



Effect of hot water extract of a glycyrrhizin-deficient strain of *Glycyrrhiza uralensis* on contact hypersensitivity in mice

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Abstract

To evaluate the medicinal properties of a glycyrrhizin (GL)-deficient strain of *Glycyrrhiza uralensis*, we investigated the anti-allergic effect of the hot water extract obtained from its roots on contact hypersensitivity in mice, and compared it with that of the hot water extract of a commercial crude drug, Glycyrrhiza Radix. The hot water root extract of the GL-deficient strain contained glucoglycyrrhizin (GGL) and rhaoglycyrrhizin (RGL) instead of GL, and it showed anti-allergic activity against contact hypersensitivity in a fashion similar to that of the crude drug extract. We further confirmed the presence of glycyrrhetic acid (GA), a major metabolite of GL, in mice serum after oral administration of the hot water root extract of a GL-deficient strain. We demonstrated that GGL underwent hydrolysis by intestinal microflora of mice to form GA. These results suggest that a GL-deficient strain of *G. uralensis* is a useful medicinal resource since the glycosides of GA work in a fashion similar to that of GL when orally administered.

Keywords *Glycyrrhiza uralensis* · Glycyrrhizin (GL) · Glucoglycyrrhizin (GGL) · Glycyrrhetic acid (GA) · Contact hypersensitivity · Intestinal microflora

Introduction

A triterpene glycoside, glycyrrhizin (GL), is present in the roots and stolons of *Glycyrrhiza uralensis* Fisher and *Glycyrrhiza glabra* Linne [1]. It has various pharmacological actions such as anti-inflammatory and anti-allergic effects [2–7]. Glycyrrhizae Radix is the most frequently used crude drugs for Kampo medicine, while the roots and stolons of *Glycyrrhiza uralensis* are also used for the same purpose. In a previous study, comparative analysis of *G. uralensis* strains was undertaken to characterize variations in *G. uralensis* [8]. During this analysis, a GL-deficient (83-555) strain of *G. uralensis* was identified, and was shown to produce glucoglycyrrhizin (GGL) instead of GL [9]. Furthermore, rhaoglycyrrhizin (RGL), a minor saponin reported

from European licorice, was isolated from this 83-555 strain [10]. GGL is a component of the GL sugar chain in which glucuronic acid at the end is replaced with glucose, while RGL has rhamnose added to the end of the GGL sugar chain.

In this study, we evaluated and compared the anti-allergic action of the hot water extracts obtained from the roots of GL-deficient strain *G. uralensis*, 83-555, on contact hypersensitivity in mice, against that of *Glycyrrhiza uralensis*, commercially available as Glycyrrhizae Radix.

Materials and methods

Reagents

The glycyrrhizin standard (GL, purity > 94%, HPLC) and glycyrrhetic acid (GA, purity > 98% HPLC) were kindly provided by Dr. Yukio Ogihara, emeritus professor at Nagoya City University. Glucoglycyrrhizin (GGL, purity > 98%, HPLC) and rhaoglycyrrhizin (RGL, purity > 90%, HPLC) were isolated from underground parts of a GL-deficient strain, 83-555 [9, 10]. Isolation of RGL from this strain will be reported elsewhere. HPLC-grade

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acetonitrile, methanol, and other chemicals were purchased from WAKO (Osaka, Japan).

Plant materials

Glycyrrhiza samples used in this study were obtained as follows. A commercial crude drug, *Glycyrrhiza Radix* (lot no. F01721), was purchased from Tsumura & Co. (Ibaraki, Japan). A GL-deficient strain of *Glycyrrhiza uralensis*, 83-555, was derived from a seed obtained from the former East Germany in 1983, and was cultivated at the Herbal Garden of Iwate Medical University (Iwate, Japan), as reported previously [9].

Preparation and characterization of hot water extracts of *Glycyrrhiza* samples

Each cut *Glycyrrhiza* sample (5 g) was decocted with 600 mL of ion exchanged and distilled water using an electric heater (HMJ-1000N, HARIO Co., Ltd., Tokyo, Japan) for 60 min. The decoctions were filtered and then cooled to room temperature. The filtrate was lyophilized to a powder and stored at -35°C until use.

