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Fractionation of apple procyanidins by size-exclusion chromatography

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Abstract

Oligomeric constituents of apple procyanidins were fractionated by size-exclusion chromatography using a TSKgel Toyopearl HW-40F column. The best separation was obtained using a mobile phase of acetone–8 *M* urea (6:4; adjusted to pH 2) at a flow-rate of 1.0 ml/min. In this chromatographic system, the use of 8 *M* urea in the mobile phase resulted in a molecular sieve effect without any surface affinity interaction between the gel beads and the procyanidin molecules. Each fraction obtained was examined by reversed-phase high-performance liquid chromatography and time-of-flight mass spectrometry. The order of elution of the procyanidins from the column was coincident with their degree of polymerization.

Keywords: Apples; Fruits; Fruit juices; Food analysis; Procyanidins; Tannins

1. Introduction

Proanthocyanidins (i.e., condensed tannins) are generally present in large amounts in members of the plant kingdom as secondary metabolites. Edible fruits are rich sources of proanthocyanidins [1,2], and we consume these compounds every day from natural foods including fresh fruits, juices, wines and the other processed foods made from edible plants.

As shown in Fig. 1, the proanthocyanidins are polymers consisting of the chains of flavan-3-ol units as oligomeric constituents linked mainly through C4–C8 (or C6) bonds. Recently, proanthocyanidins have attracted attention in the fields of pharmacology and food chemistry, because they display various types of physiological activity, such as antioxidant activity [3–6], antibiotic activity [7], anti-hypertensive activity [8,9], anti-allergy activity [10], hair growth-promoting activity [11] and inhibitory activity against some physiological enzymes and receptors [12–15]. All of these properties of proanthocyanidins largely depend on their structure and particularly their degree of polymerization. To facilitate further studies on the physiological activity of proanthocyanidins, it is necessary to establish simple

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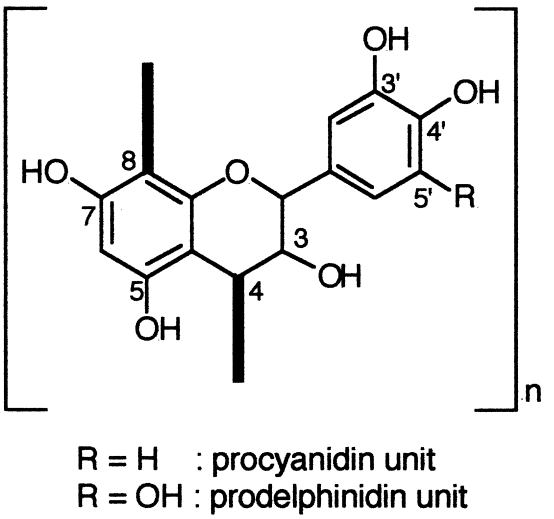

Fig. 1. Structural formulae of plant proanthocyanidins.

methods for separation and accurate quantitative determination of the oligomeric constituents with different degree of polymerization.

In general, the molecular mass (M_r) distribution of the oligomeric constituents of plant proanthocyanidins has been determined only by mass spectrometry (MS) [16,17]. MS provides a full account of molecular mass information through detection of the ionic peaks derived from each of the oligomeric constituents in the mixture. We have reported previously that matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis of apple procyanidins provided direct evidence of oligomeric constituents up to the size of pentadecamers (15-mer) [16]. Likewise, data from ^{13}C-nuclear magnetic resonance (NMR) analysis of proanthocyanidins gives us information on the number-average molecular mass (M_n) and the PC/PD ratio (procyanidin/prodelphinidin unit ratio) of the mixture, and information on the kind of terminal units in the polymer [18,19]. Otherwise, in order to calculate the M_n of proanthocyanidins conveniently, size-exclusion chromatography (SEC) has often been applied [20–22]. In the usual SEC system employed for this purpose, derivatization (such as methylation and acetylation) of proanthocyanidins before SEC analysis is necessary to achieve elution of the solute from the SEC column using tetrahydrofuran as the mobile phase because the native proanthocyanidins are very polar due to the presence phenolic hydroxyl groups. However, under such SEC conditions, the separation of proanthocyanidins is incomplete and native-form oligomers cannot be collected, and the calibrated M_r is not accurate.

In the present study, the direct fractionation of native-form proanthocyanidins (i.e., apple procyanidins) by SEC was investigated without derivatization using an aqueous eluent as the mobile phase. Under these conditions, the separation of proanthocyanidins based on the molecular sieve effect might be strongly interfered with any surface affinity interaction such as hydrogen-bonds and hydrophobic adsorption between the gel beads and the proanthocyanidins. The best separation of apple procyanidins was achieved by SEC using an aqueous mobile phase containing 8 *M* urea which serves to block without such electrostatic interactions.

