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Apple Procyanidin Oligomers Absorption in Rats after Oral Administration: Analysis of Procyanidins in Plasma Using the Porter Method and High-Performance Liquid Chromatography/Tandem Mass Spectrometry

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In this study, we investigated the absorption of apple procyanidins, namely, apple condensed tannins (ACTs), in rats using the Porter method and high-performance liquid chromatography/tandem mass spectrometry. The apple procyanidin concentrations in the rat plasma reached a maximum 2 h after administration and decreased thereafter. To investigate the limits of the absorption of apple procyanidins in the polymerization degree, we administered the procyanidin oligomer fraction, which was separated from ACT using normal-phase chromatography according to the degree of polymerization. Procyanidins from each dimer to pentamer group were detected in the plasma by the Porter method. Moreover, by the study using reconstituted procyanidins, polymeric procyanidins influenced the absorption of procyanidin oligomers. These results suggest that ACTs are absorbed and directly involved in physiological functions in the rats.

KEYWORDS: Absorption; apple procyanidins; HPLC/MS/MS; procyanidin dimers; procyanidin trimers

INTRODUCTION

Polyphenols are common secondary metabolites of plants, which are classified according to the nature of their carbon skeletons as phenolic acids, flavonoids, stilbenes, or lignans. Flavonoids, which are the most abundant polyphenols in human foodstuffs, are divided into the following classes: flavones, flavonols, flavanols, isoflavones, anthocyanins, proanthocyanidins, and flavanones. Proanthocyanidins are among the most abundant polyphenols. These polymeric flavanols are categorized as procyanidins or prodelphinidins based on the type of the flavan-3-ol units, such as (+)-catechin and (−)-epicatechin or (+)-gallocatechin and (−)-epigallocatechin. Proanthocyanidins have many isomers according to the degree of polymerization, the combinations and the types of flavan-3-ol unit, and the linkage positions (*1*). The most widely studied proanthocyanidins are the procyanidin B type, which are linked through 4→8 or 4→6 interflavanoid bonds (**Figure 1**). Proanthocyanidins are present in various beverages [e.g., red wine (*2*), green tea (*3*), and beer (*4*)], foods [e.g., grape seeds (*5*), hops (*6*), and cocoa (*7*)], and fruits [e.g., grapes (*8*) and apples (*9*−*11*)].

Proanthocyanidins are reported to have various physiological functions, including antioxidative activity. In vitro studies have shown that proanthocyanidins inhibit melanogenesis in B16 cells (*12*) and histamine release in RBL-2H3 cells (*13*). In vivo studies have revealed various properties, such as radical scavenging, antiulcer (*14*), antiallergy (*15*), antidental caries (*16*), antitumor activity (*17*), and the inhibition of food allergy (*18*) as well as functions in the activation of hair follicle growth (*19*) and protection against ultraviolet (UV) rays (*20*). Because the biological properties of proanthocyanidins depend on their bioavailability, it is important to investigate their absorption and distribution in order to understand their mechanisms of action.

The absorption of flavanols such as (+)-catechin and epigallocatechin gallate has been previously reported in both rats and humans (*21*−*23*). Other polyphenols, including phenol carboxylic acids such as chlorogenic acid (*24*), isoflavones such as genistein (*25*), anthocyanins such as cyanidin glucoside (*26*), and flavonols such as quercetin glucoside (*27*), have also been investigated. As discussed by Scalbert and Williamson (*28*), polyphenols are poorly absorbed as compared with other nutrient compounds. After absorption mainly in the digestive tract or partial degradation by the intestinal microflora, they are transported to the liver where they form glucuronide and/or sulfate or methyl conjugates, followed by transport to body tissues. However, relatively little is known about the bioavailability of proanthocyanidin. Previous studies employing ^{14}C-labeled proanthocyanidins detected these radioactive compounds in plasma and various organs after their administration (*29*, *30*).

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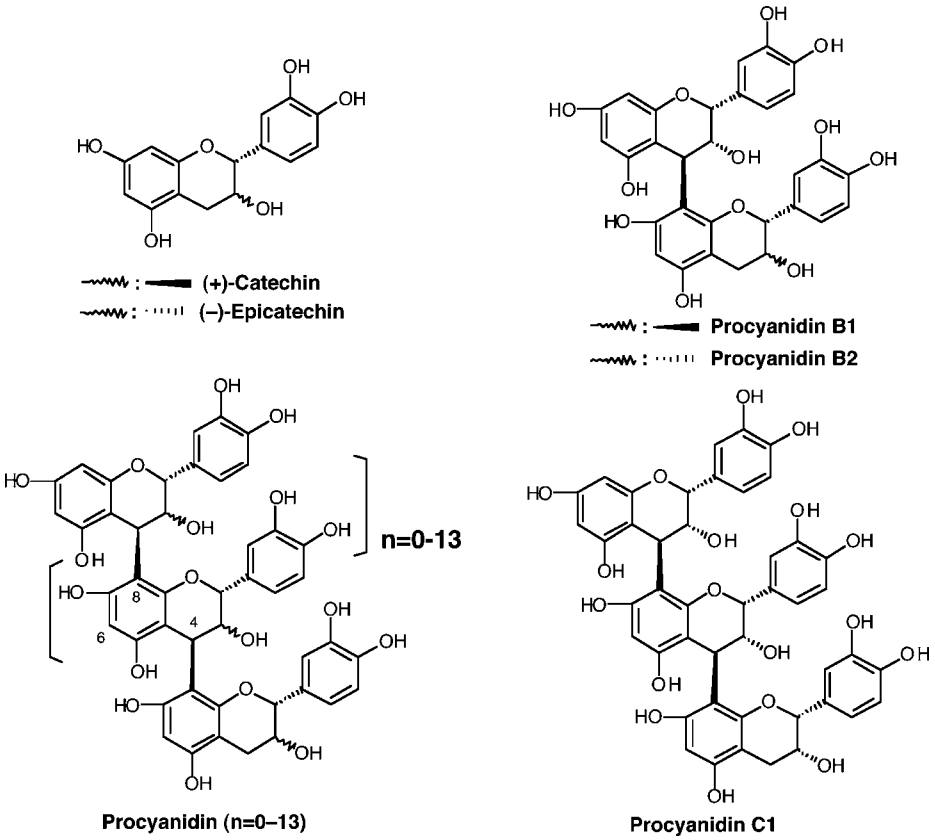

Figure 1. Structures of major apple procyanidins and basic procyanidins. Key: procyanidin B1 [epicatechin-(4β→8)-catechin]; (+)-catechin; procyanidin B2 [epicatechin-(4β→8)-epicatechin]; (–)-epicatechin; and procyanidin C1 [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin].