Each 30 mg of hot water extract was extracted with 6 mL of 80% methanol at 60°C for 120 min. An aliquot (2 μL) of the extract was analyzed by Prominence HPLC system with a photodiode array detector (Shimadzu, Kyoto, Japan). Separations were carried out in an Inertsil ODS-SP column (3 μm , 2.1 mm I.D. $\times$ 250 mm; GL Sciences, Tokyo, Japan) with acetonitrile/water (containing 0.1% formic acid) gradient of 40–90% acetonitrile eluent for 25 min. The flow rate was 0.1 mL/min, and the column temperature was 40°C . The quantities of the GL (Retention time (Rt): 18.8 min), GGL (Rt: 19.5 min) and RGL (Rt: 17.4 min) were determined on the basis of their peak area of UV absorption at 254 nm.

Animals

Six-week-old female BALB/c mice, purchased from Japan SLC (Shizuoka, Japan), were used for the anti-allergy activity test. The mice were housed in standard plastic cages in a temperature and humidity controlled environment, with food and water available ad libitum. A period of at least 7 days for acclimation was allowed prior to experimentation. All experiments were carried out according to the guidelines for the care and use of experimental animals of the Animal Care Committee of the Faculty of Pharmacy, Meijo University.

Contact hypersensitivity in mice

For the evaluation of anti-allergic effect of a GL-deficient strain of *Glycyrrhiza uralensis*, a hapten induced contact

hypersensitivity model was used [11]. Briefly, the mice were sensitized by painting their shaved abdominal skin with 100 μL of 5% 2,4,6-trinitrochlorobenzene (TNCB) in acetone. The contact hypersensitivity (CHS) response was elicited 7 days later by painting both surfaces of the right ear of each mouse with 20 μL of 1% TNCB in olive oil. The ear thickness was measured before and after (24 and 48 h) the challenge using a dial thickness gauge (G-1A; Ozaki Mfg. Co., Ltd., Tokyo Japan). A once daily oral administration of the hot water extract of *Glycyrrhiza* sample was started right after the mice were sensitized for a period of 7 days. Dexamethasone was used as a positive control drug (5 mg/kg).

Serum concentration of glycyrrhetic acid (GA) after oral administration of *Glycyrrhiza* extract

A high dose of each *Glycyrrhiza* extract was used to confirm the presence of glycyrrhetic acid (GA) in mice serum after oral administration of the hot water extract of GL-deficient strain roots or the commercial crude drug. In the case of isolated compounds such as GL or GGL, we administered a dose of 12 mmol/kg (ca. 10 mg/kg) each. The mice were deprived of food, but given free access to water approximately 18 h prior to the experiment. Each *Glycyrrhiza* extract was administered orally and blood samples were collected by exsanguination from the inferior vein under anesthetization with isoflurane at the indicated time. The blood samples were allowed to stand at room temperature for 30 min and then centrifuged at 3000 rpm for 15 min to obtain serum. Serum (0.2 mL) was transferred to a 2 mL plastic tube, to which an internal standard (2-methylantraquinone, 10 ng) was added. Acetonitrile (1.8 mL) was added to the mixture, mixed well, and allowed to stand on ice for 45 min and then at 4°C for 10 min for sufficient deproteination. The mixture was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant (1.8 mL) was transferred to a glass tube and evaporated to dryness. The residue was dissolved in 100 μL of methanol; a 30 μL aliquot was injected into the HPLC column, as described previously [12]. A Shimadzu LC-10AD HPLC series with UV–VIS detector and C-R6A (Kyoto, Japan) was used for data acquisition and integration. Separations were carried out in a COSMOSIL 5C18-AR-II (5 μm , 4.6 mm I.D. $\times$ 150 mm; Nacalai Tesque, Inc., Kyoto, Japan) with 2% acetic acid–acetonitrile–methanol (48:42:10) as the eluent. The detection wavelength was 254 nm, the flow rate was 1.0 mL/min, and the column temperature was 40°C .

