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Fractionation and Structural Characterization of Polyphenolic Antioxidants from Seed Shells of Japanese Horse Chestnut (*Aesculus turbinata* BLUME)

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Seed shells of the Japanese horse chestnut (*Aesculus turbinata* BLUME) contain high levels of polyphenolic antioxidants. These compounds were extracted, fractionated, and finally separated into three fractions, F1, F2, and F3, according to their degrees of polymerization. The structures of the isolated fractions were characterized by a combination of mass spectrometric analyses. F1 contained mainly low molecular weight phenolic substances, including procyanidin trimers. The predominant fractions F2 and F3 consisted of polymeric proanthocyanidins having a series of heteropolyflavan-3-ols, (+)-catechin/(−)-epicatechin units, and polymerization degrees of 19 and 23, respectively. The polyphenol polymers had doubly linked A-type interflavan linkages in addition to single B-type bonds without gallic acid esterified to them. The isolated polyphenolic compounds exhibited potent antioxidative activities comparable to monomeric (+)-catechin and (−)-epicatechin, or more efficacious than those monomers. The results suggest the potential usefulness of polyphenol polymers from seed shells as a source for nutraceutical factors.

KEYWORDS: Polyphenolic compounds; antioxidants; polymeric proanthocyanidins; polyphenol polymers; flavan-3-ols; catechin; epicatechin; Japanese horse chestnut; *Aesculus turbinata* BLUME

INTRODUCTION

Free radicals and reactive oxygen species generated in the body are well-known to be critical risk factors for various diseases, such as cardiovascular diseases, cancers, aging, and inflammation, among others. Recently, for the purpose of preventing these disorders through the consumption of foods, a great amount of attention has been paid to phenolic antioxidants. Of these, polyphenolic compounds called proanthocyanidins are found in abundance in a wide variety of foods such as grapes (*1*), apples (*2*), cranberries (*3*), brown soybeans (*4*), and red wine (*5*); they exhibit antioxidative activities and other biological actions.

The seeds of the Japanese horse chestnut (*Aesculus turbinata* BLUME) have been used as an emergency provision since ancient times. Recently, the seeds have been utilized traditionally in Japan as a confectionery ingredient in rice cakes and rice balls. More recently, our group has identified different types of saponins from edible seeds, which were prepared by removing the seed shells and then treating the natural seeds with wood ashes, to remove bitter materials (*6*). Using the oral glucose tolerance test in mice, the resulting saponin components were found to attenuate significantly the elevation of blood-glucose levels (*7*). Moreover, we found that saponins from edible seeds were appreciably effective in inhibiting pancreatic lipase in vitro (*7*) and exhibiting antiobesity effects in mice that had been fed high-fat diets (*8*). The resulting observations led us to explore additional nutraceutical food factors from the seeds of the Japanese horse chestnut.

The present study focused on the separation and characterization of polyphenolic compounds isolated from seed shells of the Japanese horse chestnut. Earlier, Sato et al. described the presence of phenolic antioxidants in whole seeds of the Japanese horse chestnut, but they did not elucidate the chemical structures of the active components (*9*). Alternatively, another group isolated and elucidated four types of procyanidin trimers from the seeds of European horse chestnut (*Aesculus hippocastanum* L.) (*10*). However, the polyphenol polymers have not been identified until now. Thus, in this study, we sought to fractionate and isolate polyphenolic compounds from the seed shells of

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the Japanese horse chestnut, which were generated as wastes of food processing. The structures of the polyphenolic antioxidants were characterized through analyses combining high-performance liquid chromatography (HPLC), matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and liquid chromatography electrospray−ionization mass spectrometry (LC-ESI/MS). In addition, we evaluated the antioxidative activity of the isolated polyphenol polymers.

MATERIALS AND METHODS

Materials. The seeds of the Japanese horse chestnut (*A. turbinata* BLUME) were collected from the forests of northern Hyogo Prefecture in Japan and identified as described earlier (*7*). Folin−Ciocalteu reagent and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were supplied by Sigma (St. Louis, MO). Tannase was provided by Sankyo (Tokyo, Japan). Diaion HP-20 and Chromatorex ODS 1024T for column chromatography were obtained from Nippon Rensui (Tokyo, Japan) and Fuji Silysia (Kasugai, Japan), respectively. Sephadex LH-20 for column chromatography was purchased from GE Healthcare (Buckinghamshire, U.K.). Analytical columns of YMC-Pack SIL-06 for normal-phase HPLC and YMC-ODS Pro C18 RS for reverse-phase HPLC were the products of YMC (Kyoto, Japan). The LUNA C18 (2) column for reverse-phase HPLC was obtained from Phenomenex (Torrance, CA). All other reagents and chemicals were of analytical grade and purchased from Wako (Osaka, Japan), unless otherwise stated.