2. Experimental

2.1. Materials

Epicatechin (EC) was purchased from Wako (Osaka, Japan). Procyanidin B2 [epicatechin-(4β,8)-epicatechin: PB2] and procyanidin C1 [epicatechin-(4β,8)-epicatechin-(4β,8)-epicatechin: PC1] were isolated from apple juice by chromatography on a TSKgel Toyopearl HW-40EC column (285 mm×25 mm I.D.) (Tosoh, Tokyo, Japan) using 40% EtOH as the mobile phase, followed by chromatography on a Unisil ODS column (250 mm×20 mm I.D., 5 μm particle size) (GL Sciences, Tokyo, Japan) using 20% EtOH as the mobile phase. Isolated procyanidins were further analyzed by fast atom bombardment MS [16], reversed-phase high-performance liquid chromatography (RP-HPLC) and acid catalysis degradation with phloroglucinol [23]. Folin–Ciocalteu's phenol reagent was purchased from Kanto (Tokyo, Japan). *trans*-3-Indoleacrylic acid and the silver salt of trifluoroacetic acid were obtained from Sigma–Aldrich Japan (Tokyo, Japan). All other chemicals were of analytical reagent grade. All chromatographic solvents were of HPLC grade.

2.2. Preparation of apple procyanidins from unripe apples

The method used for preparation of apple procyanidins (apple condensed tannins: ACTs) from unripe apples has been described in detail elsewhere [16]. Briefly, 3 kg of apples (the average mass of each was about 10 g) were homogenized in 0.1% (w/w) potassium pyrosulfite solution, and the homogenate was allowed to stand for 24 h at 4°C. The supernatant was centrifuged at 3500 *g* and filtered through a glass filter funnel. The filtrate (1.8 l) was applied to a Sepabeads SP-850 (Mitsubishi Kasei, Tokyo, Japan) column (285 mm×25 mm I.D.). The column was washed with 300 ml of distilled water and thereafter the crude apple polyphenol (CAP) fraction was eluted with 200 ml of 80% EtOH. The eluted fraction was evaporated to 65 ml under reduced pressure.

Next, a 25-ml portion of the concentrated CAP fraction was loaded on a TSKgel Toyopearl HW-40EC (extra course grade, Tosoh) column (285 mm×25 mm I.D.). The column was first washed with 200 ml of distilled water, then phenolic compounds were eluted with 250 ml of 40% EtOH followed by 100 ml of 60% acetone. The fraction eluted with 40% EtOH, containing monomeric catechins and oligomeric procyanidins, was applied to a Sep-Pak C_{18} ENV cartridge (Waters, Milford, MA, USA) to eliminate phloretin and quercetin glycosides. The resulting solution and the latter 60% acetone eluate (containing mainly polymeric procyanidins) were mixed together to obtain the fraction referred to as the ACT fraction, used in the subsequent experiments. The organic solvent was removed from the ACT fraction by evaporation and the remaining solution was lyophilized.

2.3. Fractionation of apple procyanidins by SEC

TSKgel Toyopearl HW-40F (fine grade, Tosoh) was suspended in the mobile phase and, after swelling, it was slurry-packed in a column (950 mm×25 mm I.D.) at room temperature. The mobile phases tested were acetone–water (6:4), acetone–8 *M* urea (6:4) and acetone–8 *M* urea (6:4) adjusted to pH 2 with HCl. A 0.5-ml portion of sample solution, prepared by dissolving 10 mg of the lyophilized ACTs in the mobile phase was loaded on the column and elution was performed at a flow-rate of 1.0 ml/min with a L-6000 pump (Hitachi, Tokyo, Japan). Fractions of 3 ml each were collected using a type SF-2120 fraction collector (Advantec Toyo, Tokyo, Japan). Analysis of total phenolic compounds in the fractions was performed by the modified Folin–Ciocalteu method [24]. A 0.2-ml portion of each fraction was mixed with an equal volume of four-fold diluted Folin–Ciocalteu's phenol reagent and 2 ml of 0.4 *M* sodium carbonate solution was added to the above mixture. After, 30 min, a deep blue color appeared and the absorbance of each fraction was measured at 760 nm using a type U-2000 spectrophotometer (Hitachi).

To remove the remaining urea from each fraction, the fractions were combined and the acetone was evaporated in vacuo. The concentrated aqueous fraction was loaded on a Sep-Pak C_{18} ENV cartridge (Waters) and washed with a distilled water. Finally, the adsorbed procyanidin oligomers were eluted with 80% methanol. Thereafter the methanol was removed by evaporation, the remaining material was lyophilized, and the procyanidin oligomers were obtained in powder form.