Preparation of an enzyme solution of the intestinal flora and determination of hydrolysis of GL or GGL into GA

The mice cecum was excised and the fresh contents were collected and suspended in 10 times weight of 100 mM sodium acetate buffer (pH 5.6). It was then incubated at 37°C under

an anaerobic condition with argon gas. After 60 min, the suspension was centrifuged at 9000×g for 10 min at 4 °C, and finally the enzyme solution of intestinal flora was obtained. An aliquot of enzyme solution (0.2 ml) was transferred to a 1.5 ml plastic tube, to which 12 nmol (ca. 10 ng) of GL or GGL was added. The mixture was incubated for 15, 30, 60, 90, 120 and 180 min at 37 °C and the enzyme reaction was stopped by the addition of 0.1 ml of 1 N HCl. The internal standard (2-methylantraquinone, 50 ng) was added and then the mixtures were extracted with ethyl acetate (0.5 ml). After centrifugation at 9000×g for 10 min at 4 °C, the supernatant (0.4 ml) was collected in a glass tube. The extraction was carried out twice and the combined solution (0.8 ml) was evaporated to dryness. The residue was dissolved in 100 µl of methanol and a 30 µl of aliquot was injected into the HPLC column. The analytical conditions were identical to what was described above.

Statistical analysis

Results are represented as mean ± SEM. Statistical analysis was conducted by ANOVA with Bonferroni correction for multiple comparison.

Results and discussion

Content characteristics of hot water extracts of *Glycyrrhiza* samples

Chemical determination of GL, GGL and RGL was carried out. It was revealed that a hot water extract prepared from the commercial crude drug *Glycyrrhizae Radix* contained GL alone, while the hot water extract obtained from a GL-deficient strain of *Glycyrrhiza uralensis* contained GGL and RGL rather than GL (Fig. 1; Table. 1). Furthermore, we also analyzed the GA contents in hot water extracts and we could not detect GA at all. The total amount of glycosides of glycyrrhetic acid in the GL-deficient strain extract was about one third compared with that in the extract of the commercial crude drug *Glycyrrhizae Radix*, used in this study.

Effects of hot water extracts of *Glycyrrhiza* samples on contact hypersensitivity in mice

The doses of hot water extracts were calculated based on the human daily doses of *Glycyrrhiza uralensis* decoction. The low dose was equal to ten times the human daily dose (222 mg/kg for strain 83-555 and 282 mg/kg for crude drug) and high dose was twenty times the human daily dose (444 mg/kg for strain 83-555 and 564 mg/kg for crude drug). As shown in Fig. 2, both the *Glycyrrhiza* samples significantly reduced ear swelling at 24 h after the elicitation of

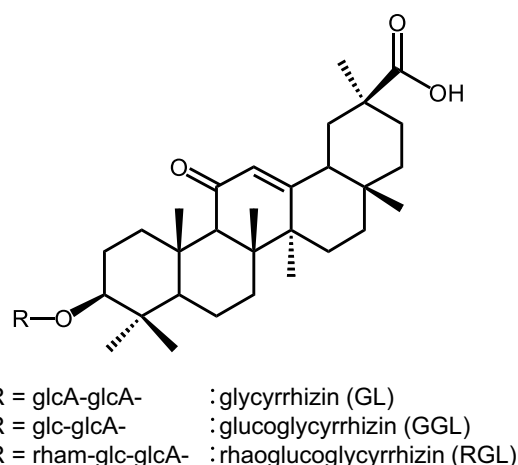


Fig. 1 Chemical structures of glycosides of glycyrrhetic acid

Table 1 Content characteristics of hot water extracts of *Glycyrrhiza* samples

	Yield (g)	GL (%)	GGL (%)	RGL (%)	Total (%)
83-555 (1)	1.39	0.5	2.3	1.9	4.7
83-555 (2)	1.26	0.5	2.7	2.3	5.5
83-555 (3)	1.33	0.3	2.1	1.4	3.9
Crude drug (1)	1.79	12.1	0.0	0.0	12.1
Crude drug (2)	1.74	13.7	0.0	0.0	13.7
Crude drug (3)	1.69	13.1	0.0	0.0	13.1

contact hypersensitivity. These results suggest that a GL-deficient strain of *Glycyrrhiza uralensis* has similar anti-allergic efficacy as the commercial crude drug derived from *Glycyrrhiza uralensis*.