Extraction, Fractionation, and Isolation of Polyphenolic Compounds. Seed shells of the Japanese horse chestnut (10 g as fresh weight with 11.5% water) were ground into powder and refluxed by boiling for 2 h in 200 mL of water. The mixture was filtered through Advantec no. 5 filters, to obtain the extracts of the polyphenolic compounds. The residues were subjected to repeated extraction by dissolving in 200 mL of water, as above. The solvent was removed by rotary evaporation in vacuo, resulting in a 1.56 g yield of dried material. To remove sugars, the extracted material was applied to column chromatography on the Diaion HP-20 (300 mm × 30 mm i.d.) and eluted with 500 mL of methanol after washing with 500 mL of distilled water. The resulting methanol extracts were evaporated to dryness and dissolved in 5% methanol. The aliquots were then applied to column chromatography on Chromatorex ODS 1024T (300 mm × 30 mm i.d.) for further purification. After the column had been washed with 500 mL of 5% methanol, polyphenolic antioxidants were eluted with 500 mL of 50% methanol. The dry materials were dissolved in ethanol and subjected to separation by column chromatography with the Sephadex LH-20 (100 mm × 10 mm i.d.), as described previously (*4*). The column was serially eluted with 40 mL of ethanol, 40 mL of methanol, and 40 mL of 70% acetone, to derive fractions F1, F2, and F3, respectively. During the chromatographic separation of polyphenolic compounds, the elution was monitored by determining the amount of total polyphenols using (−)-epicatechin as a standard according to the Folin−Ciocalteu method, as previously described (*11*). In addition, other colorimetric procedures of the vanillin−hydrochloric acid assay for flavan-3-ol (*12*) and the butanolic−HCl assay for procyanidins (*13*) were employed for the determination of polyphenolic substances, using (−)-epicatechin and cyanidin chloride as standard equivalents, respectively.

Analytical HPLC. To obtain information regarding the components of polyphenolic compounds, normal-phase and reverse-phase HPLC analyses were conducted on a Shimadzu LC-2010A system equipped with Chromatopac C-R8A as a recorder. Normal-phase HPLC was operated with an analytical column of YMC-Pack SIL-06 (250 mm × 4.6 mm i.d.) at 25 °C, essentially according to the method of Sudjaroen et al. (*14*). The column was eluted at a flow rate of 1 mL/min with a mixture of dichloromethane/methanol/50% acetic acid, using a linear gradient from 82:14:4 (v/v) at 0 min to 67.6:28.4:4 (v/v) at 30 min, followed by additional linear gradients reaching 56.8:39.2:4 (v/v) at 45 min and 10:86:4 (v/v) at 50 min, after which the column was eluted isocratically with the same mobile phase until 60 min. The elution of the polyphenolic compounds was detected by monitoring the absorbance at 280 nm. To analyze with reverse-phase HPLC, a LUNA C18 (2) (250 mm × 4.6 mm i.d.) column was used at 35 °C and eluted at a flow rate of 1.3 mL/min, with a mobile phase consisting of a mixture of 0.1% formic acid in water/0.1% formic acid in acetonitrile (90:10, v/v) for the initial 5 min. Then, the elution was continued in a linear gradient mode from a volume ratio of 90:10 (v/v) at 5 min to 84:16 (v/v) at 11 min, 83:17 (v/v) at 21 min, and 20:80 (v/v) at 22 min. The polyphenolic compounds in the eluates were detected by monitoring the absorbance at 280 nm.

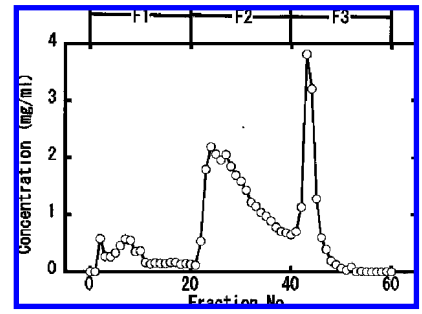

Figure 1. Sephadex LH-20 column chromatogram of polyphenolic compounds prepared from seed shells of the Japanese horse chestnut. The polyphenolic fraction obtained by column chromatography on Diaion HP-20 and Chromatorex ODS 1024T was applied to column chromatography on Sephadex LH-20 and eluted with ethanol (F1), methanol (F2), and 70% acetone (F3). The eluate was collected every 2 mL as one fraction and subjected to the determination of the amount of total polyphenols as described under Materials and Methods.

