

Note

Procyanidin B1 Is Detected in Human Serum after Intake of Proanthocyanidin-rich Grape Seed Extract

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To confirm the absorption of proanthocyanidin (PA) into the human body, four healthy adults were administered 2.0 g of PA-rich grape seed extract (GSE). Blood were drawn before intake and 2 h after intake. Through the enzymatic treatment of sulfatase and β -glucuronidase, blood samples were analyzed by HPLC coupled with mass-spectrometry (LC/MS). Procyanidin B1 [epicatechin-(4 β →8)-catechin] was detected in human serum 2 h after intake. Its concentration was 10.6 ± 2.5 nmol/l.

Key words: proanthocyanidin; procyanidin B1; absorption; grape seed extract; human

Proanthocyanidin (PA) is known as condensed tannins, a part of a specific group of polyphenolic compounds, the flavonoids. PA exists as oligomers or polymers of flavan-3-ol such as (+)-catechin and (–)-epicatechin, and has been reported to have a powerful antioxidant activity *in vitro*^{1,2)} and *in vivo*.³⁾ Much research on beneficial effects for human health has also been done.^{4,5)} Although the bioavailabilities of low molecular weight polyphenolic compounds such as catechins, gallic acid, and isoflavones have been reported,^{3,6)} the bioavailabilities of PA are limited, particularly absorption in human has been unclear.⁵⁾ This study is an attempt to identify the absorption of PA orally administered to the human body.

Because grape seed extract (GSE) is one of the richest sources of PA in nature, GSE was used as a PA sample for intake. GSE was provided by Kikkoman Co. (Chiba, Japan). Briefly, the grape seeds (*Vitis vinifera* L.) were washed with water, then extracted with water and ethanol under reflux conditions. The extract was condensed to remove solvents, and the concentrate was spray-dried to yield the GSE as a brown powder. The extract was composed of 89% PA (including 0.9% procyanidin B1 measured by HPLC analysis), 6% monomers, and 5% others. The amount of PA in the GSE was measured by both

a colorimetric assay and HPLC analysis. At first, total flavanols were estimated by the vanillin-HCl method using (+)-catechin as a standard.^{7,8)} Second, the sum of catechins was measured as total monomeric flavanols by HPLC analysis using 8 commercial available catechins as the standard.⁹⁾ Then the amount of PA was obtained by subtracting the amount of the monomers from total flavanols.

Each of four healthy adults was administered 2.0 g of the GSE in hard capsules in the morning of the experimental day. They had not taken any food that morning. The four volunteers, who were recruited under the Helsinki Declaration, were requested not to take any food or drink rich in polyphenols such as red wine, vegetables, fruits, fruit juice, teas, nuts, chocolates, and supplements, from two days before the experimental day. Their blood was collected twice at 0 and 2 h after PA intake. In the previous reports, it was shown that the maximum concentration of phenolic metabolites in blood was 1–4 h after a single intake of red wine.¹⁰⁾ And it was reported that consumption of procyanidin-rich chocolate significantly increased plasma levels of epicatechin 2 h after ingestion.¹¹⁾ Richelle *et al.* also reported a similar time to reach the maximum after chocolate consumption.¹²⁾ From these reports, we decided to draw the blood 2 h after intake. With respect to the dose, because no PA could be detected in serum after 400 mg intake of the GSE in our pre-test (data not shown), 2.0 g of the GSE was administered.

