The Influence of Rhein 8-O-β-D-Glucopyranoside on the Purgative Action of Sennoside A from Rhubarb in Mice

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Rhubarb is one of the most well-known herbal medicines that constitute daiokanzoto (DKT), which is clinically effective for constipation. Sennoside A is transformed into an active metabolite, rheinanthrone, by intestinal bacteria. Sennoside A in rhubarb showed significantly accelerated metabolic activity in intestinal bacteria in comparison with sennoside A alone. In this study, we investigated the influence of rhubarb constituents on the metabolism and purgative activity of sennoside A. The 20% MeOH-eluted fraction separated by MCI-gel CHP-20P column chromatography from the water extract of rhubarb showed sennoside A metabolic activity similar to that of rhubarb extract. The 20% MeOH elute was further purified and rhein 8-O-β-D-glucopyranoside (RG) was isolated. The metabolic activity of sennoside A was significantly accelerated by increasing the level of RG. Moreover, rhein, emodin and aloe-emodin also accelerated sennoside A metabolism. The purgative activity of sennoside A was significantly accelerated when RG or rhein was concomitantly given with sennoside A in a dose-dependent manner. These results suggest that anthraquinones contribute to the purgative action of sennoside A in rhubarb. Therefore, it is assumed that the influence of anthraquinones on the fate of rheinanthrone transformed from sennoside A may promote the purgative action of sennoside A.

Key words rhubarb; sennoside A; rhein 8-O-β-D-glucopyranoside; anthraquinone; daiokanzoto

Kampo is traditional Japanese medicine that was developed from traditional medicine originating in ancient China. Kampo medicine involves drugs based on multiple constituents, for which prescriptions are based on the combination of two or more herbal medicines. It is thought that the interaction between each herbal medicine is important for their activity, such as by reinforcing the effect of a medicine and relieving side effects, by pharmacologic or pharmacokinetic action. For example, calcium sulfate from gypsum raises ephedrine alkaloid dissolution in makyokansekito decoction. A lower glycyrrhizin content in shoseiryuto decoction is attributable to the pH of organic acids in schisandra fruit. In addition, the purgative activity increases 1.7-fold compared with that with each medication alone when the mixture ratio of sennosides A and C is 7 : 3.

Rhubarb is one of the most well-known herbal medicines that constitute daiokanzoto (DKT), which was demonstrated to be useful for constipation in a clinical double-blind study. The purgative effect of rhubarb is due to sennoside A as the main laxative constituent. Sennoside A is an inactive glycoside, and is transformed to an active metabolite, rheinanthrone, by intestinal bacteria. We have reported an HPLC method for the determination of sennoside A. This method is applicable for determination of the activity of sennoside A. In this study, we investigated the influence of rhubarb constituents on the metabolism and purgative activity of sennoside A. The 20% MeOH-eluted fraction separated by MCI-gel CHP-20P column chromatography from the water extract of rhubarb showed sennoside A metabolic activity similar to that of rhubarb extract. The 20% MeOH elute was further purified and rhein 8-O-β-D-glucopyranoside (RG) was isolated. The metabolic activity of sennoside A was significantly accelerated by increasing the level of RG. Moreover, rhein, emodin and aloe-emodin also accelerated sennoside A metabolism. The purgative activity of sennoside A was significantly accelerated when RG or rhein was concomitantly given with sennoside A in a dose-dependent manner. These results suggest that anthraquinones contribute to the purgative action of sennoside A in rhubarb. Therefore, it is assumed that the influence of anthraquinones on the fate of rheinanthrone transformed from sennoside A may promote the purgative action of sennoside A. The present study was undertaken to examine the active component in rhubarb that affects the purgative activity of sennoside A.

MATERIALS AND METHODS

Materials Rhubarb (kinnon-dao) was purchased from Tochimototenkaido (Osaka, Japan). Sennoside A and aloe-emodin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Rhein, emodin and chrysophanol were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Ultrapure distilled water was prepared with deionized-distilled water. All other chemicals were analytical reagent- or HPLC-grade commercial products.