To determine whether the galloyl moiety was esterified in polyphenolic compounds, the compounds were prepared from the extracts by open column chromatography using the Diaion HP-20 and Chromatorex ODS 1024T. The resulting materials (10 mg) were allowed to react with 0.5 unit of tannase for 30 min at 25 °C. The enzyme reaction was stopped by boiling for 5 min. For the detection of gallic acid, the reaction products were subjected to analytical reverse-phase HPLC, as described as above. (−)-Epigallocatechin was also treated with tannase under the same conditions, for use as a positive control.

Analysis by MALDI-TOF/MS. To obtain structural information regarding polyphenolic compounds, a MALDI-TOF/MS analysis was performed on an Applied Biosystems Voyager-DE RP TOF mass spectrometer (Foster City, CA) equipped with a delayed extraction system and an N_2 laser at 337 nm, the latter of which had a pulse width of 2 ns in linear mode. An accelerating voltage of 20 kV was used for the detection of positive or negative ions of mass spectra, which were the sum of 200 shots and calibrated with substance P having an average molecular weight of 1347.73. Each of the samples (each 1 mg of dry weight) was dissolved in 1 mL of a solution consisting of aqueous 0.1% trifluoroacetic acid/acetonitrile (2:1, v/v). The sample solution was mixed with the matrix solution containing 10 mg/mL 2,5-dihydroxybenzoic acid in 20% ethanol, at a ratio of 3:1 (v/v). The resulting 1 μL was placed on an analytical plate and dried, for measuring mass spectra.

Analysis by LC-ESI/MS. A structural analysis of the components of polyphenolic compounds was conducted by LC-ESI/MS, after polyphenolic compounds had been subjected to the acid hydrolysis of the linkages and the subsequent preparation of dodecyl sulfide derivatives, as described earlier (*15, 16*). Briefly, to prepare the derivatives, fraction F2 or F3 (2 mg), isolated by Sephadex LH-20, was allowed to react at 40 °C for 30 min with 4 mL of methanol containing 0.83% HCl and 0.13% 1-dodecanethiol. The analysis using LC-ESI/MS was performed on a ThermoQuest ion trap-type LC-ESI/MS spectrometer, model LCQ Deca XP (Waltham, MA), to which an analytical column of YMC-ODS Pro C18 RS (150 mm × 4.6 i.d.) had been connected. The column was eluted at a flow rate of 0.2 mL/min, with a linear gradient system using a mobile phase of 0.1% formic acid/acetonitrile from 35:65 (v/v) to 15:85 (v/v). The elution of polyphenolic compounds was detected by monitoring both the absorbance at 280 nm and the total ions of the mass spectra as analyzed by ESI/MS in positive-ion

Table 1. Purification Step and Polyphenolic Contents of Seed Shells of *Aesculus turbinata* BLUME

purification step	dry wt[a] (mg)	polyphenolic content[b] (mg)
hot water extract	1560	580
Diaion HP-20	670	480
Chromatorex ODS1024T	540	440
Sephadex LH-20		
ethanol eluate (F1)	20	10
methanol eluate (F2)	270	290
70% acetone eluate (F3)	100	90

[a] For fractionating polyphenolic compounds, 10 g of seed shells was used initially. [b] Polyphenolic contents were determined as (−)-epicatechin equivalents.

mode. Moreover, the terminal residues without the dodecyl sulfide derivatives were separated and characterized under the analytical HPLC condition, as described above.

Antioxidative Activity of Polyphenolic Compounds. Isolated polyphenolic compounds were tested for their in vitro radical-scavenging activity and their protective effect on photobleaching of β-carotene, on the basis of total amounts of polyphenols, which were quantified as those being equivalent to (−)-epicatechin. The radical-scavenging activity was assayed using DPPH, essentially according to the methods described previously (*4, 17*). Briefly, the sample to be tested was incubated for 20 min at 25 °C in 1.2 mL of 50 mM MES buffer (pH 6) containing 100 μM DPPH and 50% ethanol. After the reaction, the absorbance at 520 nm was determined. As an alternative assay of antioxidative activity, the photobleaching of β-carotene was monitored as described by Tsushida et al. (*18*). A 980 μL suspension of 20 mM sodium phosphate buffer (pH 6.8) with 0.02% linoleic acid, 0.5×10^{-3}% β-carotene, and 0.2% Tween 20 was mixed with 20 μL of a sample solution. The resulting mixture was incubated for the indicated time at 50 °C while being shaken. The photobleaching of β-carotene was assayed by monitoring the decrease in absorbance at 470 nm.