At first, we attempted to analyze PA in serum using electrochemical detection coupled to HPLC (ECD-HPLC).⁶⁾ However, so many and such broad peaks were detected that PA could not be separated from other substances, and the quantitative analysis of PA could not be done by the ECD-HPLC method. Next we tried HPLC coupled with mass-spectrometry (LC/MS), which was more effective to analyze procyanidin in serum as follows. Blood samples were treated as described in previous papers^{3,13)} with slight modifications. In brief, a serum sample (500 μ l) was

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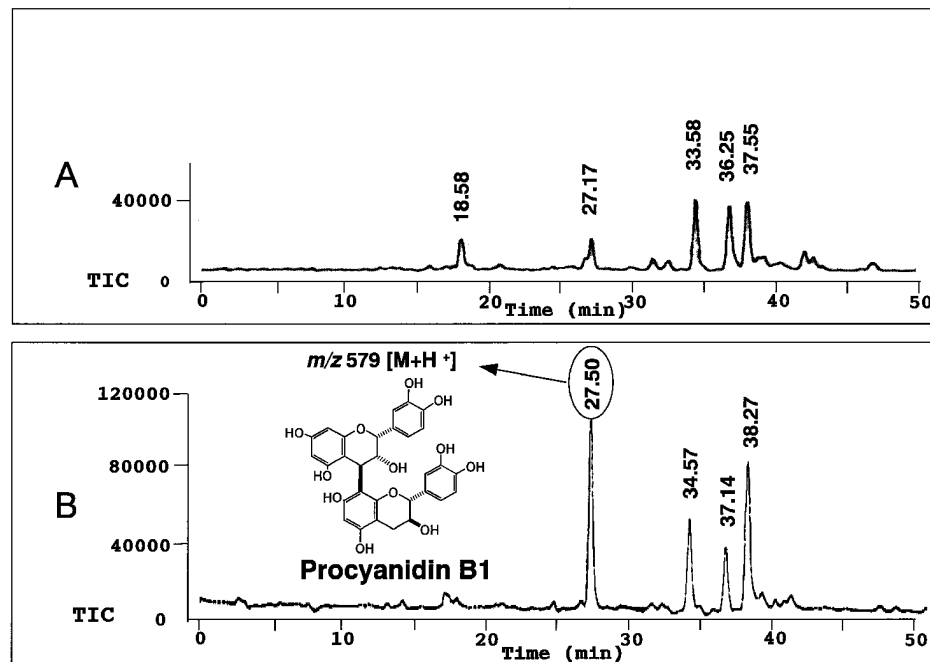


Fig. 1. LC/MS Analysis of PA Dimer of Human Serum before and after Single GSE Intake.

A; a total ion LC/MS chromatogram of a serum before consumption by one male volunteer. B; a total ion LC/MS chromatogram of serum 2 h after 2.0 g of GSE consumption by the same volunteer.

mixed with 1 mg/ml EDTA and 50 μ l of 0.1 M acetate buffer (pH 5.0) containing sulfatase (150 U) and β -glucuronidase (2500 U) (sulfatase type H-5 from Sigma Chemical Co., St. Louis, USA) and incubated at 37°C for 45 min to hydrolyze conjugated metabolites into free forms. After the hydrolysis, a part of the serum (550 μ l) was extracted with 1200 μ l of methanol/formic acid (99.95:0.05, v/v). The mixture was vortexed for 30 s, sonicated for 20 s, vortexed again for 20 s, and centrifuged for 10 min at 2300 \times g at 5°C. The supernatant was filtered using a DISPO COLUMN C18H050 (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The resulted supernatant was analyzed by LC/MS, using a reversed phase column (Wakosil-II 5C18 HG: 2.0 \times 250 mm, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and JMS-LCMATE LCMS system (JEOL Ltd., Tokyo, Japan). The flow rate was 0.2 ml/min. The mobile phase was comprised of two solvent solutions that were mixed according to the detection method used: solvent A; water/formic acid (99.95:0.05, v/v), solvent B; methanol/water/formic acid (90:9.95:0.05, v/v/v). The composition of the mobile phase was first set at 0% B, which was linearly increased to 50% B in 60 min. This was followed by another linear increase to 100% B in 115 min. LC/MS conditions were that the ionizing mode was electro spray ionization (ESI) in the positive ion mass, selected ion monitoring (SIM; m/z 579, 731, and 867), desolving plate 220°C; orifice 170°C; and ring lens voltage 50 V. These selected masses represent procyanidin

