Absorption and metabolism of cyanidin 3-*O*-β-D-glucoside in rats

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Abstract We have clarified for the first time how cyanidin 3-*O*-β-D-glucoside (C3G), which is a potent antioxidant anthocyanin, is absorbed and metabolized in vivo. Rats were orally administered C3G (0.9 mmol/kg body weight), and C3G rapidly appeared in the plasma. However, the aglycon of C3G (cyanidin; Cy) was not detected, although it was present in the jejunum. Protocatechuic acid (PC), which may be produced by degradation of Cy, was present in the plasma and the concentration was 8-fold higher than that of C3G. These results suggest that plasma PC and C3G may contribute to the antioxidant activity of the plasma. In the liver and kidney, C3G was metabolized to methylated C3G (methyl-C3G), suggesting that C3G and/or methyl-C3G act as antioxidants in the tissues.

Key words: Anthocyanin; Cyanidin 3-*O*-β-D-glucoside; Antioxidant; Absorption; Metabolism

1. Introduction

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. They are widely distributed in the human diet through crops, beans, fruits, vegetables and red wines [1], suggesting that we ingest considerable amounts of anthocyanins from plant-based daily diets. The average intake of flavonoids by humans was estimated to range from 25 mg/day to 1 g/day [2,3], however, the intake of the anthocyanins has not been estimated.

There is now an increasing interest in the in vivo protective function of natural antioxidants contained in dietary plants against oxidative damage caused by free radical species [4,5]. The antioxidant activity of phenolic phytochemicals has been widely investigated [6,7]. Anthocyanins, which are included in the phenolic phytochemicals, are stable under acidic conditions but not stable and rapidly broken down under neutral condition [8]. From this background, anthocyanins have not been recognized as a physiological antioxidant [8]. However, we demonstrated in an in vitro study that anthocyanins have antioxidative activity under physiological conditions [9,10]. Among the anthocyanins tested, cyanidin 3-*O*-β-D-glucoside (C3G) (Fig. 1) had a significant antioxidative activity [11]. We recently reported in an in vivo study using rats that C3G feeding lowered the serum thiobarbituric acid-reactive substance (TBARS) concentration, and increased the oxidation resistance of the serum against lipid peroxidation [12].

Recent reports showed that phenolic compounds contained in red wine may play an important role as inhibitors of low-density lipoprotein (LDL) oxidation [13]. Red wine contains a large amount of anthocyanins, such as C3G [14], indicating that these anthocyanin pigments may contribute to the inhibitory effect of the oxidation of LDL. However, it has not been clarified how anthocyanins are metabolized in vivo.

We also reported that C3G was able to react with peroxyl radicals, and then convert itself in vitro to the oxidation products 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran and protocatechuic acid (PC) (Fig. 1) [15]. PC can scavenge the radicals, suggesting that C3G would produce another radical scavenger by its reaction with biological radicals in vivo. However, it has not been clarified that these oxidation reactions of C3G occur in vivo, and how C3G is effectively absorbed, distributed, and metabolized in some tissues.

The purpose of this study was to demonstrate the absorption, distribution, and metabolism of C3G in rats, and clarify the mechanism for the antioxidant activity of C3G in vivo.

2. Materials and Methods

2.1. Chemicals

β-Glucuronidase from *Ampullaria* was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Authentic PC was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Authentic cyanidin (Cy) and peonidin 3-*O*-β-D-glucoside were obtained from Extrasynthèse (Genay, France). Sulfatase (type VIII) was purchased from Sigma Chemical (St. Louis, MO, USA). C3G (purity more than 96%) was purified using the commercial food coloring material (San Red No. 5F, San-Ei Gen F.F.I., Inc., Osaka, Japan) derived from purple corn (*Zea mays* L.) using previously described high-performance liquid chromatography (HPLC) [15]. The structure and purity were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance, fast atom bombardment mass spectrometry (FAB-MS), UV-VIS, and infrared spectral data before use.

2.2. Animals and diets

Eighteen 7-week-old male Wistar rats (Japan SLC Inc., Hamamatsu, Japan; ~170 g) were used, and individually housed in stainless-steel wire-mesh cages at 23 ± 0.3°C with a 12-h light cycle. The rats were allowed free access to tap water and a semi-purified diet. All animals were fed the control diet containing (g/kg diet) casein, 250; mineral mixture (AIN93G-MX) [16], 35; vitamin mixture (AIN93-VX) [16], 10; choline chloride, 2; corn oil, 50; cellulose powder, 40; and the remainder carbohydrate, 613 (sucrose:starch = 1:2) for 7 days before the experiment. Rats were maintained in accordance with the Guidelines for Animal Experimentation of Nagoya University.