RESULTS AND DISCUSSION

Fractionation and Preparation of Polyphenolic Compounds from Seed Shells of the Japanese Horse Chestnut. The extracts of polyphenolic compounds were fractionated by column chromatography using the Diaion HP-20 and Chromatorex ODS 1024T. They were further separated by column chromatography with Sephadex LH-20 into three fractions, including F1 with ethanol, F2 with methanol, and F3 with 70% acetone (**Figure 1**). Using 1.56 g of the crude extracts with boiling water, the aforementioned chromatographic procedures derived 20, 270, and 100 mg yields of fractions F1, F2, and F3, respectively (**Table 1**). The chromatographic profile of Sephadex LH-20, as determined for total phenolic content, was also similar to those monitored by detecting flavanol-type tannins and procyanidins (data not shown). Earlier, Takahata et al. reported the separation of extracts from brown soybean seed coats with 70% acetone and elution patterns of polyphenolic compounds that were similar to ours (*4*). The observation led us to consider that the main components of extracts from the seed shells of the Japanese horse chestnut would be polyphenolic substances that consist of higher polymers.

Commonly consumed beverages and foodstuffs such as red wine, black tea, coffee, and apples have been shown to contain typically 1.8 mg/mL, 1.0 mg/mL, 0.9 mg/mL, and 2.2 mg/g of polyphenolic substances, respectively, as estimated by the Folin−Ciocalteu colorimetric assay (*19*). Our studies showed that fresh seed shells included total polyphenolic substances at the levels of 58 mg/g. The observation suggests that the seed shells of the Japanese horse chestnut are a considerably useful source of polyphenolic compounds. Generally, the assay of total polyphenolic content by the Folin−Ciocalteu method is known

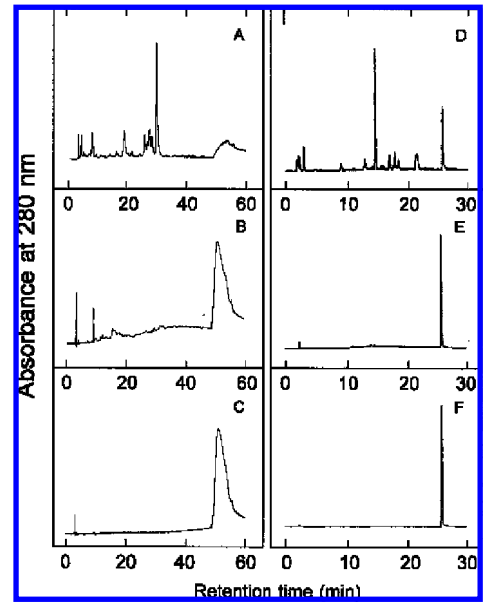

Figure 2. HPLC profiles of polyphenolic compounds. Fractions F1, F2, and F3, prepared by column chromatography on Sephadex LH-20, were separated by normal-phase and reverse-phase HPLC. The left panels show the normal-phase HPLC profiles of F1 (**A**), F2 (**B**), and F3 (**C**). The right panels represent the reverse-phase HPLC profiles of F1 (**D**), F2 (**E**), and F3 (**F**).

to be affected by the presence of ascorbic acid in the samples. However, ascorbic acid was not detected by reverse-phase HPLC analysis in our extracts (data not shown).