In contrast, ^{14}C-labeled proanthocyanidins from sorghum (*31*), *Lotus pedunculatus* (*32*), and *Ceratonia siliqua* (*33*) were reported not to be absorbed in chickens, sheep, and rats, respectively. The discrepancies between these results are probably due to the insufficient purity of the proanthocyanidins used in the assays because of the complexity of their chemical structures, the difficulties involved in fractionating proanthocyanidins, the sensitivity and specificity of the measurements used to detect them in plasma and urine, and differences between the animals used in the assays.

Scalbert et al. (*34*) reported that (+)-catechin, procyanidin dimer B3, and procyanidin trimer C2 passed through the human intestinal epithelial Caco-2 cell monolayer, whereas polymers with an average degree of polymerization of 7 did not. Spencer et al. (*35*) showed that a mixture of procyanidin dimers B2 and B5 was degraded in vitro using an intestinal preparation; a large amount of nonconjugated epicatechin and small amounts of the dimers were subsequently detected on the serosal side. These studies were performed using cell monolayers and rat intestinal model systems. More recent studies using liquid chromatography/mass spectrometry (HPLC/MS) have suggested that procyanidin dimers are absorbed in both rats (*36*) and humans (*37*). However, the fate of proanthocyanidins with a molecular weight greater than a trimer and the limits of absorption in vivo with respect to the degree of polymerization have remained unclear.

In the current study, we investigated the absorption of procyanidins fractionated from apples and the limits in terms of the degree of polymerization using a rat model. Apples contain many types of procyanidin ranging from dimers to pentadecamers (**Figure 1**) as well as flavan-3-ols, phenolcarboxylic acid derivatives, and flavonols (*9*, *11*). Apple procyanidins, namely, apple condensed tannins (ACTs), can be fractionated from monomers to octamers according to the degree of polymerization using normal-phase chromatography (*38*). They are therefore suitable for investigating the absorption of procyanidins and determining the limits according to the degree of polymerization.

MATERIALS AND METHODS

Apparatus. High-performance liquid chromatography (HPLC)/MS was performed using an API 3000 (Applied Biosystems, CA) with electrospray ionization (ESI) as an ion source operated in the negative-ion mode. For the HPLC-ESI/MS analysis, HPLC was performed using a HPLC system equipped with an L-2100 intelligent pump (Hitachi Ltd., Tokyo, Japan) and an L-2200 autosampler (Hitachi).

Reagents. (+)-Catechin, (−)-epicatechin, β-glucuronidase, and sulfatase type H-5 were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). Procyanidin B1, procyanidin B2, and procyanidin C1 were obtained from Funakoshi Ltd. (Tokyo, Japan). Unless otherwise stated, all reagents and chemicals were commercially available extrapure grade products.

Preparation of Apple Procyanidins. ACTs were prepared from apple polyphenol extracts using the methods described by Shoji et al. (*17*). The procyanidins were measured by the modified method of Porter et al. (*39*), which degraded proanthocyanidins to anthocyanidins in boiling water under acidic conditions. Briefly, 0.5 mL of a 50 μg/mL (w/v) solution of ACTs was added to 1.5 mL of *n*-buthanol/HCl (95:5, v/v) and 50 μL of a 2% (w/v) solution of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ dissolved in 2 M HCl. The reaction mixture was capped and then thoroughly mixed and heated in a water bath at 95 °C for 40 min. Similarly, procyanidin B2 as standard was also analyzed and the procyanidin concentration in ACTs was expressed as procyanidin B2. For the analysis of individual procyanidins and other polyphenols, reversed- and normal-phase HPLC were performed by the method of Shoji et al. (*40*). The polyphenol profiles of the ACTs analyzed by reversed- and normal-phase HPLC are summarized in **Table 1**. The procyanidins accounted for 75.6% of the ACTs by Porter method and comprised dimers (14.1%), trimers (8.4%), tetramers (6.8%), pentamers (3.1%),

Table 1. Components of Polyphenols in Apple Procyanidins

components	% (w/w)	analytical methods
procyanidins	75.6	Porter method
dimers	14.1	RP-HPLC[a]
procyanidin B1	5.0	RP-HPLC
procyanidin B2	9.1	RP-HPLC
trimers	8.4	NP-HPLC
procyanidin C1	6.3	RP-HPLC
tetramers	6.8	NP-HPLC
pentamers	3.1	NP-HPLC
hexamers	2.9	NP-HPLC
polymers	40.3	NP-HPLC
flavan-3-ols	13.3	
(+)-catechin	5.3	RP-HPLC
(−)-epicatechin	8.0	RP-HPLC
phenol carboxylic acids	1.7	
chlorogenic acids	0.6	RP-HPLC
p-coumaroyl quinic acid	1.1	RP-HPLC
chalcones	4.0	
phloridzin	0.4	RP-HPLC
phloretin-2′-xyloglucoside	3.6	RP-HPLC
total	94.6	

[a] RP-HPLC, reversed phase HPLC; NP-HPLC, normal phase HPLC.

hexamers (2.9%), and other polymers (40.3%). The ACTs also contained 13.3% flavan-3-ols (monomers), 4.0% other flavonoids, and 1.7% nonflavonoids (phenol carboxylic acid derivatives). To prepare each procyanidin fraction according to the degree of polymerization, a preparative normal-phase chromatography using a 250 mm × 20 mm i.d. silica gel packed column (GL Science Inc., Tokyo, Japan) with hexane/methanol/ethyl acetate as the mobile phase was performed (*38*). Each procyanidin oligomer fraction was then concentrated by rotary evaporation at 45 °C and lyophilized. Each procyanidin oligomer fraction was characterized by HPLC/MS and MALDI-TOF/MS, and the purity was measured by HPLC/MS: dimers, 95.2%; trimers, 94.2%; tetramers, 96.8%; and pentamers, 96.3%.