2.3. Experimental design and tissue preparation

After 7 days of feeding the control diet, food was withheld for 24 h, then C3G (0.9 mmol/kg body weight) dissolved in 0.1% citric acid was orally administered to 15 rats by direct stomach intubation. The rats were killed 15, 30, 60, 120, or 240 min after the administration by withdrawing blood by heart puncture using heparinized needles and syringes under anesthesia with diethyl ether. The plasma was imme-

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Abbreviations: C3G, cyanidin 3-*O*-β-D-glucoside; COMT, catechol-*O*-methyltransferase; Cy, cyanidin; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; PC, protocatechuic acid; TFA, trifluoroacetic acid; UV, ultraviolet; VIS, visible

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diately obtained from the collected blood by centrifugation at 1600×*g* for 15 min at 4°C. The plasma separation was finished within 30 min. An aliquot of the plasma was acidified with one-tenth volume of phosphoric acid and stored at −80°C until use. The liver and kidneys were rapidly excised after being perfused with ice-cold physiological saline. The stomach and jejunum were washed with ice-cold physiological saline to remove any C3G that adhered to the surface of the mucosa. The tissue samples were kept at −80°C until use.

2.4. Determination of C3G and its metabolites in the plasma

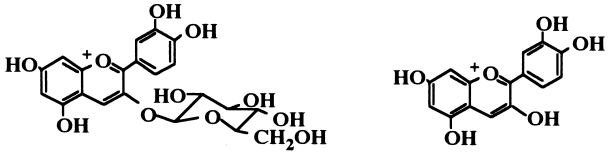
The plasma (400 μl) was mixed with an equal volume of 10 mM oxalic acid, and the mixtures were subjected to Sep-Pak C_{18} cartridges (Waters, USA) [17]. After washing with 10 mM oxalic acid, C3G and the metabolites were eluted with methanol containing 1% trifluoroacetic acid (TFA). The eluent was carefully evaporated to dryness in vacuo below 35°C. The dried extract was dissolved using 100 μl of 15% acetonitrile in water containing 50 mM sodium phosphate (pH 1.7). An aliquot (20 μl) of this solution was injected into an HPLC system. HPLC was carried out on a Develosil ODS-HG-5 column (Nomura Chemical Co. Ltd., Aichi, Japan, 4.6×250 mm) using 15% acetonitrile in water containing 50 mM sodium phosphate (pH 1.7) for C3G and PC or 20% acetonitrile in water for Cy as a solvent at a flow rate of 1.0 ml/min, with a photodiode array detector (MD-910, Jasco, Tokyo, Japan). The recovery of the method has been checked (>88%) using authentic C3G and Cy and PC.

2.5. Determination of C3G and its metabolites in the tissues

Frozen tissue samples (0.5 g) were homogenized in 4 volumes of 0.4 M sodium phosphate buffer (pH 3.9) containing 0.1% EDTA using a Potter-Elvehjem homogenizer. An aliquot (400 μl) of homogenate was mixed with 2.0 ml of acetone containing 0.1% TFA and centrifuged at 3000×*g* for 5 min at 4°C. The supernatant was collected and 20 μl of TFA was added. The extract was carefully evaporated to dryness in vacuo below 35°C. The dried extract was dissolved in 100 μl of methanol containing 1% TFA. An aliquot (20 μl) of this solution was analyzed by HPLC using 18% or 12% acetonitrile in water containing 50 mM sodium phosphate (pH 1.7) as the solvent.

2.6. Identification of C3G and its metabolites

The identification of the C3G metabolite in the plasma was performed by UV-VIS spectrometry using the photodiode array detector, retention time and HPLC-MS. For HPLC-MS, the plasma samples were applied to the HPLC system, which was connected to a mass spectrometer (PLATFORM II, Fisons Instruments, UK), on a Develosil ODS-HG-5 column (4.6×250 mm) using 15% acetonitrile containing 0.01% acetic acid as the solvent. The electrospray ionization (positive) mode was used for the detection. The identification of C3G and its metabolites in the plasma, liver and kidneys was performed by UV-VIS spectrum using the photodiode array detector, retention time and FAB-MS. For the FAB-MS, C3G and the metabolite were isolated using preparative HPLC with a Develosil ODS-HG-5 column (4.6×250 mm) monitored by a spectrophotometric detector (530 nm). The solvent was 18% acetonitrile containing 0.1% TFA. The isolated samples were applied to FAB-MS using a JEOL JMS-DX-705L (JEOL, Tokyo, Japan).