Fractions F1, F2, and F3 separated by the Sephadex LH-20 column were each further analyzed by normal- and reverse-phase HPLC (**Figure 2**). Fractions F2 and F3, as major constituents, were eluted as single peaks at the same position and detectable at later retention times in comparison to F1, as minor components. Previous studies have reported that normal-phase column chromatography can separate polyphenolic compounds on the basis of the size of polymers (*2, 14*). Compounds with lower molecular weights are eluted earlier, depending on the degree of polymerization. Hence, F1 in our sample is regarded as a polyphenolic substance with a lower degree of polymerization than F2 or F3. According to previous papers, normal-phase chromatography is limited in its ability to separate compounds with molecular weights higher than those of procyanidin decamers (*14*); therefore, F2 and F3 are likely to correspond to the polyphenolic compounds having molecular weights higher than those of procyanidin decamers and the related substances. Although the retention times of F2 and F3 were almost similar in the analyses by normal- and reverse-phase HPLC, these compounds are considered to have different degrees of polymerization, because the separation of these compounds is clearly evident by column chromatography using Sephadex LH-20. Taken together, our results suggest the presence of a mixture of polymers with flavan-3-ols such as (+)-catechin and (−)-epicatechin as polyphenolic compounds in our isolated samples. In an effort to determine whether gallic acid might be esterified to the flavan-3-ol moieties, the isolated fractions F1, F2, and F3 were treated with tannase. The released free gallic acid was not detected in the reaction mixtures when analyzed by reverse-phase HPLC.

Structural Elucidation of Isolated Polyphenolic Compounds by MALDI-TOF/MS and LC-ESI/MS. To elucidate the structure of polyphenolic compounds and the degree of polymerization, a MALDI-TOF/MS analysis was performed in

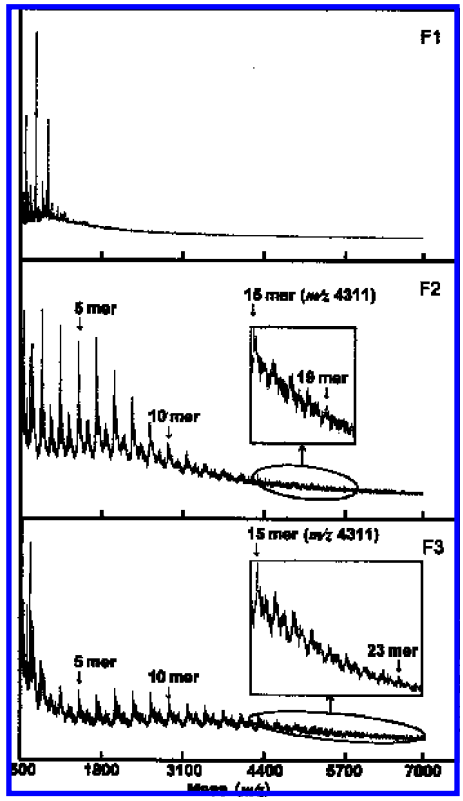

Figure 3. Negative-ion mode MALDI-TOF/MS spectra of each fraction F1, F2, and F3. Each fraction of polyphenolic compounds was isolated by column chromatography on Sephadex LH-20 and analyzed by MALDI-TOF/MS in the negative-ion mode.

both the positive- and negative-ion modes. This analysis provided evidence for polymeric proanthocyanidins, as reflected in the variation in the number of (+)-catechin/(−)-epicatechin (C/EC) units and hydroxyl substitutions. The mass spectral data of F1, F2, and F3 isolated from the extracts are represented in the negative-ion mode (**Figure 3**). F1 was composed of oligomers with molecular weights of less than 1000, as characterized by a prominent ion at *m/z* 865 of proanthocyanidin trimers consisting of C/EC units only. We also detected monomeric forms of (+)-catechin and (−)-epicatechin in the preparation of F1. In contrast, F2 and F3 were found to be polymeric proanthocyanidins having at least nonadecamers and tricosamers, respectively. To reveal the structural features of F2 more precisely, we determined the mass spectra in the negative-ion and positive-ion modes corresponding to $[M - H]^-$ and $[M + Na]^+$ (**Figure 4**; **Table 2**). Mass spectra of F2 gave characteristic ions with a mass difference of 288 unified atomic mass units (u), reflecting the variation in the number of the C/EC unit, which in turn indicates a series of polyflavan-3-ol polymers termed the B-type linkage (*1, 2*). In addition, the mass spectra were detectable at ions with a mass difference of 286 u, due to the presence of the doubly linked A-type interflavan bonds, which were earlier described in cranberry proanthocyanidins by Howell et al. (*3*). Moreover, the mass spectra in both the positive- and negative-ion modes exhibited the variation in the degree of hydroxylation, as characterized by the mass difference of 16 u. This supports the assertion that F2 and F3 are a class of proanthocyanidins. The observed masses in the positive- and negative-ion modes are consistent with the calculated masses, when one considers the numbers of C/EC units and A-type linkages, as well as the degree of polymerization from 4 to 9, in which one A-type linkage was detectable in the oligomer every 3.5−6 C/EC units, depending on the polymerization