Animals and Diets. The test compounds were orally administered to 7 week old male Wister (Crj) rats from Charles River Japan Inc. (Kanagawa, Japan) at the Institute for Animal Reproduction (Ibaraki. Japan). The body weights of the animals were ∼250 g. The rats were maintained in air-conditioned quarters under a 12 h light/dark cycle. All animals were fed a standard diet of CRF-1 (Oriental Yeast Ltd., Tokyo, Japan) for a week prior to the experiment. Before the administration of the test compounds, the rats were starved for 15 h with access to distilled water only. The samples were administered to the rats by intragastric injections at a dose of 1000 mg/kg/10 mL via direct stomach intubation. Blood samples were collected from the abdominal aorta of the rats under diethyl ether anesthesia at the intervals of 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h after the administration. The blood was centrifuged (1000*g* × 15 min at 4 °C) after adding 1.2% ethylenediaminetetraacetic acid (EDTA), and 0.2 mL of 1.2% $K_2S_2O_5$ samples was added to 1 mL of plasma before storage at −20 °C.

Analyses of Procyanidins Concentrations in Rat Plasma. To analyze the conjugated forms, 0.2 mL of 100 mM phosphate buffer (pH 5.0) with 2.9 mM EDTA was added to 2 mL of rat plasma. The samples were hydrolyzed using β-glucuronidase type VII and sulfatase type VIII and then incubated at 37 °C for 1 h before the solid-phase extraction (SPE).

The procyanidin concentration in the plasma was measured after SPE. Briefly, 8 M urea was added to rat plasma in order to solubilize the protein−procyanidin complex. To extract the procyanidins from the rat plasma, SPE was performed using C_{18} Sep-Pak cartridges (Waters Associates, Tokyo, Japan). The cartridges were preconditioned with methanol and 5% HCOOH, followed by the application of 1.5 mL of rat plasma. The sample was extracted using 1% HCl−methanol (1.5 mL) after washing with 5 mL of 5% HCOOH. The extracted sample was then measured using the modified Porter method (*39*) described above. The procyanidin concentration in the plasma was expressed as procyanidin B2.

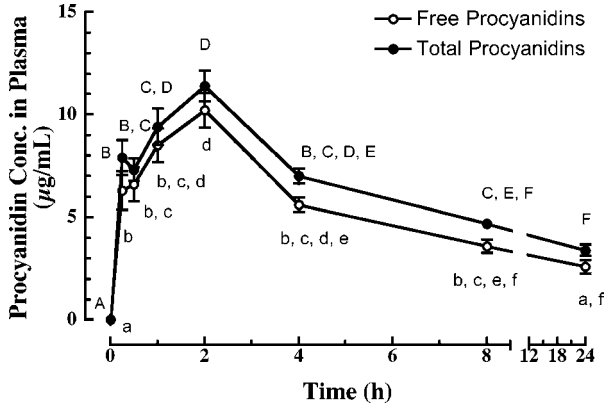

Figure 2. Changes in free and total procyanidin concentrations in rat plasma after administration at an ACT dose of 1000 mg/kg. Free and total procyanidins were expressed as procyanidin B2. All data are expressed as the mean ± standard error of the mean (SEM; $n = 7$–8 per group). Different letters indicate statistically significant differences at $P < 0.01$.

Liquid Chromatography/Tandem Mass Spectrometry (HPLC/MS/MS) Analyses. A 0.5 mL amount of plasma was extracted twice with 1 mL of ethyl acetate at ambient temperature. The ethyl acetate fraction was concentrated under N_2 gas and dissolved with 0.5 mL of 10% CH_3CN. HPLC/MS/MS was then performed as described below. The recovery of the procyanidins from the plasma was achieved by adding (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, procyanidin C1, and ethyl gallate (internal standard) to the plasma.

Each procyanidin was measured by HPLC/MS/MS in the multiple reaction monitoring (MRM) mode. The ion spray voltage was set at −40 eV. HPLC was performed with a 50 mm × 2.1 mm i.d. (3 μm) Inertsil ODS III (GL Science) column (GL Science) at 35 °C. A mixture of 0.1% HCOOH and CH_3CN was used as the mobile phase, and the flow rate was 0.2 mL/min. The initial eluent was 5% CH_3CN, followed by a linear gradient from 5 to 40% CH_3CN for 12 min. Subsequently, the CH_3CN concentration was held at 50% for 4 min and then returned to the initial conditions (5% CH_3CN) to reequilibrate for 10 min. The injection volume was 20 μL of the sample solution.

The mass spectra of the SPE sample from the plasma were measured using HPLC/MS. The injection volume was 100 μL of sample solution. The initial eluent was 20% CH_3CN, followed by a linear gradient from 20 to 30% CH_3CN for 75 min. Subsequently, the CH_3CN concentration was held at 30% for 5 min, and then, the initial conditions (20% CH_3CN) were restored to allow reequilibration for 15 min.

Statistical Analysis. All data are presented as the means ± standard error. The data were analyzed by Tukey's test after one-way analysis of variance (ANOVA). Statistical significance was recognized at $P < 0.01$.