Serum concentration of glycyrrhetic acid after oral administration of hot water extracts of *Glycyrrhiza* samples and their ingredients

It is well-known that GL, when administered orally, is hydrolyzed by β-D-glucuronidase originating from intestinal microflora in the alimentary tract to form its aglycone, GA. The GA is then absorbed into the bloodstream to exert its pharmacological activity [13–15].

Since a GL-deficient strain of *G. uralensis* contains glycosides of GA such as GGL and RGL, these glycosides are hydrolyzed into GA by intestinal flora.

Thus, we next investigated the serum concentration of GA after oral administration of both *Glycyrrhiza* samples at high doses of hot water extracts. We first observed bimodal peaks for GA concentration at 8 h and 12 h after administration of hot water extract prepared from the commercial crude drug *Glycyrrhizae Radix* (Fig. 3a). When the hot water

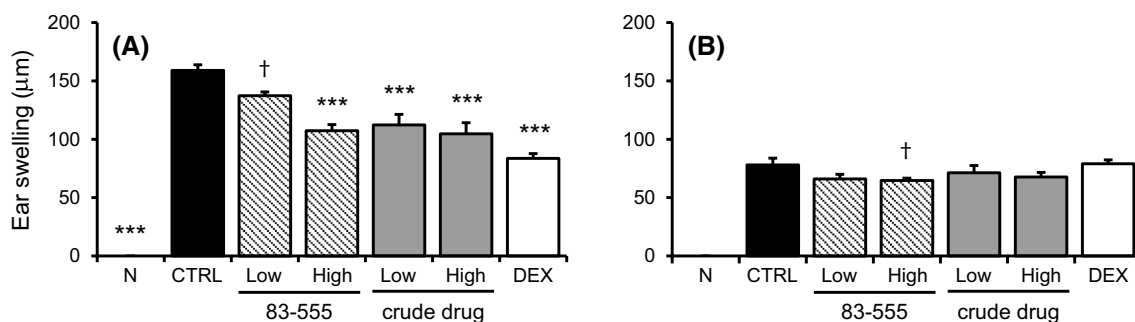


Fig. 2 Effects of hot water extracts of a GL-deficient strain, 83-555 or commercially available *Glycyrrhiza uralensis* roots on 2,4,6-trinitrobenzene (TNCB)-induced contact hypersensitivity in mice. **a** Ear swelling at 24 h after topical application of 1% TNCB, **b** ear swelling at 48 h after topical application of 1% TNCB. Each column represents

the Mean $\pm$ SEM of 3–5 mice. *** $p < 0.001$ vs. the control group by ANOVA with Bonferroni correction for multiple comparisons. † $p < 0.05$ vs. the control group by ANOVA with Bonferroni correction for selected two comparisons

extract of the GL-deficient strain was administered orally, we observed a single peak for GA at 6 h (Fig. 3b). These results suggested that GGL could be hydrolyzed to form GA by intestinal microflora in the alimentary tract and then absorbed into the bloodstream in a similar fashion as the metabolic fate of GL. According to their pharmacokinetic parameters, area under the curve (AUC)_{0–24} values for the administration of the GL-deficient strain sample was almost one third compared to that of the commercial crude drug sample which seemed to match to the total contents as the glycosides of GA. Interestingly, the maximum serum concentration ($C_{\max}$) values were almost comparable in both cases and we therefore speculated that both extracts showed anti-allergic actions in the same manner. Furthermore, we compared the serum concentration of GA after oral administration of the major ingredient such as GL or GGL at the same dose. As shown in Fig. 4, the time course of serum GA concentration was similar for each compound when administered orally in the form of hot water extract. With respect to the pharmacokinetic properties when these compounds

were administered orally, the $C_{\max}$ values of both cases were almost equal, but the AUC_{0–24} values were quite different. The AUC_{0–24} value for administration of GGL was about one third that of GL even though the equivalent amount was administered in both cases. We also could not observe a second peak when GGL was administered. Additional studies are needed to explain the differences of the time-course of serum GA concentration, especially observed after 12 h.