2.4. Analysis of SEC fractions by RP-HPLC

The phenolic compounds in the SEC fractions were characterized by RP-HPLC using a Hitachi system consisting of a L-6200 intelligent pump, an AS-2000 autosampler, a L-4200 UV–Vis detector and a D-2500 integrator. The column used was an Inertsil ODS-3 column (150 mm×4.6 mm I.D., 5 μm particle size) purchased from GL Sciences and the column temperature was controlled by means of a Model 556 column oven (GL Sciences). The mobile phase solvents A and B were each a mixture of methanol–0.01 *M* KH_2PO_4 (adjusted to pH 2 with phosphoric acid) at volume ratios of (A) 2:8 and (B) 5:5. A 10-ml sample solution, prepared by dissolving the dried powder in solvent A, was injected through the sample port, and eluted with solvent A for 10 min. Then, linear gradient elution from 0 to 100% solvent B, was applied for 40 min. The components that remained in the column were then eluted by applying solvent B for 15 min. The above chromatography was performed at a flow-rate of 1 ml/min

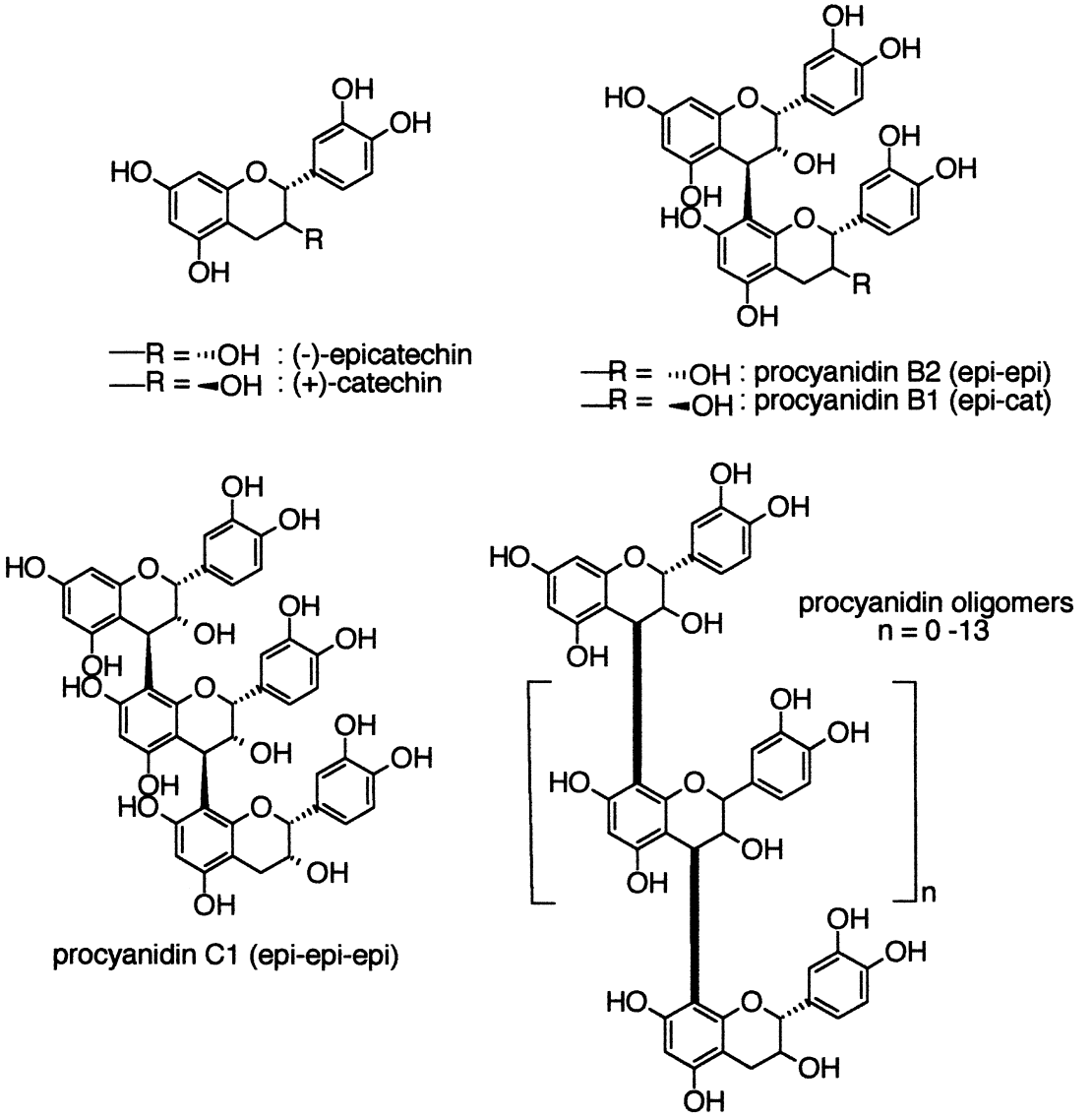

Fig. 2. Structures of monomeric flavan-3-ols and procyanidin oligomers in apples.

and the absorbance of the eluate was monitored at 280 nm.