RESULTS

Procyanidin Concentrations in the Rat Plasma. The free nonconjugated procyanidin concentration in the plasma increased dose dependently with ACT concentrations ranging from 500 to 2000 mg/kg in our preliminary study by the Porter method. However, the free procyanidin concentration reached a plateau at an ACT dose of 1000 mg/kg. Thus, at higher ACT doses of 1000 mg/kg, the free nonconjugated procyanidin concentrations in rat plasma ($n = 8$) were measured using the Porter method (**Figure 2**). At 0 h, procyanidins were not detected in the rat plasma. However, after 2 h, the free procyanidins concentrations in the plasma had increased to a maximum of 10.2 ± 2.2 μg/mL as measured as procyanidin B2 equivalents. Similarly, the total procyanidins concentration in the plasma after enzymatic treatment reached a maximum of 11.4 ± 2.1 μg/mL 2 h after administration. The total procyanidin concentrations in the plasma were slightly higher than those of

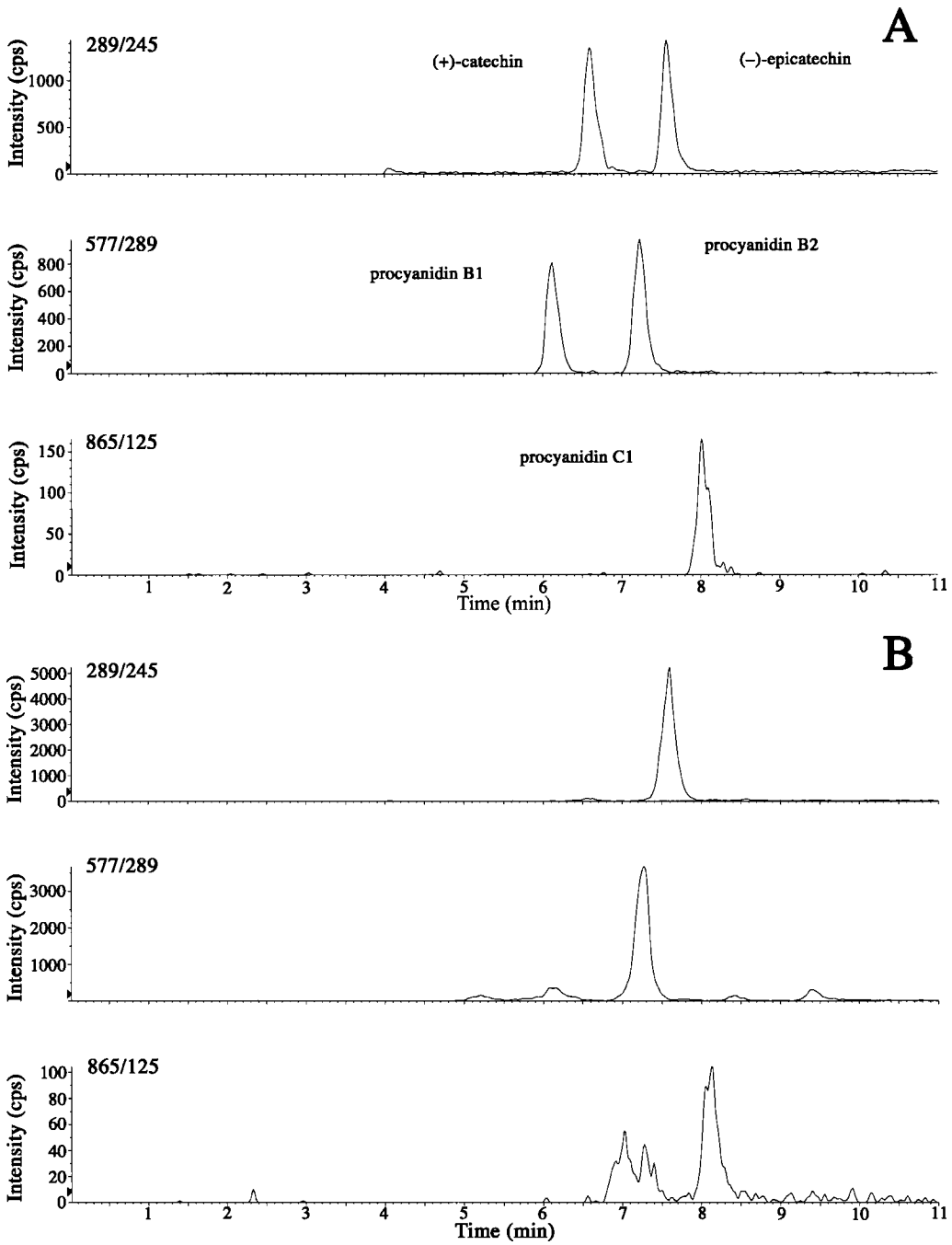

Figure 3. HPLC/MS/MS chromatograms in the MRM mode of rat plasma 2 h after administration. (**A**) Standard flavan-3-ols, procyanidin dimers, and trimers. (**B**) Rat plasma 2 h after administration of ACTs. HPLC/MS/MS was performed to analyze flavan-3-ols, procyanidin dimers, and trimers in the MRM mode by monitoring parent and product ion pairs of *m*/*z* 289/245, 577/289, and 865/125, respectively.

the nonenzymatic free procyanidins. In addition, the nonconjugated and conjugated procyanidins in the plasma had decreased to the basal level 24 h after administration.

Identifications of Flavan-3-ols, Procyanidin Dimers, and Procyanidin Trimers in Rat Plasma. Flavan-3-ols, procyanidin dimers, and trimers were detected using a HPLC/MS/MS in the MRM mode by monitoring parent and product ion pairs (*m*/*z* 289/245, 577/289, and 865/125, respectively). Typical HPLC/MS/MS chromatograms in the MRM mode are shown in **Figure 3**. The peaks detected at 6.6 and 7.6 min with *m*/*z* 289/245 were identified as (+)-catechin and (−)-epicatechin, the peaks detected at 6.1 and 7.2 min with *m*/*z* 577/289 were procyanidin B1 and procyanidin B2, and the peak detected at 8.0 min with *m*/*z* 865/125 was procyanidin C1. Analyses of the flavan-3-ols, procyanidin dimers, and procyanidin trimers by HPLC/MS/MS showed strong correlations in the range of 4−1000 nM. The specific parent and product ions for each procyanidin in the MRM mode allowed procyanidins to be analyzed in the plasma with high sensitivity and specificity.