Comparison of hydrolysis of GL or GGL to form GA by intestinal microflora in cecum

Next, we studied the hydrolysis of GGL by intestinal microflora using the enzyme solution prepared from murine cecal contents under anaerobic conditions. The preparation of enzyme solution and enzyme reaction was carried out at an optimum pH of 5.6 in human [16]. As a preliminary experiment, no difference was observed in the production of GA when GL hydrolysis reaction was carried out in the presence or absence of mouse cecal contents after 60 min

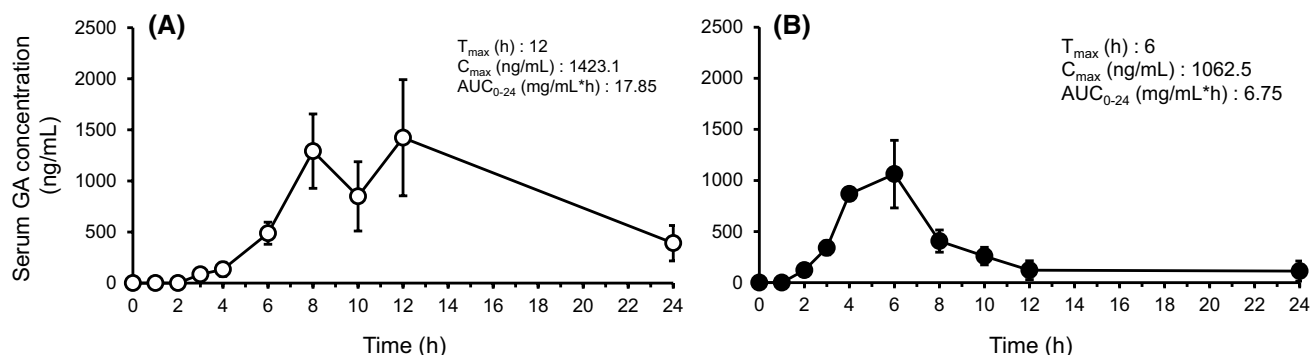


Fig. 3 Serum concentration of glycyrrhetic acid (GA) after oral administration of *Glycyrrhiza* extract. **a** A hot-water extract of commercially available *Glycyrrhiza uralensis*. **b** A hot-water extract

of a GL-deficient *Glycyrrhiza uralensis*. Each point represents the Mean $\pm$ SEM of 3–6 mice

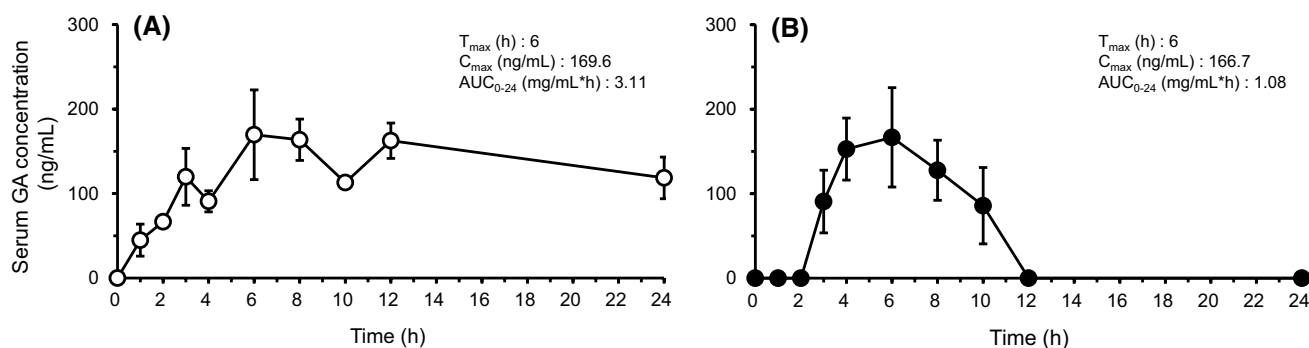


Fig. 4 Serum concentration of glycyrrhetic acid (GA) after oral administration of GL or GGL. **a** GL, **b** GGL. Each point represents the Mean $\pm$ SEM of 3–6 mice