Purification of Rhein 8-O-β-D-Glucopyranoside (RG) Chopped dried rhizomes of rhubarb (100 g) were extracted three times with water (1 L) under reflux for 30 min. The combined extract was subjected to MCI-gel CHP-20P column chromatography (CC) (75–150 µm, Mitsubishi Chemical Industries) to give H2O-eluated (24.75 g), 20% MeOH-eluated (5.52 g), 40% MeOH-eluated (9.33 g), 60% MeOH-eluated (6.70 g) and MeOH-eluated fractions (3.19 g). The 20% MeOH-eluated fraction was subjected to MCI-gel CHP-20P CC eluting with 20% MeOH and Sephadex LH-20 CC (25–100 µm, GE Healthcare Life Sciences) eluting with H2O to give RG (69.7 mg) as orange-colored needle crystals. 1H- and 13C-NMR spectra were recorded at 35°C on a JEOL JMN-LA500 (Tokyo, Japan) operating at 500 MHz using a 5 mmø sample tube. Hexadeuterodimethylsulfoxide (DMSO-d6) was used as a solvent. Chemical shift values are expressed in ppm downfield using tetramethylsilane (TMS) as an internal standard. RG: mp 262–265°C. 1H-NMR (DMSO-d6) δ: 3.15–3.39 (1H, m, H-3'), 3.15–3.39 (1H, m, H-4'), 3.40–3.58 (1H, m, H-2'), 3.40–3.58 (1H, m, H-5'), 3.40–3.58 (1H, m, H-6'), 3.72 (1H, dd, J2,3'=11.6 Hz, H-6'), 5.18 (1H, d, J1,2'=7.6 Hz, H-1'), 7.73

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shown in Fig. 1 by comparison of 1H- and 13C-NMR, HMBC, and H-6 correlations were of H-1 with C-11, H-7 with C-6 and C-8, H-1′ with C-8, C-3′ and C-5′, and H-3′ with C-4′ and C-5′. The 1H–1H correlation spectroscopy (COSY) correlations were of H-1′ with H-2′, H-5′ with H-6′, and H-6′ with H-6′. The structure of RG was identified as shown in Fig. 1 by comparison of 1H- and 13C-NMR, HMBC, 1H–1H COSY spectral data and the melting point measurement with those reported in the literature. 11,12)

Animal Preparation Animal experiments were all carried out in accordance with the Guidelines for Animal Experimentation of Fukuyama University. Male ddY mice, weighing 30–40 g, were obtained from SHIMIZU Laboratory Supplies (Kyoto, Japan) and housed in a 12 h light–dark cycle at 21 to 23°C for at least one week before the experiments. They were given free access to food and water before experiments.

Chromatographic Condition The HPLC apparatus was an Agilent Technologies 1100 Series system (Waldbronn, Germany) consisting of a binary pump, an autosampler, a thermostatted column compartment and a photodiode array (PDA) detector. All modules and data collection were controlled by Agilent ChemStation software. The column was a TSKgel ODS-80TsQA reversed-phase column, particle size of packing 5 μm, 150×4.6 mm i.d. ( Tosoh, Tokyo, Japan). The mobile phase was a gradient system with 200 μL·L⁻¹ (ca. 0.017%) phosphoric acid in water (A) and acetonitrile (B), which were deaerated by sonication before use. The elution program was performed from 19 to 20% B for 3 min, 20 to 21% B for 5 min, a stepwise increase to 43% B for 8 min and a stepwise increase to 90% B for 5 min to wash. A re-equilibration period of 12 min was used between individual runs. The flow rate was 1.0 mL·min⁻¹. The injection volume was 5 μL. The column temperature was 50°C. The detection wavelength was set at 265 nm for determination and in the range of 200 to 500 nm for validation of peak purity.

Assay of Activity of Sennoside A Metabolism by Mouse Intestinal Flora Fresh feces obtained from mice were homogenized in 20 volumes of 0.01 M potassium phosphate buffer (pH 7.4) by bubbling with CO₂ gas to eliminate air, and the sediments were removed by filtration through gauze. A fecal suspension was incubated at 37°C for 24 h under anaerobic conditions. Anaerobic procedures were carried out using an anaerobic jar with an AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan). Sennoside A was present at 0.5 mg/mL in rhubarb extract. Sennoside A (0.2 mM), rhubarb extract (17 mg/mL), H₂O Fr. (8.5 mg/mL), 20% MeOH Fr. (1.9 mg/mL), 40% MeOH Fr. (3.2 mg/mL), 60% MeOH Fr. (2.3 mg/mL) and MeOH Fr. (1.1 mg/mL) were prepared with 0.01 M potassium phosphate buffer. The concentrations of these fractions were decided on the basis of the dried weight of each fraction. RG, rhein, emodin and aloe-emodin were prepared with 0.5% sodium hydrogen carbonate. The assay samples were mixed with sennoside A. Tubes containing the assay samples (0.25 mL) and the fecal suspension (1 mL) were incubated at 37°C for 4 h under anaerobic conditions. The reaction was immediately stopped by adding 0.425% v/v phosphoric acid in methanol (1.25 mL). After centrifugation at 1500 g for 5 min, the supernatant was passed through Minisart RC 15 (Japan Sartorius, Tokyo, Japan) and subjected to HPLC. The control process involved an incubation mixture at 0 min, as mentioned above. Metabolic ratio was calculated by the percentage of the content of sennoside A in the incubation mixture compared with that in the control.