Changes in Flavan-3-ols, Procyanidin Dimers, and Procyanidin Trimers in Plasma. At the ACT dose of 1000 mg/kg, changes in the concentrations of flavan-3-ols, procyanidin dimers, and procyanidin trimers in the rat plasma ($n = 6$) during the 24 h observation period are shown in **Figure 4**. The concentrations of procyanidin dimers and procyanidin trimers in the plasma reached a maximum 2 h after administration. By contrast, the flavan-3-ol levels peaked 1 h after administration, and these compounds were absorbed faster than the procyanidin dimers and trimers. Subsequently, the flavan-3-ol, procyanidin dimer, and trimer concentrations in the plasma decreased.

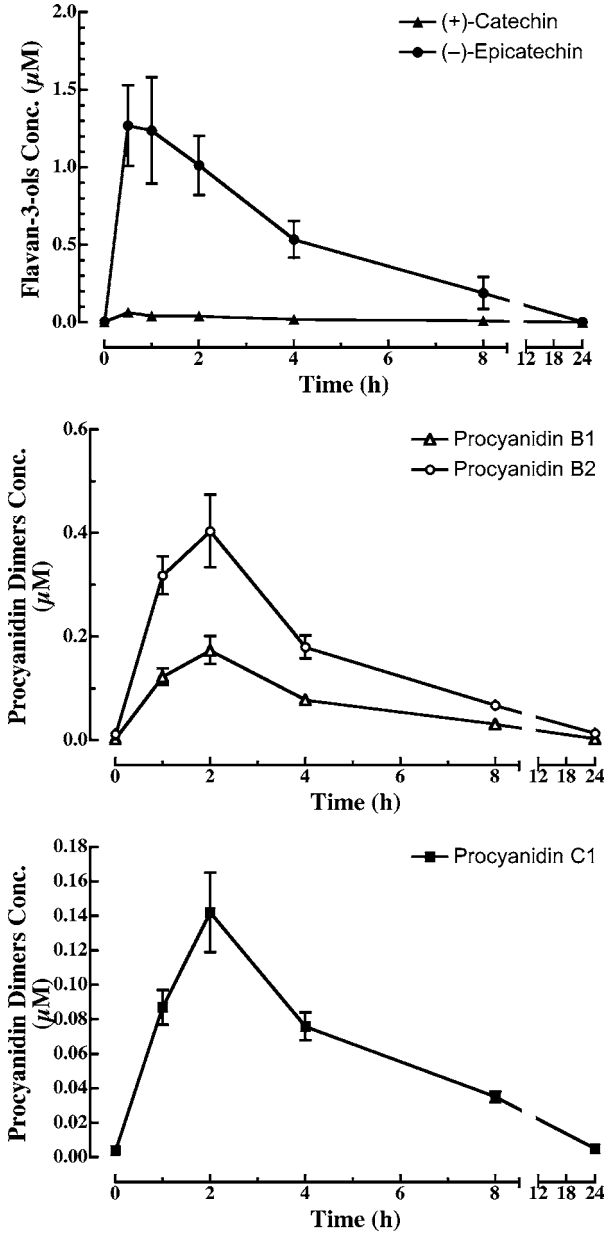

Figure 4. Changes in flavan-3-ols, procyanidin dimers, and trimers in rat plasma after administration at an ACT dose of 1000 mg/kg. Flavan-3-ols, procyanidin dimers, and trimers in rat plasma were analyzed by HPLC/MS/MS in the MRM mode by monitoring parent and product ion pairs of *m*/*z* 289/245, 577/289, and 865/125, respectively.

Absorption Procyanidin Oligomer Fractions Separated According to the Polymerization Degree. To determine the limits of the absorption of procyanidins in the rats, we further fractionated the ACTs according to the degree of polymerization using preparative normal-phase chromatography. Each procyanidin dimer to pentamer fraction was then administered to rats ($n = 7$) at a dose of 1000 mg/kg, and plasma samples were collected 2 h after administration. As shown in **Figure 5**, free nonconjugated procyanidins of each dimer to pentamer group were detected in the rat plasma using the Porter method. There were statistically significant differences in the free procyanidin concentrations between the dimer group and each procyanidin trimer to pentamer group ($P < 0.01$). Moreover, the free procyanidins in each procyanidin dimer to pentamer group were smaller than those observed in the ACT group ($P < 0.01$). However, no significant differences were observed in the free

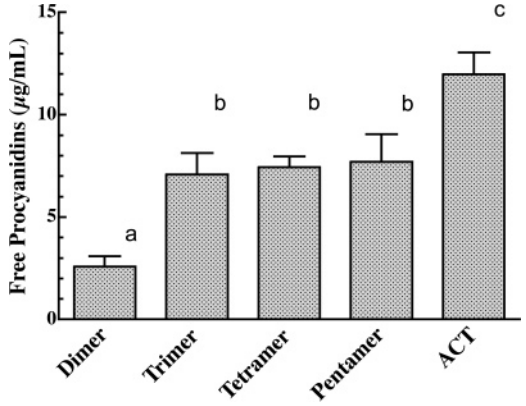

Figure 5. Free nonconjugated procyanidins in rat plasma 2 h after administration of each procyanidin fraction separated according to the degree of polymerization using normal phase chromatography. Free procyanidins were analyzed by the Porter method, and data were expressed as procyanidin B2. All data are expressed as the mean ± SEM ($n = 6$ per group). Different letters indicate statistically significant differences at $P < 0.01$.

procyanidin levels in the plasma between each procyanidin trimer to pentamer group.