2.5. MS analyses of SEC fractions

MALDI-TOF-MS analyses of the procyanidins in the SEC fractions were carried out as described previously [16]. The lyophilized sample was dissolved in acetone (500 mg/l). A 10 μl portion of sample solution was mixed with 5 μl of matrix solution (1% *trans*-3-indoleacrylic acid dissolved in acetone) and a 1 m*M* solution of the silver salt of trifluoroacetic acid (in acetone). The mixture (ca. 1 μl) was put on the stainless steel target tray and crystallized at room temperature.

The MALDI-TOF mass spectra were acquired on a Reflex II (Bruker-Franzen Analytik, Bremen, Germany) instrument, in which samples were irradiated

with a nitrogen laser (wavelength 337 nm; 3 ns pulse) in vacuo. The reflection mode of operation used 20 kV ion acceleration and 21.1 kV postacceleration. Spectra obtained in the linear mode were measured using 20 kV ion acceleration without postacceleration. All spectra were recorded with a detector voltage of 1.6 kV, and were the averaged result of at least 100 laser shots. Laser intensity was variable, providing maximum flexibility in collecting spectra. The singly charged molecular ions of insulin and angiotensin II were used as external standards and a mass calibration procedure was employed prior to the analysis of each sample.

3. Results and discussion

3.1. UV spectra of apple procyanidins

Plant proanthocyanidins are classified on the basis of the hydroxylation patterns of the A- and B-rings in the flavan-3-ol unit. Those of apple are only procyanidin-form (hydroxylation pattern: 3,3′,4′,5,7), and the structural unit compounds are mainly epicatechin and catechin [25]. Fig. 2 shows the structures of the monomeric and oligomeric constituents in the ACT fraction. In our previous study, monomeric flavan-3-ols and procyanidin oligomers ranging in size from dimers (2-mer) to pentadecamers (15-mer) were detected in ACTs by mass spectrometric analysis [16].

Fig. 3 shows the UV absorption spectra of the CAPs, ACTs and EC. The spectrum of the ACTs was very similar to that of EC. This result also indicates that the ACTs purified from CAPs are a mixture of EC oligomers (i.e., procyanidin).

3.2. Size-exclusion chromatography of apple procyanidins

Because Toyopearl HW gel beads are made from a vinyl polymer, the surface of the gel beads is weakly hydrophilic. In the case of SEC using these gel beads as the column packing, it is known that aromatic compounds including polyphenols are adsorbed on the gel surface when an aqueous solvent is used as

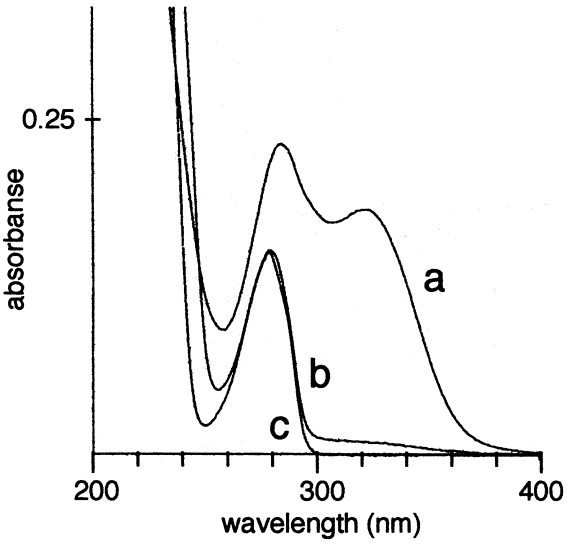

Fig. 3. UV absorption spectra of apple condensed tannins (ACTs) and related compounds. Spectra: (a) crude apple polyphenols (CAPs), (b) ACTs, (c) (−)-epicatechin. Each sample was dissolved in MeOH (10 μg/ml final concentration) and the spectral measurement was carried out using a MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan) at room temperature.

the mobile phase. The elution of polyphenols (especially, polymerized materials) from the column was facilitated using a high concentration of acetone above 40%, in the eluent [26], but in such case the chromatographic mode of separation is adsorption rather than size exclusion.