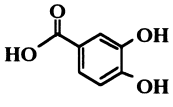


Fig. 1. Chemical structures of C3G, Cy, and PC.

2.7. Determination of conjugated C3G and its metabolites in the plasma and tissues

The plasma or tissue homogenate (400 μl) was mixed with β-glucuronidase (550 U/ml) and sulfatase (25 U/ml) in 1 M acetate buffer (pH 4.5) and incubated at 37°C for 20 min. The reaction mixture was centrifuged at 8000×*g* for 10 min at 4°C and the supernatant was analyzed by HPLC according to the determination procedure of C3G and its metabolites as already described.

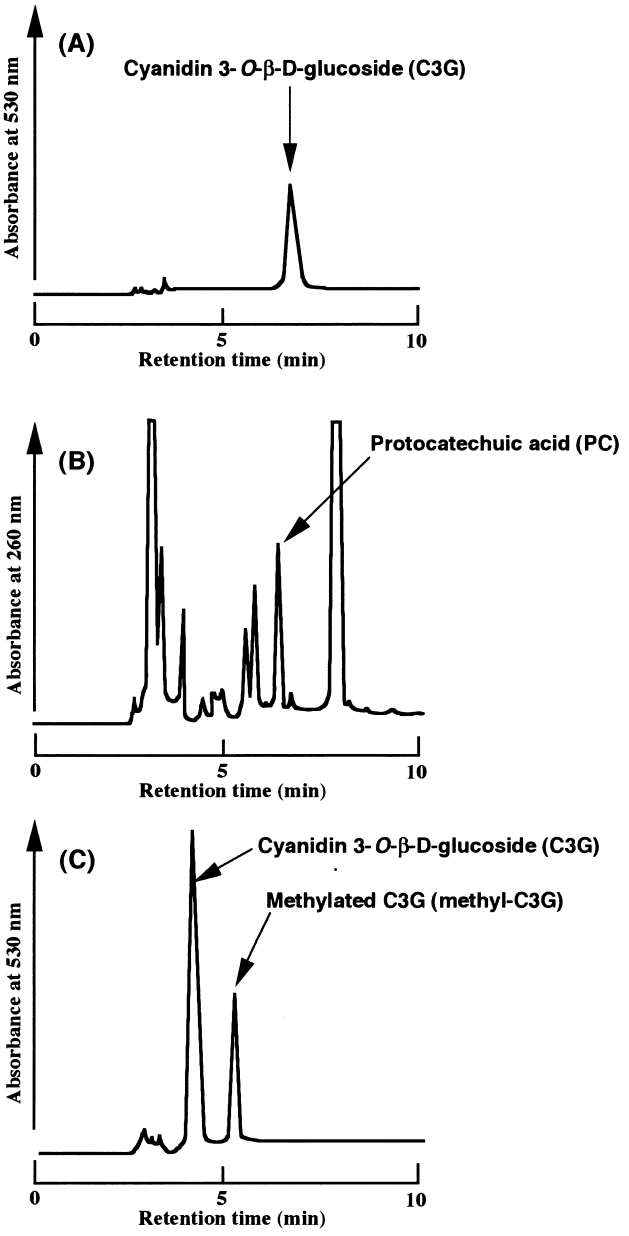


Fig. 2. Typical HPLC chromatogram of rat plasma and kidney extracts after oral administration of C3G. A: HPLC chromatogram of rat plasma extracts at 30 min detected at 530 nm using 15% acetonitrile in water containing 50 mM sodium phosphate (pH 1.7) as the solvent. B: HPLC chromatogram of rat plasma extracts at 60 min detected at 260 nm using 15% acetonitrile in water containing 50 mM sodium phosphate (pH 1.7) as the solvent. C: HPLC chromatogram of rat kidney extracts at 30 min detected at 530 nm using 18% acetonitrile in water containing 50 mM sodium phosphate (pH 1.7) as the solvent.