$[M - H]^-$ ion peaks at *m*/*z* 577 and 865 in the dimer and trimer groups were detected by HPLC/MS, respectively. These peaks were identified as procyanidin dimers B1 and B2 and trimer C1, based on direct comparison of the retention time and $[M - H]^-$ ion peaks with an authentic standard. A few $[M - H]^-$ ion peaks at *m*/*z* 865 were detected in the trimer group, with the exception of procyanidin C1 and other procyanidin trimers. $[M - H]^-$ ion peaks at *m*/*z* 1154 in the tetramer group corresponded to the peak profile of the tetramer fraction before administration. However, $[M - H]^-$ ion peaks at *m*/*z* 1442, which corresponded to procyanidin pentamers, were not detected in pentamer group. In addition, procyanidins in each dimer to pentamer group were not reduced to compounds with molecular weights lower than those of the native procyanidins using LC/MS (**Figure 6**). $[M - H]^-$ ion peaks at *m*/*z* 591 and 879, which corresponded to methylated procyanidins, were also detected in the dimer and trimer groups by HPLC/MS/MS. However, $[M - H]^-$ ion peaks were not detected at *m*/*z* 657and 945, corresponding to sulfate form or $[M - H]^-$ ion peaks at *m*/*z* 753 and 1041, corresponding to glucuronide forms (data not shown). These results suggest that the native and methylated procyanidins were detected by the Porter method in the rat plasma.

Absorption of Reconstituted Apple Procyanidins. We further investigated the absorption of highly polymerized procyanidins. Dimer to pentamer fractions separated using normal-phase chromatography were reconstituted according to the original component ratio of the ACTs, with or without the procyanidin fraction with a molecular weight greater than an octamer. As the procyanidin dimer to pentamer fractions were administered at doses corresponding to the original composition ratio (dimer group, 141 mg/kg; trimer group, 84 mg/kg; tetramer group, 68 mg/kg; and pentamer group, 31 mg/kg), free nonconjugated procyanidins were not detected, respectively (data not shown). Reconstituted dimer to pentamer procyanidins (2−5-mer group), greater than octamer procyanidins (≥8-mer group), and a mixture of both 2−5-mer and ≥8-mer group were administered to rats at doses of 324, 432, and 756 mg/kg, respectively. The free nonconjugated procyanidin concentrations in the rat plasma 2 h after administration are shown in **Figure 7A**. In the separate reconstituted 2−5-mer and ≥8-mer groups,

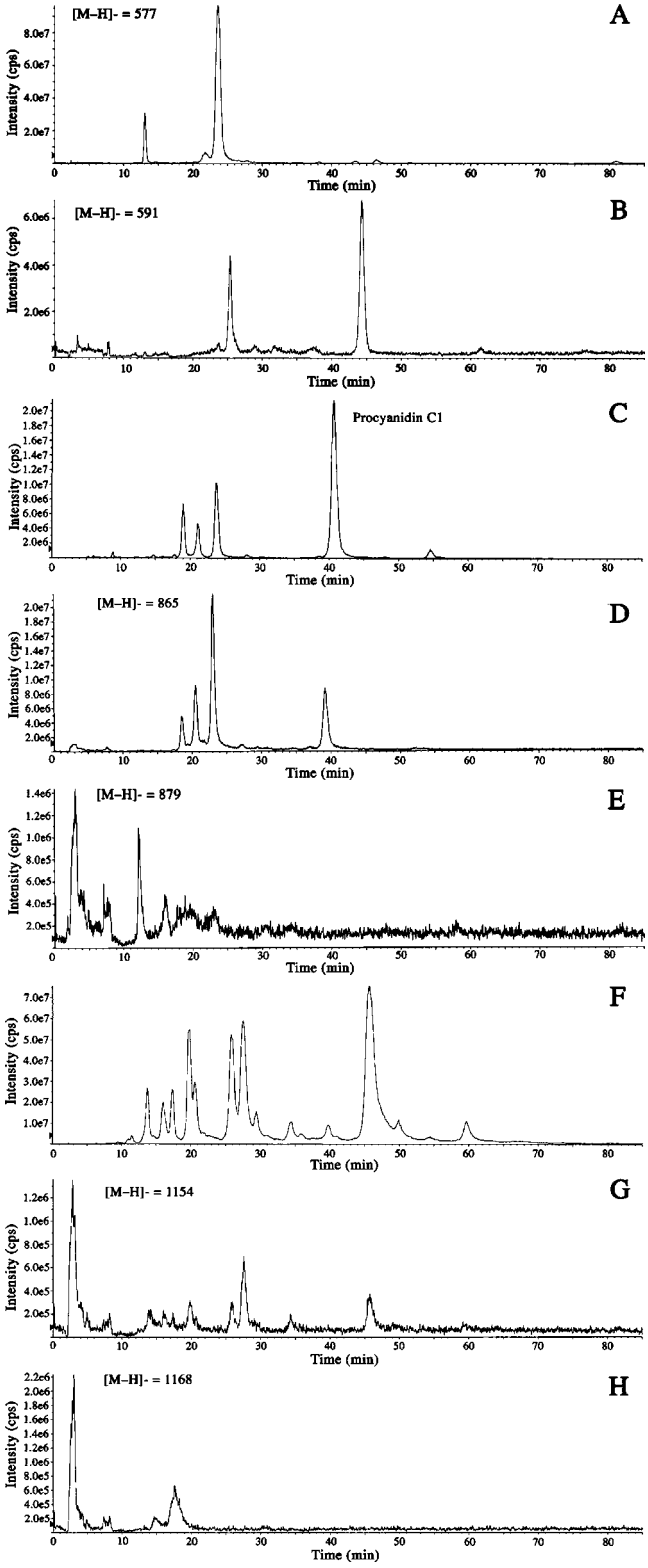

Figure 6. HPLC/MS/MS chromatograms of rat plasma 2 h after administration of each procyanidin (1000 mg/kg) fractionated by normal phase chromatography. (**A**) $[M - H]^-$ ion peaks at 577 in dimer group after administration; (**B**) $[M - H]^-$ ion peaks at 591 in plasma of dimer group after administration; (**C**) $[M - H]^-$ ion peaks at 865 in trimer fraction before administration; (**D**) $[M - H]^-$ ion peaks at 865 in plasma of trimer group after administration; (**E**) $[M - H]^-$ ion peaks at 879 in plasma of trimer group after administration; (**F**) $[M - H]^-$ ion peaks at 1154 in tetramer fraction before administration; (**G**) $[M - H]^-$ ion peaks at 1154 in plasma of tetramer fraction after administration; and (**H**) $[M - H]^-$ ion peaks at 1168 in plasma of tetramer fraction after administration.