In view of the above, we tried to separate the ACTs by SEC. Fig. 4a shows the elution profile of ACTs on a Toyopearl HW-40F column (950 mm× 25 mm I.D.). The sample applied consisted of 10 mg of ACTs dissolved in 0.5 ml of the mobile phase solvent, acetone–water (6:4), and elution was performed at a flow-rate of 1.0 ml/min. However, the components of the ACTs were not separated. This finding suggested the occurrence of interactions such as hydrogen bond formation between hydroxyl groups on the surface of the gel beads and the proanthocyanidin molecules, which prevented the molecules from permeating into the Toyopearl HW gel matrix.

In an effort to prevent the formation of hydrogen bonds between the procyanidin molecules and the surface on the gel beads, urea was added to the mobile phase at a high concentration (8 *M* urea). As

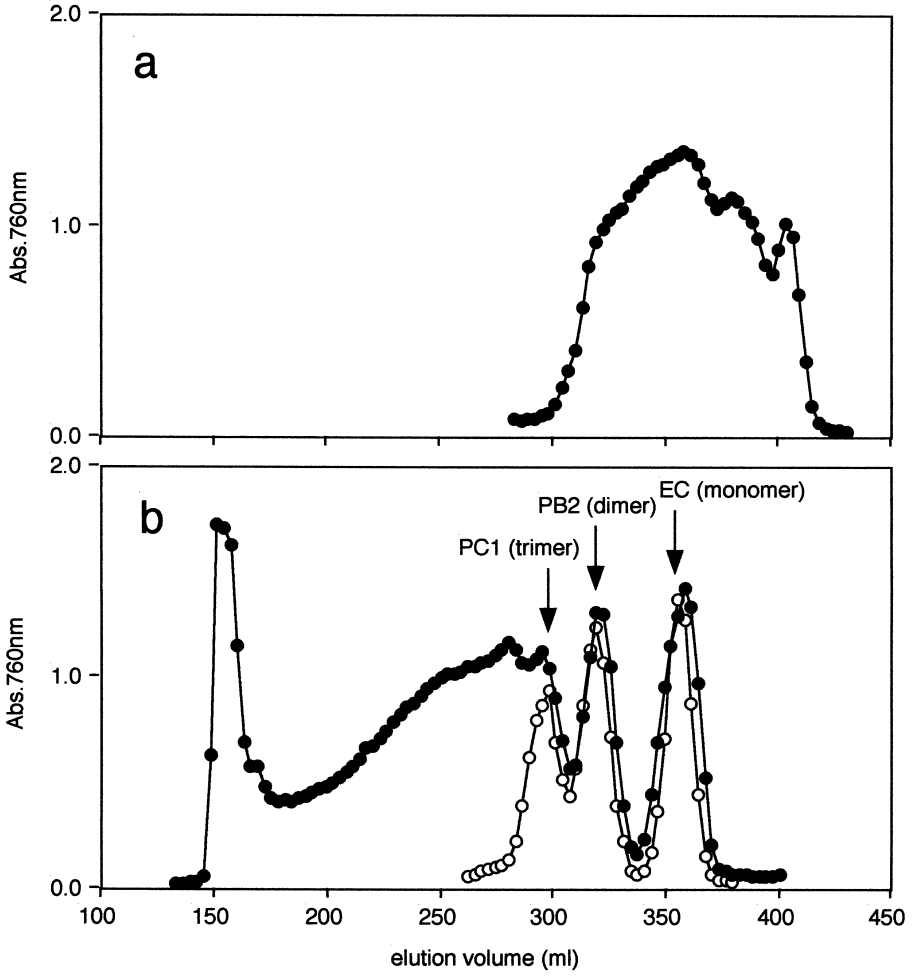

Fig. 4. SEC elution profiles of ACTs and standard oligomers applied to a Toyopeal HW-40F column and eluted at flow-rate of 1.0 ml/min. Column size: 950 mm×25 mm I.D. Mobile phases: (a) acetone–water (60:40), (b) acetone–8 *M* urea (60:40). ●=Elution pattern of ACTs; ○=elution pattern of a mixture of flavan-3-ol (epicatechin, EC) and isolated procyanidins (procyanidin B2, PB2; procyanidin C1, PC1) as a molecular mass standard. A 10-mg portion of ACTs and 2 mg of each of standard oligomers were dissolved in 0.5 ml of the mobile phase solvent, and loaded on the HW-40F column. SEC separation was carried out at room temperature.

shown in Fig. 4b, under these chromatographic separation conditions, the monomer (epicatechin or catechin), dimer (procyanidin B) and trimer (procyanidin C) forms of procyanidin were isolated. It seems that the urea molecules disrupted any surface affinity interaction such as hydrogen bonds and hydrophobic adsorption, and the mode of separation was only size exclusion. The order of elution of the procyanidins from the column was oligomer, trimer, dimer and monomer, that is, the procyanidins were eluted in order of decreasing molecular mass.