The Purgative Action of Sennoside A When Adding Rhein 8-O-β-D-Glucopyranoside or Rhein Mice were isolated in a wire-bottomed cage covered with a beaker (11×15 cm), which was placed on blotting paper. The condition of feces was observed 1 h before administration of each sample, and only the mice that excreted normal feces were used. Sennoside A (15 mg/kg) was prepared with 0.01 M potassium phosphate buffer. RG at doses of 2.1, 4.2, 8.4 and 16.8 mg/kg and rhein at doses of 1.3, 2.7, 5.4 and 10.7 mg/kg were prepared with 0.5% sodium hydrogen carbonate. These were mixed and orally administered to mice in a single dose. After the oral administration of sample, the condition of feces was observed at intervals of 1 h for 10 h. The feces in the worst condition was graded into three consistency levels as follows: 0: normal, 1: soft and 2: unformed. 13) The feces score was the mean value for the total consistency level of every hour in each mouse.

Statistical Analyses Data are shown as the mean±S.D. Statistical comparisons between two groups were made using unpaired t test (KyPlot, Kyence Inc.). To compare more than two groups, Dunnett’s test or Steel’s test (KyPlot, Kyence Inc.) was used. A probability value of p<0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Purification of RG as Rhubarb Constituent Affecting Sennoside A Metabolism DKT is mainly used as a laxative with a Kampo medicine that consists of two herbal medicines, rhubarb and glycyr rhiza. Sennoside A, which is the major purgative active constituent of rhubarb, is metabolized by intestinal bacteria and shows purgative activity. Many constituents besides sennoside A that show mild purgative activity are contained in rhubarb. 14) However, the influence that those constituents present in rhubarb have on the metabolism of sennoside A by intestinal bacteria has not been reported. Therefore,
in order to investigate the influence of rhubarb constituents on sennoside A metabolism, the metabolic ratio of sennoside A in rhubarb was compared with that of sennoside A alone. As shown in Fig. 2, the metabolic ratio of sennoside A in rhubarb was significantly accelerated compared with that of sennoside A alone. That is, the existence of a constituent in rhubarb that accelerates sennoside A metabolism was suggested.

In order to investigate which constituent of rhubarb is associated with sennoside A metabolism, rhubarb water extract was subjected to MCI-gel CHP-20P CC using stepwise gradient elution with H₂O–MeOH. The effect of these fractions on the metabolic activity of sennoside A was tested. The fraction of 20% MeOH and MeOH eluate showed sennoside A metabolic activity similar to that of rhubarb extract, as shown in Fig. 3.

The HPLC profile of the 20% MeOH-eluted fraction is shown in Fig. 4. RG was isolated as a major peak in the 20% MeOH-eluted fraction, and the structure of RG was identified on the basis of spectroscopic data of ¹H- and ¹³C-NMR, HMBC and ¹H–¹H COSY and the comparison of spectral data with those in the literature. For the MeOH-eluted fraction, it was identified by HPLC that rhein, emodin and aloe-emodin were present as major constituents. The rhubarb water extract contained RG, rhein, emodin and aloe-emodin at rates of 5.9, 1.2, 0.7 and 0.3 compared with sennoside A.

**Effect of RG and Rhein on Activity of Sennoside A Metabolism** To examine the effect of RG, investigation of sennoside A metabolism upon addition of RG was carried out. In Fig. 5, the metabolic ratio of sennoside A is shown. The metabolic activity of sennoside A was significantly accelerated by increasing the level of RG. Therefore, the constituent of RG that affected this metabolic activity of sennoside A was examined. RG is known as one of the anthraquinone glycosides of rhubarb and is transformed to rhein, which is abundant in rhubarb, by intestinal bacteria. The metabolic ratio of sennoside A is also significantly accelerated by increasing the level of rhein. These results indicated that the metabolism of sennoside A was accelerated by the anthraquinone part of RG.