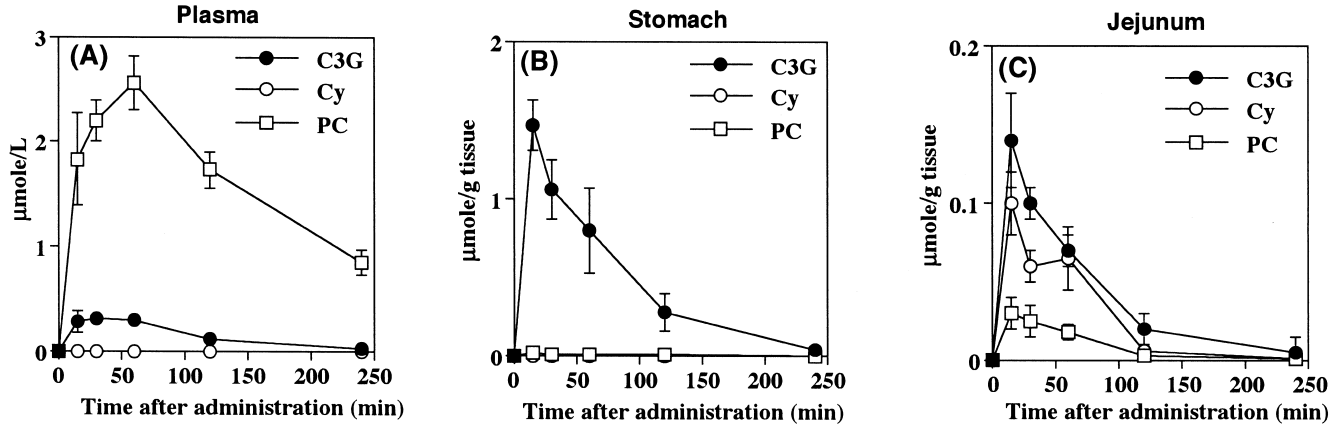


Fig. 3. C3G, Cy, and PC concentrations in rat plasma (A), stomach (B) and jejunum (C) after oral administration of C3G. Values are means ± S.E.M., $n = 3$.

3. Results and discussion

3.1. C3G and its metabolites in the plasma

The HPLC chromatogram of the plasma obtained from rats administered C3G showed the peak which was assumed to be anthocyanins (Fig. 2A). The peak was identified as C3G by comparison with the authentic compound based on the retention time in the HPLC analysis, UV-VIS spectrum, and FAB-MS. The plasma C3G concentration reached a maximum at 30 min after the administration (0.31 ± 0.03 μmol/l), and began to fall from 60 min (Fig. 3A). These results demonstrate that C3G is absorbed and rapidly enters the circulatory system as a glucoside. However, Cy, which is the aglycon of C3G (Fig. 1), was not detected in the plasma during 4 h after the administration.

The HPLC chromatogram of the plasma also showed a peak for a compound assumed to be a metabolite of C3G (Fig. 2B). The metabolite was identified as PC (Fig. 1) by comparison with the authentic compound based on retention time in the HPLC analysis, UV spectrum and HPLC-MS. The plasma PC concentration reached a maximum at 60 min (2.56 ± 0.26 μmol/l), and it is noteworthy that the concentration was 8 times that of C3G (Fig. 3A). These results suggest that plasma PC rather than C3G contributes to the increase in oxidation resistance of the serum obtained in our previous study [12].

The enzymatic treatment of the plasma using β-glucuronidase and sulfatase did not cause the appearance of additional C3G, Cy and PC (data not shown). It seems that these compounds in the plasma are neither conjugated glucuronides nor sulfates.

3.2. C3G and its metabolites in the tissue

Fig. 3B,C shows the time course of the concentrations of C3G and its metabolites in the stomach and jejunum after the administration of C3G. C3G was detected in the stomach throughout the 4-h period and the concentration reached a maximum at 15 min (Fig. 3B). On the other hand, Cy and PC were not detected throughout the 4-h period after the administration of C3G.

In the jejunum, C3G, Cy and PC were detected throughout the 4-h period (Fig. 3C). It must be emphasized that Cy was detected in the jejunum, although it was not detected in the plasma. Cy would be produced from C3G by β-glucosidase reactions in the intestines.