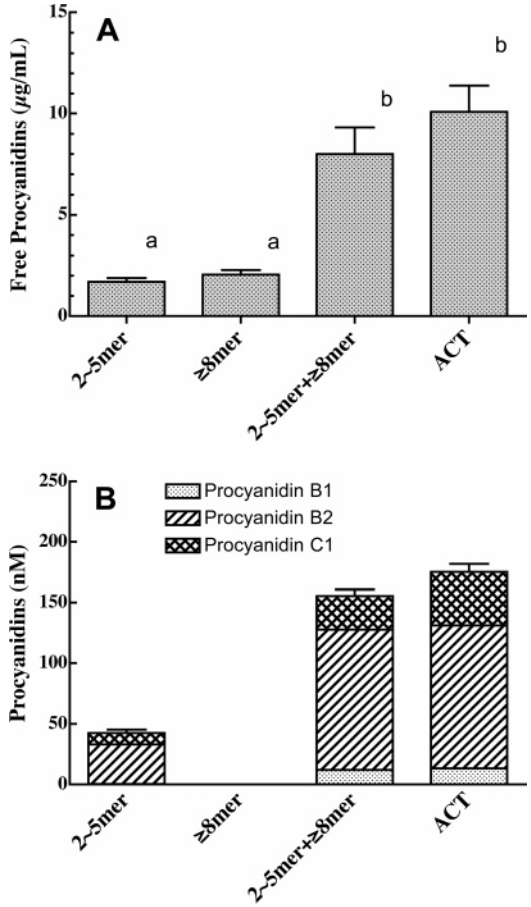

Figure 7. Free nonconjugated procyanidins in rat plasma 2 h after administration of reconstituted procyanidin dimer to pentamer (2–5-mer group, 324 mg/kg), procyanidins with a molecular weight greater than an octamer (≥8-mer group, 432 mg/kg), a mixture of both (2–5-mer plus ≥8-mer group, 756 mg/kg), or apple procyanidins (ACT group, 1000 mg/kg). (**A**) Free procyanidins analyzed using the Porter method and expressed as procyanidin B2. (**B**) Total amounts of procyanidin B1, B2, and C1 analyzed using HPLC/MS/MS. All data are expressed as the mean ± SEM (n = 5–6 per group). Different letters indicate statistically significant differences at $P < 0.01$.

low concentrations of free procyanidins were detected, although there were statistically significant differences in the free procyanidin levels between the separate reconstituted 2−5-mer and ≥8-mer groups and the ACT group ($P < 0.01$). However, the free procyanidin concentrations in the mixture of 2−5-mer and ≥8-mer groups were similar to those of the ACT group. Procyanidin B1, B2, and C1 concentrations in the plasma analyzed using HPLC/MS/MS in the MRM mode are shown in **Figure 7B**. In separated, reconstituted 2−5-mer and ≥8-mer groups, free procyanidins B1, B2, and C1 were not detected. However, the total quantity of procyanidins detected in the mixture of 2−5-mer and ≥8-mer groups was similar to that observed in the ACT group.

DISCUSSION

Proanthocyanidins have been reported to possess some physiological functions in vitro and in vivo studies similar to those of other polyphenols. However, bioavailability of proanthocyanidins remains poorly understood for the several reasons, including the existence of multiple isomers, the lack of pure fractionated samples, the formation of complexes with a range of proteins, and the requirement for sensitive and specific analytical methods for procyanidins in the plasma.

It is difficult to separate proanthocyanidins as natural compounds, particularly according to the degree of polymerization on the large scale, because numbers of polyphenols and proanthocyanidin isomers rise as the degree of polymerization, the combinations, and types of units increase in such mixtures (*41*). Furthermore, most previous studies used samples in which the proanthocyanidin contents were not defined because of the lack of pure standards. Here, we investigated the absorption of ACTs using high-purity samples (**Table 1**) and each procyanidin fraction separated using normal-phase chromatography (*38*).

Proanthocyanidins form complexes with proteins (e.g., salivary protein and bovine serum albumin) (*41*). These interactions are generally reversible and are reinforced by hydrogen bonds between the phenolic groups of the proanthocyanidins and the carbonyl groups of the peptides. If absorbed, the procyanidins can also form complexes with plasma proteins. When ACTs were added to the plasma in the current study, the complex with plasma protein and procyanidins was formed and the recovery rate of procyanidin was relatively low. Thus, to dissolve plasma protein−procyanidin complexes, urea was added to the plasma samples before SPE.

Generally, polyphenols in the plasma were not detectable using HPLC with a UV and electrochemical detectors. Because additional compounds in the plasma such as proteins could interrupt the HPLC analysis and the proanthocyanidin content was relatively small after administration, ^{14}C-labeled proanthocyanidins were used due to sensitivity. As the detection of proanthocyanidins using these techniques was not specific, the results needed to be treated with caution. However, we were able to detect procyanidin in the plasma with high specificity in vivo using the Porter method, which degraded proanthocyanidins to anthocyanidins in boiling water under acidic conditions. In the Porter method, flavan-3-ols and other polyphenols were not measured, and the yield of anthocyanidins generated from proanthocyanidins should increase, as the polymerization degree of proanthocyanidins becomes higher. In contrast, HPLC/MS/MS in the MRM mode by monitoring parent and product ion pairs could detect procyanidin dimers and trimers in the plasma with high specificity and sensitivity. However, a standard with high purity is needed for the analysis and the standard of procyanidin tetramer and pentamer should be purified for the quantification.