3.3. Effect of pH on SEC separation of apple procyanidins

Using the Toyopearl HW column for SEC separation, anionic compounds are excluded from entering the gel matrix because of the presence of ether bonds and hydroxyl groups on the surface of the HW gel beads. In the case of apple polyphenols, the elution of phenolic acids (PAs) containing carboxyl groups, such as caffeic acid and chlorogenic acid, is strongly influenced by this effect. Under the SEC

conditions mentioned above, their elution volume depended on the pH of the 8 *M* urea in the mobile phase. For example, when the pH of the 8 *M* urea was nearly neutral (about 8), the PAs were eluted at near the void volume of the column (data not shown).

To confirm the influence of the pH of the mobile phase, two different mobile phase solvents containing 8 *M* urea, adjusted to either pH 2 or pH 8, were tested in elution of the ACTs, as shown in Fig. 5. The elution behavior of the monomeric flavan-3-ols and dimeric procyanidins in ACTs was scarcely affected by pH. However, in the case of highly polymerized oligomers containing many phenolic hydroxyl groups, the elution profile was slightly affected by pH, and the elution volume decreased to some degree when the pH of the 8 *M* urea in the eluent was around 8. This result indicates that the SEC separation of ACTs on HW-40F involves only the molecular sieve effect under acidic pH conditions, but not under neutral conditions.

3.4. Molecular mass distribution of procyanidin oligomers in the SEC fractions

Fig. 5 shows the chromatographic fractionation pattern of ACTs obtained using the HW-40F column and a mobile phase of acetone–8 *M* urea (6:4) adjusted at pH 2. The oligomeric constituents in fractions 1 to 7 were found to differ in size, having been eluted in increasing order of degree of polymerization. The components of these fractions were further purified by Sep-Pak C_{18} treatment and then lyophilized. The composition of the procyanidin oligomers in the lyophilized samples was determined by RP-HPLC and MALDI-TOF-MS.

Fig. 6 shows the RP-HPLC profiles of ACTs and the oligomeric constituents in the above SEC fractions (fractions 1–7). In the chromatogram of native ACTs, two kinds of monomeric flavan-3-ols (epicatechin and catechin) and a few oligomeric procyanidins (such as procyanidin B1, B2, C1) appeared as obvious peaks. Upon analysis of the SEC fractions 1–7, the constituents of fraction 1 were found to be monomers (epicatechin and catechin), those of fraction 2 were dimers (procyanidin B1 and B2), and those of fraction 3 were trimers (mainly, procyanidin C1). However, in the chromatograms of fractions 4, 5, 6 and 7 there were many unidentified peaks which were due to the presence of many isomeric high-M_r oligomers larger than tetramers.

Furthermore, for accurate determination of the M_r distribution, MALDI-TOF-MS analysis of the con-

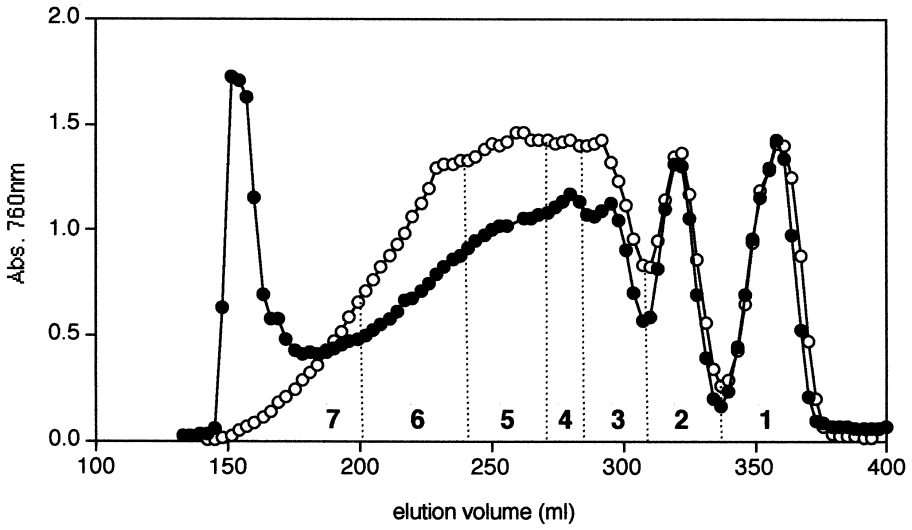

Fig. 5. Effect of the pH of the 8 *M* urea in the mobile phase on the SEC elution profile of ACTs. ●=The 8 *M* urea was adjusted to near neutral condition (about pH 8.0); ○=the 8 *M* urea was adjusted to pH 2.0 with HCl. The fractions obtained under the pH condition (2.0) are numbered from 1 to 7. Other SEC conditions were as described in Fig. 4.