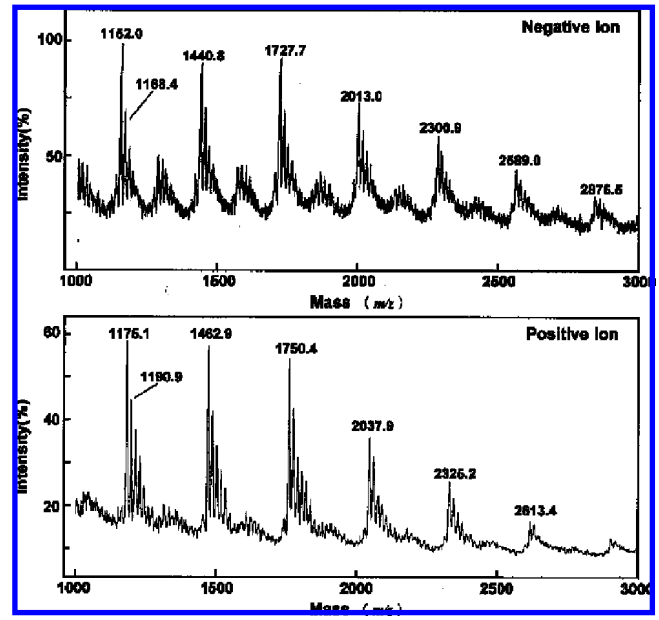

Figure 4. MALDI-TOF/MS spectra of the isolated F2 fraction in the negative-ion and positive-ion modes. The mass spectra represent the range of polyphenol polymers in the F2 fraction with polymerization degrees of 4−9.

Table 2. Observed and Calculated Masses Detemined by MALDI-TOF/MS Spectra of the Isolated F2 Fraction in Negative-Ion and Positive-Ion Modes

DP[a]	no. of C/EC	no. of A-type	obsd mass[b] ($[M - H]^-$)	calcd mass ($[M - H]^-$)	obsd mass ($[M + Na]^+$)	calcd mass ($[M + Na]^+$)
4	4	1	1152.0	1151.3	1175.2	1175.3
5	5	1	1440.9	1439.3	1462.9	1463.3
6	6	1	1727.7	1727.4	1750.4	1751.4
7	7	2	2013.0	2013.4	2037.0	2037.4
6	8	2	2300.9	2301.5	2325.2	2325.5
9	9	2	2589.0	2589.6	2613.4	2613.6

[a] Abbreviations: DP, degree of polymerization; C/EC, (+)-catechin/(−)-epicatechin equivalent unit; A-type, A-type interflavan bond. [b] The mass spectra represent the range of polyphenol polymers in the F2 fraction with polymerization degrees of 4−9.

(**Table 2**). On the other hand, the polymer with 15 C/EC units—which was found in F2 and F3—gave a negative ion of *m/z* 4311; it was calculated to contain 10 C/EC units and 5 A-type linkages (**Figure 3**). The increase in the number of A-type linkages should result in an attenuation of antioxidative potency, because the number of free hydroxyl groups in polyphenols is lowered, due to the double linkage of the A-type bond. A previous study identified procyanidin trimers possessing the A-type interflavan bond, a doubly linked structure, from fruit shells of European horse chestnut, *A. hippocastanum* L. (*10*). However, polymeric proanthocyanidins have not been investigated. In contrast, our studies now provide novel findings on polyphenolic compounds, with special reference to the higher polymers found in the seed shells of the Japanese horse chestnut.

The isolated fractions F2 and F3, including polymeric proanthocyanidins, were subjected to acid hydrolysis with methanolic HCl; this was followed by the preparation of dodecyl sulfide derivatives. The resulting derivatives were analyzed by LC-ESI/MS on a reverse-phase column. Each preparation derived from F2 or F3 gave similar elution patterns, through monitoring both the absorption at 280 nm and the total number of ions in the mass spectrum. **Figure 5** shows the chromatogram of F3, in which three peaks were detectable at 29 min (**I**), 34 min (**II**), and 39 min (**III**). The mass spectra of derivatives **I**,