Our investigation of the absorption of ACTs in vivo revealed that free procyanidin concentrations in the plasma peaked 2 h after administration and decreased thereafter for up to 24 h according to both the Porter and the HPLC/MS/MS methods (**Figures 2** and **4**). Déprez et al. (*42*) reported that procyanidin dimer B3 and procyanidin trimer C2 were absorbed to a similar extent as (+)-catechin in the human intestinal Caco-2 cell line in vitro. In addition, Baba et al. (*36*) and Sano et al. (*37*) detected procyanidin dimers in rat and human plasma using HPLC/MS. Here, we detected procyanidin trimer C1 in rat plasma in vivo for the first time.

Procyanidin concentrations required a longer period to peak in the rat plasma as compared with those reported for flavan-3-ols and other polyphenols [e.g., 0.5 h for epigallocatechin-3-gallate (*43*), 0.5−1 h for epicatechin (*22*, *23*), and 1 h for cyanidin-3-glucoside (*26*)]. The absorption of these compounds is influenced by their chemical, physical, and biological properties (e.g., chemical structures and partition coefficients). In the case of procyanidins, high molecular weight and the formation of complexes with mucosal proteins significantly influence the absorption rate. In addition, the partition coefficients of compounds in the aqueous and organic phases, which determine the rate of passive diffusion across a membrane, might contribute to the difference of the absorption rate from the gastrointestinal tract. The absorption rates of catechin and epicatechin significantly differed in our study, although they have the same molecular weights and mutual epimers together (**Figure 4**). Baba et al. (*23*) also reported that epicatechin were absorbed more efficiently than catechin and suggested that catechin and epicatechin might be competitively absorbed in the gastrointestinal tract. It was suggested that the difference of chemical structure and partition coefficients was important for the absorption.

Particularly, the absorption of proanthocyanidins seemed to be highly dependent on the degree of polymerization. Therefore, to determine the limits of absorption in this respect, the procyanidins were fractionated according to the degree of polymerization using normal-phase chromatography and were then administered to rats. Unexpectedly, even in the pentamer group, free procyanidins were detected in the plasma using the Porter method (**Figure 5**). Moreover, in each procyanidin dimer to tetramer group, $[M - H]^-$ ion peaks were detected by HPLC/MS at *m*/*z* 577, 865, and 1154, which corresponded to those of the native procyanidins. These results suggest that procyanidins were absorbed from the upper portions of the digestive tract such as the small intestine and were not degraded into compounds with lower molecular weights than the intact procyanidins 2 h after administration. Polymeric proanthocyanidins reported to be degraded to compounds with lower molecular weights by the colonic microflora in vitro (*44*). The degradation of procyanidins by colonic microflora after administration remains to be confirmed in vivo.

$[M - H]^-$ ion peaks at *m*/*z* 591 and 879, which corresponded to methylated procyanidins, were also detected in our study. In a previous report, after the intake of a large dose (2000 mg) of (+)-catechin, free catechin was detected in the plasma after 30 min, traces of methylated catechin were detected after 2 h, and 40% of the urinary catechin was methylated after 8 h (*45*). However, all of the catechins were conjugated and no free catechin was detected after the consumption of a relatively small initial quantity (*46*). These results suggested that the dose determines the primary site of metabolism; large doses are metabolized primarily in the liver, while small doses are metabolized by the intestinal mucosa. Metabolites such as methylated procyanidins, the compounds produced by the actions of microflora, and their bioactivity remain largely unknown. Further studies will be needed to elucidate the metabolite profile of procyanidins resulting from the actions of metabolic enzymes and degradation by microflora.

Generally, the compounds with a low molecular weight are thought to be absorbed more easily in the digestive tract than those with a high molecular weight. However, as the results shown in **Figure 5** did not correspond with this theory, we further investigated the absorption of highly polymerized procyanidins. As shown in **Figure 7**, procyanidins with a high molecular weight (≥8-mer) influenced the absorption of procyanidin oligomers, although they were not absorbed themselves. These procyanidins (e.g., ≥8-mer) bound to mucosal proteins of the digestive tract, thereby allowing the procyanidin oligomers to be absorbed rather than binding to mucosal protein. Sami-Manchado et al. (*47*) reported that when a mixture of grape seed proanthocyanidins was allowed to interact with salivary proteins, the higher proanthocyanidins with an average polymerization degree of 7 precipitated with the proteins, whereas those left in the supernatant were mainly dimers and trimers.

Here, we show that the high molecular weight of procyanidins might also influence the absorption in vivo for the first time.

Scalbert and Williamson (*28*) reported the dietary intake of total polyphenols to be approximately 1 g/day, regardless of the methods used to determine the polyphenol concentration (e.g., HPLC, Folin−Ciocalteu method). However, the daily intake of proanthocyanidins from foods and beverages in various countries could not be estimated because of the lack of commercial pure standards and the specific methods of proanthocyanidins. Recently, Gu et al. (*48*) characterized proanthocyanidins in foods collected in the United States using normal-phase chromatography with HPLC/MS. The precise determination of the proanthocyanidin contents in foods and beverages in different countries will allow the daily intake of proanthocyanidins to be estimated, which should enable the correlation between proanthocyanidins, their physiological functions, and the incidence of disease in epidemiological studies to be elucidated. To clarify this relationship, the bioavailability of proanthocyanidins must also be investigated. We believe that the current study would be useful in resolving this issue.

In conclusion, apple procyanidins oligomers were shown to be absorbed from the digestive tract and were present in the rat plasma. Procyanidins are thought to have some physiological functions in vivo. Our results indicate that procyanidins might be active compounds both in vivo and in vitro.

ABBREVIATIONS USED

ACTs, apple condensed tannins; ANOVA, analysis of variance; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; HPLC/MS, liquid chromatography/mass spectrometry; HPLC/MS/MS, liquid chromatography/tandem mass spectrometry; MRM, multiple reaction monitoring; SEM, standard error of the mean; SPE, solid phase extraction.

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