**Effect of Anthraquinones on Activity of Sennoside A Metabolism** The major constituents of rhubarb are anthraquinones. One of the representatives of anthraquinones is rhein. It was identified by HPLC that the MeOH-eluted fraction contained emodin and aloe-emodin, which are also
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anthraquinones, as major constituents along with rhein. The metabolic activity of sennoside A was significantly accelerated by increasing the levels of emodin and aloe-emodin, which were similar in potency to rhein, as shown in Fig. 6. These anthraquinones contributed as active substances in rhubarb, and promoted the metabolism of sennoside A. In rhubarb, emodin and aloe-emodin are contained at 0.13 mM and 0.06 mM, respectively, along with sennoside A (0.2 mM), and are active in its metabolism.

**Effect of RG and Rhein on Purgative Action of Sennoside A**

We demonstrated that the metabolic activity of sennoside A in intestinal bacteria was significantly accelerated when RG and rhein coexisted with sennoside A. The result of the sennoside A metabolism experiment suggested that these constituents contribute to the purgative action of sennoside A. In order to clarify which constituent of rhubarb is associated with the purgative action of sennoside A, the feces score of sennoside A with RG or rhein was compared with that of sennoside A alone. Various constituents besides sennoside A for which purgative activity has been shown are present in rhubarb, and RG and rhein also have mild purgative activity. The purgative activities of RG (4.2–66.8 mg/kg) and rhein (1.3–10.7 mg/kg) were observed over 10 h. The purgative score of RG was about 1.0 at the doses of 33.4 and 66.8 mg/kg, and its purgative activity was not observed at doses less than 16.8 mg/kg. Therefore, the dose of RG was set at 16.8 mg/kg or less. Rhein did not show purgative activity in these dose ranges. Figure 7 shows the mean feces scores of sennoside A with RG or rhein. When the influence of RG and rhein on the purgative activity of sennoside A was considered on the basis of the above results, the purgative activity of sennoside A was shown to be significantly stimulated by increasing the amounts of both RG and rhein. Compared with the purgative activity of sennoside A alone, the feces score showed marked stimulation of the purgative activity in these other cases, and the synergistic purgative activity-promoting effect of sennoside A by RG or rhein was verified.

The intestinal bacteria related to the metabolism of orally administered glycosides are important to understand the pharmacological effects.15–17) Our screening method using mouse feces proved useful in the search for activators of glycoside transformation. We found in a previous study that the activity of sennoside A metabolism in intestinal bacteria was significantly accelerated when glycyrrhiza, liquiritin or

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**Fig. 5. Effect of Rhein 8-O-β-D-Glucopyranoside (RG) and Rhein on Metabolism of Sennoside A (SA)**

Each value represents the mean±S.D. of 3 samples. ***p<0.001, significant difference from SA (Dunnett’s test).

**Fig. 6. Effect of Emodin, Aloe-emodin and Rhein on Metabolism of Sennoside A (SA)**

Each value represents the mean±S.D. of 3 samples. ***p<0.001, significant difference from SA (Dunnett’s test).
liquiritin apioside coexisted with sennoside A. In addition, the purgative action of sennoside A was significantly increased when glycyrrhiza, liquiritin or liquiritin apioside was co-administered orally to mice. In this study, we revealed that RG, present in rhubarb, accelerated the metabolism of sennoside A. Rhein, which is an aglycone of RG, accelerated the metabolic activity of sennoside A similarly to RG. Moreover, emodin and aloe-emodin, which are anthraquinones of rhubarb, also accelerated the metabolism of sennoside A. We also revealed that RG and rhein contribute to the purgative action of sennoside A in rhubarb, and that the accelerating metabolic activity of sennoside A is caused by the constituents that have an anthraquinone skeleton. Through this study, it was determined that the interaction between sennoside A and other constituents in rhubarb enhances the purgative activity of sennoside A. We have recognized a useful interaction in a Kampo medicine that has multiple constituents.

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Fig. 7. Effect of Rhein 8-O-β-D-Glucopyranoside (RG) and Rhein on Purgative Action of Sennoside A (SA)

Each column represents the mean±S.D. of 12 mice. *p<0.05, **p<0.01, ***p<0.001, significant difference from SA (Steel’s test).