the inhibitory actions, w noticed that the F3 fractions with higher molecular sizes from all the sources had totally more potent effects to inhibit pancreatic lipase than the F2 fractions with smaller molecular ones from the same sources. This observation indicates that higher degree of polymerization is one of the important factors to show the inhibitory effect. Moreover, these results from different food sources were compared between the same fractions of F2 or F3 to obtain the structural information relevant to the difference in the biological activities of those fractions. The comparison between the F2 fractions from different sources revealed that the AF2 fraction with the highest proportions of doubly linked A-type linkages exhibited the most potent effects to inhibit pancreatic lipase although the molecular sizes are the smallest among the fractions of F2 from three sources [11]. However, no significant difference was observed between the F3 fractions from different food sources, presumably due to much higher molecular sizes and more inhibitory effects of the F3 fractions than those of the F2 fractions. Taking these findings into consideration, both the higher molecular sizes and more abundance of A-type linkages of polymeric proanthocyanidins should be crucial determinants that contribute to more effective inhibition of pancreatic lipase. Consistent with this indication, we demonstrated that procyanidin A2 with one A-type linkage had much more potent activity to suppress pancreatic lipase than procyanidin B2 with one B-type bond.

Highly polymeric proanthocyanidins are generally considered to bind to a number of proteins nonspecifically. However, their effects to inhibit pancreatic lipase are not explained simply by the absorption to the pancreatic lipase protein because the enzyme was more potently inhibited by AF2 with higher levels of doubly linked A-type linkages than other related BF2 and CF2 in spite of the lowest size of AF2 among the F2 fractions [11]. Moreover, we have recently examined the effects of highly polymeric proanthocyanidins on the digestive enzymes of carbohydrates, including α-amylase, maltase, and sucrase [5]. According to the results regarding their dose-dependence, the fractions of AF2 and AF3 from seed shells of the Japanese horse chestnut were found to be much more effective to suppress the enzyme activity of α-amylase compared with other α-glycosidases. Considering these findings, these highly polymeric polymers should have the specificity to inhibit digestive enzymes, depending on the proportions of A-type and B-type linkages as well as the degree of the polymerization.

Earlier, Nakai et al. has reported the importance of galloyl moieties in polymerized polylphenolic compounds from oolog tea in the inhibition of pancreatic lipase [13]. We also noticed that (−)-epigallocatechin 3-O-gallate had much potent effect to inhibit pancreatic lipase in our assay conditions [6]. Since galloyl moieties were detected by the thiolytic cleavage of our fractions from highly polymeric proanthocyanidins, this galloyl group serves as an inhibitor of pancreatic lipase. As separate biological activities, A-type proanthocyanidins have been described to exhibit bacterial antiadhesion activity [3] and anticancer effects [14]. We expect further studies to find the unique and promising

biological activity of doubly linked A-type bonds and other moieties in proanthocyanidins and the related natural products.

6. Conclusions

Recently, we have performed the structural characterization of highly polymeric proanthocyanidins from three food sources including seed shells of the Japanese horse chestnut and fruits of blueberry and cranberry. Thiolytic cleavage of the single B-type linkages with 1-dodecanethiol resulted in the better resolution of the cleaved products with the doubly linked A-type bonds or the dodecylsulfide derivatives by HPLC and UPLC-ESI/MS. Thus, we were able to determine the proportions of the terminal and extension units of the isolated fractions. The fractions with higher molecular sizes and higher proportions of A-type linkages over B-type bonds exerted more potent effects to inhibit pancreatic lipase.

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