PC was also detected in the jejunum throughout the 4-h period after administration (Fig. 3C). Although the data are not shown, PC was produced by the degradation of Cy when

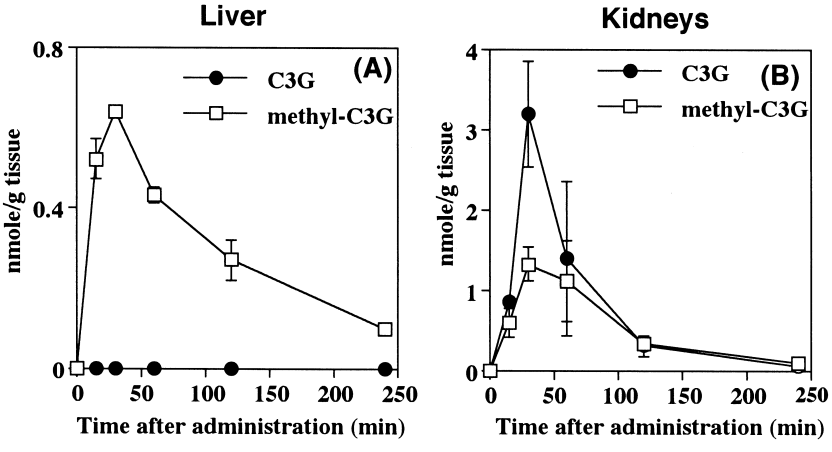


Fig. 4. C3G and methyl-C3G concentrations in rat liver (A) and kidneys (B) after oral administration of C3G. Values are means ± S.E.M., $n = 3$.

Cy was incubated with rat plasma for 15 min. It was also produced from C3G, but more than 16 h was required for its formation. Therefore, the accumulation of PC in the plasma could be derived from the degradation of Cy, but not C3G. There are two possibilities that explain the formation of PC from Cy. One is that PC produced from Cy in the intestine is absorbed and accumulated in the plasma. The other is that Cy is absorbed and degraded due to its instability in the plasma, resulting in the appearance of PC. Although it remains to be clarified which one is correct, the concentration of PC in the plasma is much higher than that of C3G or Cy throughout the 4-h period after the administration of C3G.

Fig. 4 shows the time course of C3G and its metabolite concentrations in the liver (Fig. 4A) and kidneys (Fig. 4B) after the administration of C3G. Cy and PC were not detected in the liver and kidneys throughout the 4-h period after the C3G administration. C3G was clearly detected in the kidneys (Fig. 2C), and its concentration reached a maximum at 30 min (3.20 ± 0.66 nmol/g tissue), but it was not detected in the liver. This result suggests that C3G in the plasma is efficiently transferred into the kidney tissue compared to the liver.

The HPLC analysis showed that a peak for the compound assumed to be an anthocyanin was detected in the liver and kidneys (Fig. 2C). The peak was identified as C3G methylated at the 3′ or 4′ hydroxyl moiety position (methyl-C3G) by comparison with the authentic compound as peonidin 3-*O*-β-D-glucoside based on the retention time in the HPLC analysis, UV-VIS spectrum, and FAB-MS. Methyl-C3G was not detected in the plasma suggesting that it may be formed and retained in the tissues. The concentration reached a maximum at 30 min in the liver and kidneys, and the concentration was 2-fold higher in the kidneys (1.32 ± 0.21 nmol/g tissue) (Fig. 4B) than in the liver (0.64 ± 0.01 nmol/g tissue) (Fig. 4A). It is speculated that C3G is distributed to the liver after absorption in the intestine, and most C3G in the liver is methylated at its catechol group by catechol-*O*-methyltransferase (COMT). C3G was also efficiently distributed to the kidneys, and probably methylated by COMT. The concentration of methyl-C3G in the kidneys was higher than that in the liver. As the concentration of C3G in the kidney was also significantly high, dietary C3G may selectively improve the antioxidant activity of the kidneys.

In conclusion, we show here for the first time how anthocyanin is absorbed and metabolized in vivo. After the administration of C3G, it is hydrolyzed by β-glucosidase and chemically changed in the intestines. In the plasma, C3G itself and PC are present, but Cy is not detected. Cy is unstable under physiological conditions and degraded to PC, even if Cy were absorbed into the circulatory system. It is known that PC has a protective potency in carcinogenesis [18]. C3G feeding is expected to improve the antioxidant capability and chemically prevent carcinogenesis in vivo. In tissues (liver and kidneys), methyl-C3G and/or C3G are mainly present, and these compounds may contribute to tissue antioxidant activity. In addition, glucuronides and sulfates of C3G and its metabolites were not detected in the plasma and tissues tested. This observation is completely different with respect to the other flavonoids. The fact that C3G mainly exists as a free form would be of benefit to its antioxidant activity.

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