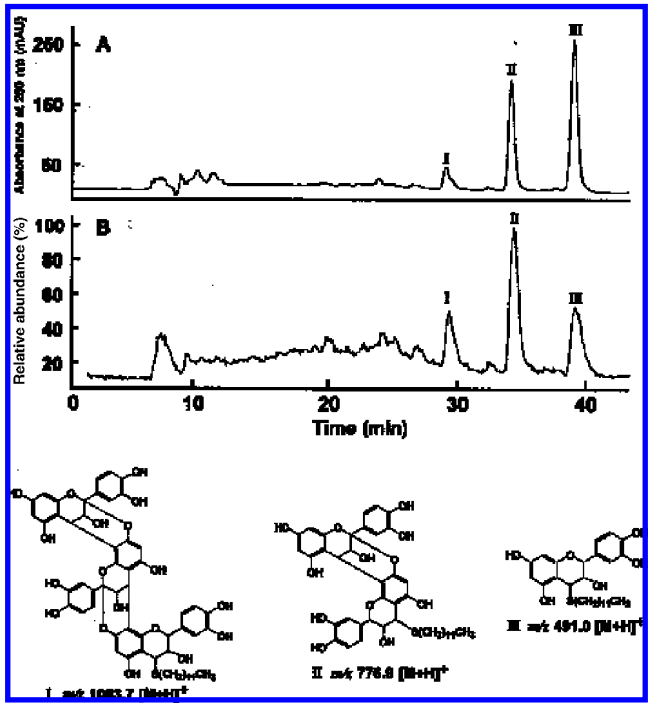

Figure 5. LC-ESI/MS spectra of dodecyl sulfide derivatives following the hydrolysis of polyphenol polymers in fraction F3. The chromatograms were recorded by monitoring the absorbance at 280 nm (**A**) and total ions of mass spectra in the positive-ion mode (**B**).

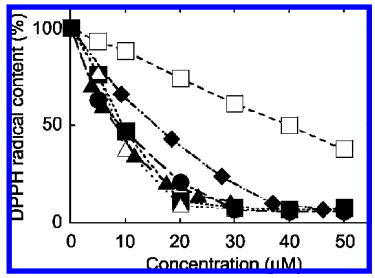

Figure 6. DPPH radical scavenging activity of isolated fractions F1, F2, and F3. The DPPH radical was incubated with increasing concentrations of standard BHA (▲), monomeric (−)-epicatechin (●), monomeric (+)-catechin (◆), or each fraction of F1 (□), F2 (■), and F3 (△) determined as an equivalent of (−)-epicatechin.

II, and **III** were characterized by molecular ions of $[M + H]^+$ at *m/z* 1063.7, 776.9, and 491.0, respectively. According to the data, compounds **I**, **II**, and **III** were identified as trimeric dodecyl sulfide derivatives with two A-type interflavan bonds, dimeric dodecyl sulfide derivatives with one A-type linkage, and the dodecyl sulfide derivative of (+)-catechin or (−)-epicatechin, respectively. An analysis of the terminal moiety of F2 and F3 by reverse-phase HPLC revealed that the proportion of (+)-catechin to (−)-epicatechin was 1:1.1 for F2 and 1:0.6 for F3.

Antioxidative Activity of Isolated Polyphenolic Compounds. The isolated polyphenolic compounds were tested for their antioxidative activity by assaying with DPPH their in vitro radical-scavenging activity. This was done to evaluate the capacity of antioxidants to stop the radical propagation reaction (**Figure 6**), and the photobleaching activity of β-carotene in the presence of linoleic acid was used as an index of a series of the initiation and propagation of radical reaction (**Figure 7**). The antioxidative activity of F1, which had low molecular weight compounds, was much lower than those of F2 or F3, when assayed with DPPH. On the other hand, no marked

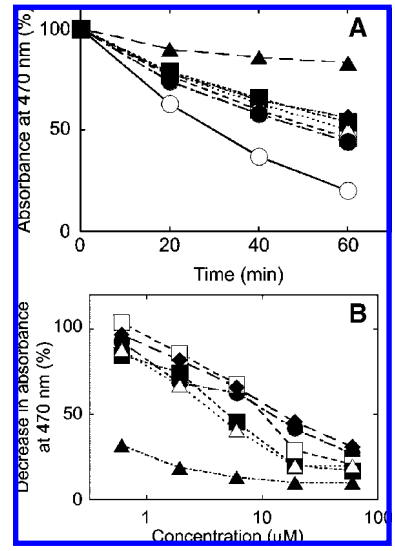

Figure 7. Antioxidative activity of isolated fractions F1, F2, and F3 as evaluated by their protecting activity on photobleaching of β-carotene together with linoleic acid. (**A**, top) Time course for photobleaching of β-carotene in the absence (○) or presence of either (−)-epicatechin (●), (+)-catechin (◆), F1 (□), F2 (■), F3 (△), or BHA (▲) at a concentration of 6 μM equivalent to (−)-epicatechin. (**B**, bottom) Dose dependence of BHA, (−)-epicatechin, (+)-catechin, F1, F2, and F3 on photobleaching of β-carotene. Symbols used are the same as (**A**).

