Isoliiquiritigenin, One of the Antispasmodic Principles of Glycyrrhiza Uralensis Roots, Acts in the Lower Part of Intestine

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Glycyrrhizae Radix is used to treat abdominal pain as a component of Shakuyakukanzoto (Shaoyao-Gancao-tang), a traditional Chinese medicine formulation. Previously, we have reported the isolation of glycycoumarin as a potent antispasmodic with an IC50 value of 2.93±0.94 μM for carbamylcholine (CCh)-induced contraction of mouse jejunum from an aqueous extract of Glycyrrhizae Radix (licorice), and clarified that its mechanism of action involves inhibition of phosphodiesterase 3. The purpose of the present study was to examine an antispasmodic principle of licorice other than glycycoumarin. Isoliiquiritigenin was isolated from an aqueous extract of licorice as a potent relaxant, which inhibited the contraction induced by various types of stimulants, such as CCh, KCl, and BaCl2, with IC50 values of 4.96±1.97 μM, 4.03±1.34 μM, and 3.70±0.58 μM, respectively, which are close to those of papaverine. However, the amount of isoliquiritigenin in the aqueous extract of licorice was very small. When the aqueous licorice extract was treated with naringinase, the amounts of glycosides such as isoliquiritin, which were abundant but had much less potent relaxant activity, were decreased while isoliquiritigenin was increased. At the time, the relaxant activity of the treated sample was increased significantly, shifting the IC50 from 358±104 to 150±38 μg/ml for CCh-induced contraction. Isoliiquiritigenin also showed the most potent inhibition of mouse rectal contraction induced by CCh with an IC50 value of 1.70±0.07 μM. These results suggest that isoliquiritigenin acts as a potent relaxant in the lower part of the intestine by transformation from its glycosides.

Key words Shakuyakukanzoto; Glycyrrhiza uralensis; antispasmodic; isoliquiritigenin; naringinase; davidigenin

Abdominal pain is commonly caused by powerful contraction of smooth muscle through the action of the enteric nerve system.2) Clinically, pain caused by gastrointestinal spasms is generally treated with drugs that induce smooth muscle relaxation.

Shakuyakukanzoto (Shaoyao-Gancao-tang), an aqueous extract of a mixture of Paeoniae Radix and Glycyrrhizae Radix (licorice) and Kanzoto (an aqueous extract of Glycyrrhizae Radix) have been used clinically to relieve gastrointestinal pain.3) Shakuyakukanzoto has also been shown to exert a relaxant effect against the actions of various types of stimulants such as CCh, KCl, and BaCl2 with IC50 values of 4.96±1.97 μM, 4.03±1.34 μM and 3.70±0.58 μM, respectively, which are close to those of papaverine. However, the amount of isoliquiritigenin in the aqueous extract of licorice was very small. When the aqueous licorice extract was treated with naringinase, the amounts of glycosides such as isoliquiritin, which were abundant but had much less potent relaxant activity, were decreased while isoliquiritigenin was increased. At the time, the relaxant activity of the treated sample was increased significantly, shifting the IC50 from 358±104 to 150±38 μg/ml for CCh-induced contraction. Isoliiquiritigenin also showed the most potent inhibition of mouse rectal contraction induced by CCh with an IC50 value of 1.70±0.07 μM. These results suggest that isoliquiritigenin acts as a potent relaxant in the lower part of the intestine by transformation from its glycosides.

MATERIALS AND METHODS

Chemical Acetylcholine (ACh) chloride was purchased from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Butylscopolammonium bromide, carbamylcholine (CCh) chloride, forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Isoliiquiritigenin and naringinase (β-D-glucosidase from Penicillium decumbens) were from Sigma-Aldrich Fine Chemicals (St. Louis, MO, U.S.A.). Liquiritigenin, isoliquiritin, and liquisirin were from Funko Co., Ltd. (Tokyo, Japan). Isoliiquiritin apioside, liquiritin apioside and davidigenin were kind gifts from Tsumura and Co. (Tokyo, Japan).

Apparatus El-MS was measured with a JMS-GC mate mass spectrometer at an ionization voltage of 70 eV (JEOL Co., Akishima, Japan). 1H- and 13C-NMR spectroscopy were performed with a JNM LA 400 NMR spectrometer (1H, 400 MHz; 13C, 100 MHz, JEOL Co.). HPLC was performed on a Jasco PU-980 (Tokyo, Japan) equipped with a ternary gradient unit: Jasco LG-1580-02 (Tokyo, Japan); Multiwavelength detector; Jasco MD-910 (Tokyo, Japan); Oven: Tosoh CO-8020 (Tokyo, Japan); Injector: Rhedyne 7725i (U.S.A.) under the following conditions: column, YMC-Pack ODS-A-303 (250×4.6 mm I.D., 5 μm, Kyoto, Japan); mobile phase, 0.05 M AcONH4 (pH 3.6): CH3CN from 90: 10 to 0:100 in 60 min; flow rate, 1.0 ml/min. Column temperature: 40°C.