dimers (m/z 579), procyanidin dimer gallate esters (m/z 731), and procyanidin trimers (m/z 867), respectively. The chromatograms "A" and "B" represent the sum of the selected three masses (Fig. 1). In chromatogram "A" before the GSE intake, there were several peaks exactly equal to the selected masses. In chromatogram "B" 2 h after the GSE intake, a characteristic peak appeared, that is, the 27.50 min (retention time) peak having m/z 579 [$M+H^+$], which is the mass of a procyanidin dimer, obviously increased after consumption of the GSE.

In order to confirm that the increased peak is that of the procyanidin dimer, we added commercial available procyanidin dimer (procyanidin B1 [epicatechin-(4 β →8)-catechin]; Funakoshi Co., Ltd., Tokyo, Japan) to the serum drawn after and before the GSE intake, and analyzed the mixture by the LC/MS method (the mass m/z 579 [$M+H^+$]). The peak detected in chromatogram "B" was coincided with authentic procyanidin B1 on the retention time and the mass m/z 579 [$M+H^+$]. It was strongly suggested that the increased peak in human blood is procyanidin B1. In quantitative analysis by the LC/MS method using authentic procyanidin B1 as a standard, the significantly increased concentration of B1 in blood which was drawn 2 h after single intake of the GSE was 10.6 ± 2.5 nmol/l (mean \pm SD, student-*t* test, $n=4$, $p<0.01$).

The content of procyanidin B1 in the GSE was confirmed as follows. Twenty grams of the GSE was chromatographed on Sephadex LH-20 (Amersham

Biosciences Co., NJ, USA) and C18 silica gel (YMC Co., Ltd., Kyoto, Japan), then the desired fractions were mixed, condensed, and freeze-dried to give 0.183 g (y. 0.9%) of pale brown powder (procyanidin B1). NMR data (^1H , ^{13}C , COSY, HMQC, HMBC, NOESY, Bruker AVANCE 500) of the powder agreed with that of the authentic sample. The melting point (210–230°C-dec.) also agreed with the value from a previous report.¹⁴⁾

The gain of a 38.27 min peak (retention time) in chromatogram "B" also suggested the increment of other procyanidin dimers having the same mass m/z 579 $[\text{M} + \text{H}^+]$ after intake of the GSE. However, the peak did not agree with any authentic procyanidin dimer samples in the LC/MS analysis.

From these results, absorption of procyanidin B1 in the GSE into the human body was strongly suggested. This shows the possibility that other PA can also be absorbed.

In past studies on absorption of catechins, intake of a single dose of 230 mg of epicatechins (epicatechin, epigallocatechin, epigallocatechin gallate) from a green tea extract led to a rise of the concentrations of catechins in plasma of 0.6–1.6 $\mu\text{mol/l}$.¹⁵⁾ A study about consumption of black chocolate containing 82 mg of epicatechin and PA demonstrated an elevation of the concentration of the epicatechin to 0.38 $\mu\text{mol/l}$, 2 h after a single intake of plasma.¹²⁾ The serum concentration of procyanidin B1 in this report was 0.0106 $\mu\text{mol/l}$ after intake of 2.0 g GSE, which contains 18 mg of procyanidin B1. Although the amount of procyanidin B1 detected in plasma was not as much as for reported catechins, the ratio of absorption by dose may be similar to them, if all detected B1 would be arisen from B1 in the GSE, not from degradation products of polymer PA.

Recently, absorption of procyanidin B2 in rats has been reported.¹⁶⁾ But our finding that procyanidin B1 is detected in human serum after oral intake of the GSE, is the first work showing that there is every chance of demonstrating the absorption of PA into the human body. We are trying to analyze other procyanidins further. Serum or plasma kinetics and total absorption of procyanidin B1 or PA into the human body also remain for the future.

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