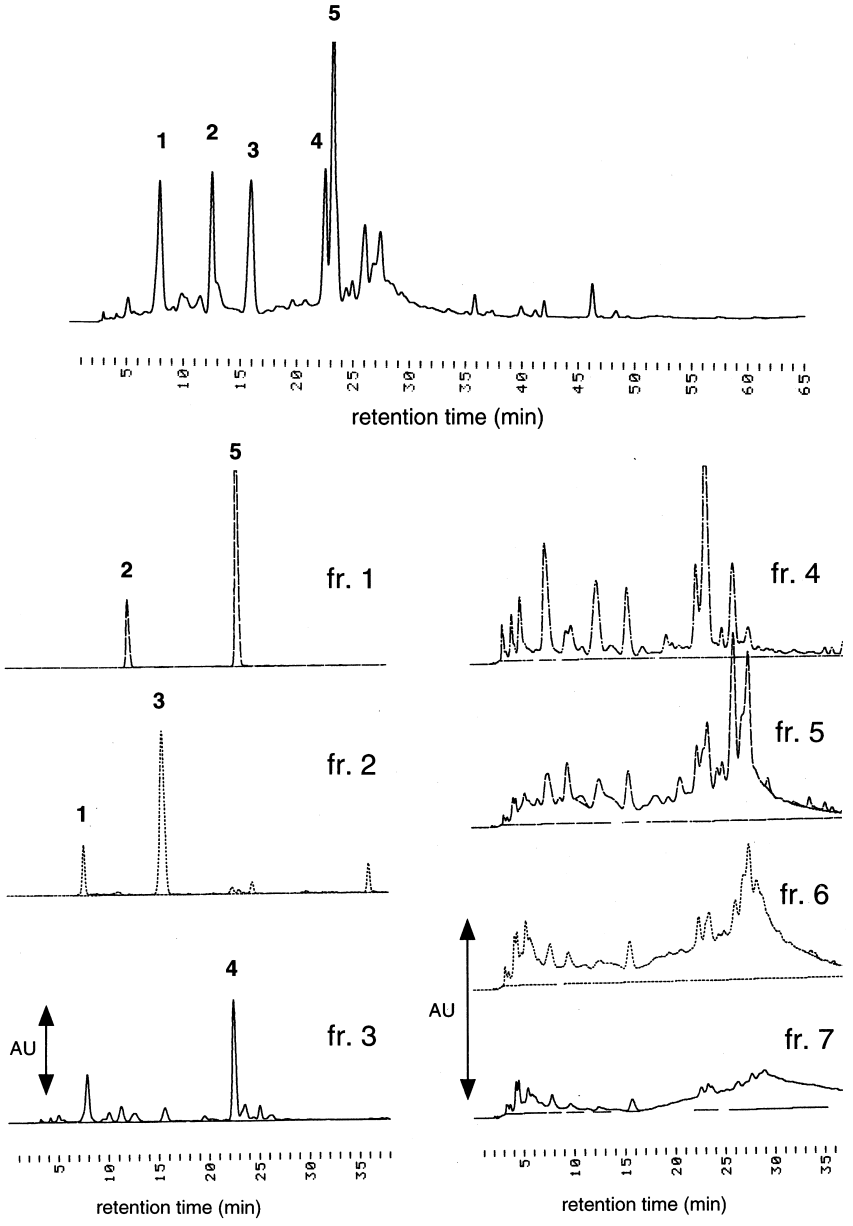

Fig. 6. RP-HPLC profiles of ACTs and SEC fractions (fr.) of ACTs. Each lyophilized sample was dissolved in water (1 mg/ml), and analyzed by RP-HPLC. Upper chromatogram: RP-HPLC profile of ACTs. Lower chromatograms with fraction numbers: RP-HPLC profiles of SEC fractions of ACTs. The numbers of the SEC fractions correspond to those in Fig. 5. The numbers of the identified peaks in each chromatogram are (1) procyanidin B1 (PB1), (2) (+)-catechin, (3) procyanidin B2 (PB2), (4) procyanidin C1 (PC1), (5) (−)-epicatechin (EC). AU means relative absorbance units (at 280 nm). For the details of the RP-HPLC conditions, see Experimental.