differences were found between F2 and F3, showing a potency comparable to that of butylated hydroxyanisole (BHA) and (−)-epicatechin or slightly greater than that of (+)-catechin. It is not known why F1 showed a lower antioxidative activity via DPPH, but one plausible explanation might be the presence of compounds that interfere with radical scavenging; these compounds may have been present due to the inclusion of some impurities. Alternatively, F2 and F3, including polymeric proanthocyanidins, exhibited almost similar antioxidative activities when monitored by photobleaching activity of β-carotene. Their inhibitory actions were slightly more potent than that of the monomer of (−)-epicatechin or (+)-catechin, although they exhibited much less potent activity in comparison to BHA, as a positive control. Thus, the antioxidative potency of the polymerized proanthocyanidins of F2 and F3 were not significantly different from monomeric (−)-epicatechin or (+)-catechin. This observation can be explained by the absence of gallic acid esterified to a series of polyflavan-3-ol polymers, termed the "B-type linkage." Earlier, Sato et al. observed the radical-scavenging activity of the crude extracts of whole seeds of the Japanese horse chestnut (*9*), reporting the concentration of 0.65 mg/mL required to scavenge the DPPH radical by 50%. The IC_{50} value was much higher than those of our isolated preparations of F2 and F3, which can reduce 50% of the DPPH radicals at concentrations of 2.4−2.6 μg/mL. The present study provides evidence that the main active components of antioxidants from the seed shells of the Japanese horse chestnut are polymeric proanthocyanidins of F2 and F3. Considering their use in foodstuffs, we first extracted polyphenolic antioxidants from seed shells using hot water, so that some autoxidation of those compounds might not be excluded during the process. However, the above data indicated that our preparations were potent enough to show antioxidative activities.

We isolated polyphenol polymers consisting of proanthocyanidins from the seed shells of the Japanese horse chestnut, and those shells appear to be useful as antioxidants that, when used as a food additive, protect against the oxidation of unstable

polyunsaturated fatty acids in some processed foods. Alternatively, the bioavailability of these polymers, including nonadecamers and tricosamers, is currently not known in humans or animals, so it remains unclear whether they can increase antioxidative capacity in vivo in the plasma after the consumption of foods containing polyphenol polymers. Recently, a great amount of attention has been paid to the other biological activities of polyphenolic compounds, activities that could affect the function of digestive tracts such as the stomach or the small intestine when the polyphenol-containing food is consumed. Indeed, proanthocyanidins from grape seed extracts have been reported to suppress the onset of a gastric ulcer (*20*) and colon cancer (*21*) in animal experimental models. Additionally, the fraction of polyphenol polymers from oolong tea has been described to inhibit pancreatic lipase in vitro (*22*) and attenuate the elevation of plasma neutral fats in vivo (*23*). Furthermore, more recent studies have reported the antibacterial effects of polyphenol polymers. These include, for example, the uropathogenic bacterial antiadhesion activity of A-type cranberry proanthocyanidins with the degree of polymerization from 4 to 10 (*3*) and the potent suppressive effects of a polymeric procyanidin with the mean polymerization degree of 17 from *Zanthoxylum piperitum* fruit on methicillin-resistant *Staphylococcus aureus* (*15*). The biological activities led us to predict additional novel functions of polyphenol polymers with different structures, as derived from a variety of food resources.

In summary, our study addressed the separation and characterization of polyphenolic compounds from the seed shells of the Japanese horse chestnut. We have identified polymeric proanthocyanidins with polymerization degrees of 19 and 23; they have potent antioxidative activities. These findings indicate the potential usefulness of the polyphenol polymers abundant in the seed shells as nutraceutical factors or for other such purposes. Our studies provide clues as to the uses of the seed shells of the Japanese horse chestnut, which have to date been regarded as waste materials in the processing of edible seeds.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; MALDI-TOF/MS, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; LC-ESI/MS, liquid chromatography electrospray−ionization mass spectrometry; DPPH, 1,1-diphenyl-2-picrylhydrazyl; C/EC unit, (+)-catechin/(−)-epicatechin unit; u, unified atomic mass unit; BHA, butylated hydroxyanisole; DP, degree of polymerization.

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