of pre-culture in an anaerobic condition. Therefore, in this study, the supernatant from which the cecum contents were removed by centrifugation was used as the enzyme solution.

As shown in Fig. 5, GL was hydrolyzed to form GA linearly until 180 min, while GGL was hydrolyzed to form GA at a much faster rate. The rate of formation of GA was almost twice when GGL was used as the substrate. Since GGL has a 2'-O- β -D-glucopyranosyl moiety instead of the 2'-O- β -D-glucuronopyranosyl moiety of GL, it can be argued that the hydrolyzed rate of saponin depends on the sugar moiety. This may explain the differences in rate of GA formation even though there is substrate specificity for the hydrolysis by intestinal microflora. According to previous studies, GL is thought to be hydrolyzed directly to form GA using human intestinal microflora such as *Eubacterium* sp. These results, however, suggested the possibility that the sugar moiety of GGL and/or GL was hydrolyzed one by one by several enzyme such as β -glucosidase and β -glucuronidase originating from murine intestinal microflora. In this study, we detected 3-O-monoglucuronide of GA (data not shown) suggesting

the need to further characterize the hydrolysis processes of GL and GGL in detail. These results suggest that GGL was also hydrolyzed to form GA, showing anti-allergic properties in murine contact hypersensitivity.

Conclusion

In this study, we confirmed that a hot water extract of a GL-deficient strain of *G. uralensis* showed anti-allergic properties, comparable to that of a hot water extract of commercial crude drug of *G. uralensis*. Moreover, we demonstrated that GGL is also hydrolyzed to form GA by intestinal microflora in the alimentary tract. These results suggest that the glycosides of GA can work the same as GL in our body when they are administered orally. We cannot conclusively say that a GL-deficient strain of *G. uralensis* is approved as *Glycyrrhizae Radix* in Japanese Pharmacopoeia but our results suggest that this is also a useful medicinal resource.

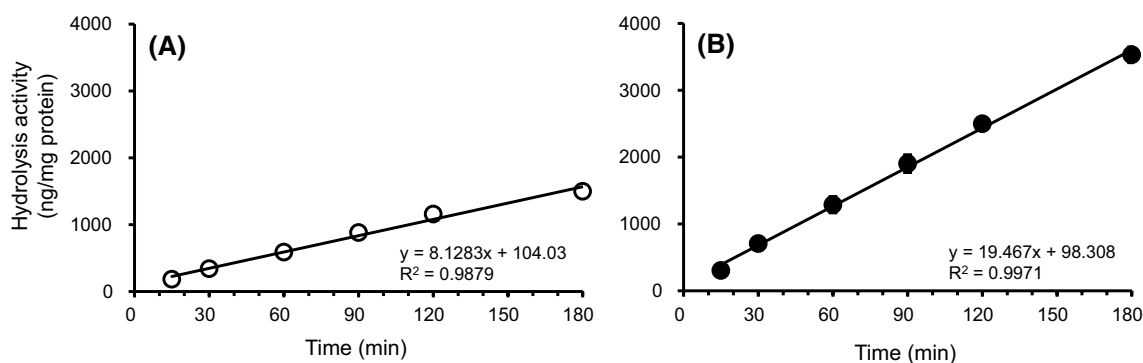


Fig. 5 Hydrolysis of GL or GGL to form GA by intestinal microflora. **a** GL, **b** GGL. Each point represents the Mean $\pm$ SEM of 3 samples

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be constructed as a potential conflict of interest.

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