stituents in latter SEC fractions (Nos. 4–7) was carried out. The mass spectra are shown in Fig. 7. The data revealed that the oligomeric procyanidins in fraction 4 were mainly tetramers, those in fraction 5 were tetramers to 7-mers (mainly pentamers and hexamers), and those in fraction 6 were mainly

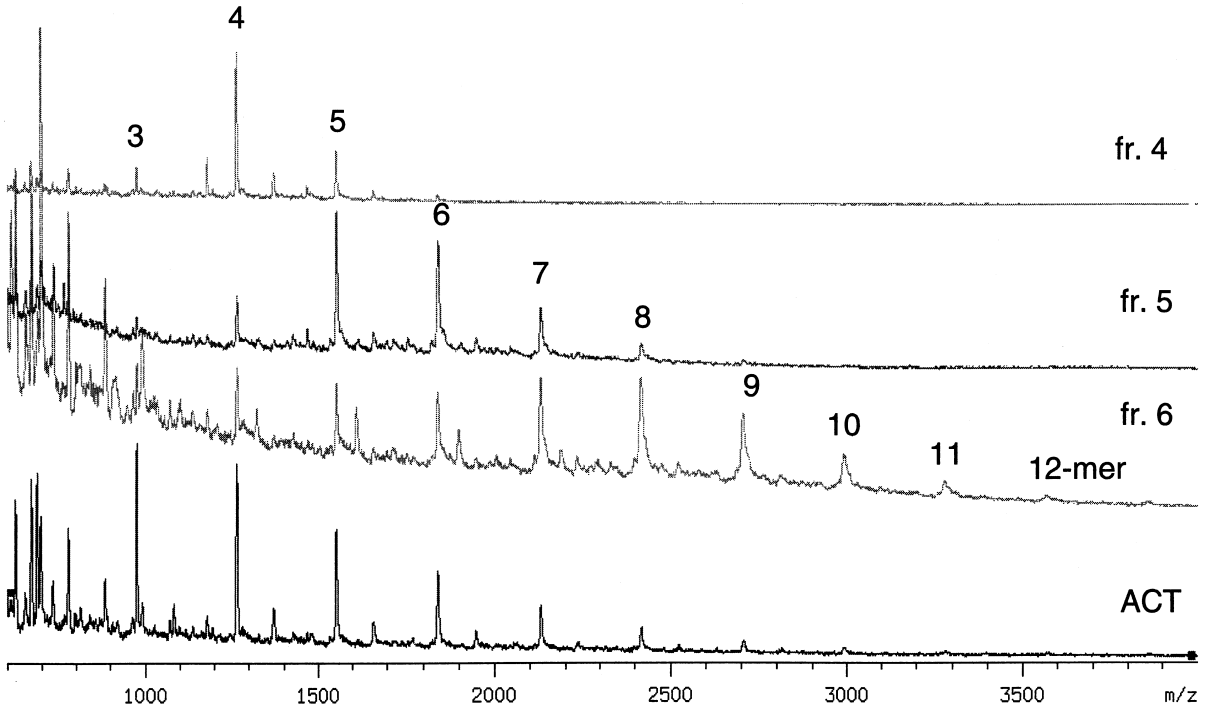

Fig. 7. MALDI-TOF mass spectra of ACTs and SEC fractions of ACTs obtained in the positive-ion mode in the presence of silver ions. Lower spectrum: MALDI mass spectrum of ACTs. Upper spectra with fraction numbers: MALDI mass spectra of SEC fractions of ACTs. The numbers of the SEC fractions correspond to those in Fig. 5. The number shown on each ionic peak ($[M+Ag]^+$) indicates the degree of polymerization (number of flavan-3-ol units in each molecular ion).

7-mers and 8-mers. However, ionic peaks derived from highly polymerized constituents were not detected in fraction 7.

The results shown in Figs. 6 and 7 prove that the order of elution of the procyanidin oligomers in ACTs from the HW-40F column is coincident with their degree of polymerization.

3.5. Possibility of other applications and improvements

The procyanidin oligomers separated by our SEC method, showing different degree of polymerization will facilitate elucidation of the structure–activity relationships in assays of physiological activity. Furthermore, our SEC method is applicable to the separation of hydrolyzable tannins, polymerized products generated by oxidation of polyphenols such as black tea tannins, and the polymerized anthocyanins formed during long-term maturation of wine. Our method has several shortcomings, as the separation of highly polymerized constituents is not always perfect, the time required for elution is relatively long, and the detection method is complicated. However, at present, there is no other suitable method available for separation of polymerized polyphenols (especially, proanthocyanins) according to their degree of polymerization. In an effort to improve the above shortcomings, we are now trying to develop a new HPLC–UV detection system with chromatographic separation on the basis of the molecular sieve effect.

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