Extraction of Licorice Roots and Isolation of Isoliiquiritigenin Licorice, the root of Glycyrrhiza uralensis, was purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). The botanical origin was confirmed by the presence of glycyccoumarin, a species-specific ingredient of G. uralensis, in HPLC-profile.7) Voucher specimens were deposited in the department of Kampo-Pharmaceuticals, Institute of Natural Medicine, University of Toyama. The dried and crushed licorice (6 g for each extract) was extracted by boiling in water (600 ml) until water volume had reduced to half, filtered and freeze-dried into powder (yield about 4 g/each extract). The extract (5 g in 30 ml water) was applied to
Sephadex LH-20 (diameter: 4.0 cm, length: 90 cm, Amer-sham Biosciences, Piscataway, NJ, U.S.A.) and eluted with water, and then with a gradually increasing proportion of ethanol (5%, 10%, 20%, 40%, 60%, 80%, 100%) as reported previously.8 The flavonoid-rich fractions eluted with H2O–
eohanol and ethanol were next chromatographed on a silica gel column (diameter: 3.2 cm, length: 15 cm, Silica gel 60 for chromatography, Nacalai Tesque Inc., Kyoto, Japan) by stepwise elution with MeOH in CHCl3 (CH3Cl/MeOH, 100 : 0, 99 : 1, 98 : 2, 95 : 5). Two eluates obtained with 1% MeOH in CHCl3 showed antispasmodic activity and the rest was extracted with methanol for chromatography, Nacalai Tesque Inc., Kyoto, Japan). A part of the fractions eluted with the 50 : 50 mixture had a strong inhibitory effect. Finally, further purification of this fraction by rechromatography on the reverse-phase column led to the isolation of isoliquiritigenin (Fig. 1), which was identified by NMR and MS spectroscopic analyses.

**Treatment of an Aqueous Extract of Licorice with Naringinase** An aqueous extract of licorice (55 mg of dried powder) was dissolved in 1 ml of 50 mM acetate buffer (pH 4.7), and treated with naringinase (10 mg/ml) at 37 °C for 3 h. A part of the mixture was used directly for antispasmodic activity and the rest was extracted with methanol for HPLC analysis. The treatment was carried out three times for statistical analysis.

**Muscle Preparation and Tension Measurement** Male ICR mice (6 weeks old, 25—30 g weight) were purchased from Japan SLC Inc., (Hamamatsu, Japan) and maintained under a 12-h light–dark cycle at 21—24 °C in the Laboratory for Animal Experiments, University of Toyama. The animal experiments were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals at the University of Toyama. The ICR mice were purchased for one to two weeks, then starved overnight with free access to water before the experiments, and killed by exsanguination. The jejunum, ileum and rectum were excised rapidly, cleaned of connective tissues, and cut into rings of 10—15 mm in length. The rings were maintained in Tyrode solution. The muscle tension was recorded isometrically as described previously.

**Assay of cAMP or cGMP Content** cAMP or cGMP content was measured by enzyme immunoassay as reported previously.

**Data Analysis** The concentrations of drugs were expressed as final concentrations in the organ bath. Data are expressed as the mean values ± standard deviation of the mean (S.D.). Statistical analysis was performed using paired and unpaired two-tailed Student’s t test. Differences at \( p < 0.05 \) were accepted as statistically significant.

**RESULTS**

**Effects of Isoliquiritigenin on Contractile Responses in Mouse Jejunum** Bio-assay-guided fractionation and purification of the aqueous extract of licorice led to the isolation of isoliquiritigenin (Fig. 1) as a potent spasmolytic. Isoliquiritigenin showed concentration-dependent inhibition of the tonic contraction of mouse jejunum induced by various types of stimulants such as CCh (1 μM), KCl (60 mM) and BaCl2 (0.3 mM) (Fig. 2) with IC50 values of 4.96 ± 1.34 μM and 3.70 ± 0.58 μM, respectively, which were close to those (1.64 ± 0.46 μM, 0.78 ± 0.51 μM and 2.35 ± 0.16 μM for CCh-, KCl- and BaCl2-induced contraction, respectively) for papaverine. Moreover, pretreatment with isoliquiritigenin inhibited non-competitively the contraction caused by the cumulative addition of ACh as strongly as papaverine, while the inhibitory effect of butylscopolammonium bromide was competitive (Fig. 3). These results indicate that isoliquiritigenin has potent relaxant effects against the actions of various stimulants similar to those of papaverine, and that the mechanism responsible may be similar to that of papaverine.

**Little Relationship between Relaxation Action and**
isoquercitrin is a potent inhibitor of PDEs, 13) we studied ammonium bromide (0.1 mM) or butylscopolammonium bromide (0.1 mM, △, 1 μM, ▽), or without agents (●), and then ACh was added cumulatively. The maximum contraction induced by ACh (0.1 μM) in the absence of agents was taken as 100%. Values are means±S.D. (n=3—4).

**PDE-Inhibitory Effect of Isoquercitrin**  As papaverine relaxes smooth muscle through inhibition of PDEs,11,12 and isoquercitrin is a potent inhibitor of PDEs,13 we studied the relationship between the antispasmodic action of isoquercitrin and its PDE inhibition. The adenyl cyclase activator, forskolin, inhibited the CCh-induced contraction of mouse jejunum in a concentration-dependent manner (IC₅₀=0.25±0.08 μM). As shown in Fig. 4, the inhibition was not significantly accelerated by pretreatment with isoquercitrin (10 μM) in spite of a shift in the IC₅₀ to 0.16±0.08 μM, whereas it was increased significantly by IBMX (10 μM), a potent non-selective inhibitor of PDEs, and glycyccoumarin (10 μM), a PDE 3 inhibitor from licorice, with IC₅₀ values of 0.06±0.02 μM and 0.11±0.04 μM, respectively. On the other hand, the inhibition of CCh-induced contraction with IBMX (IC₅₀=3.23±0.72 μM) tended to be decreased by pretreatment of isoquercitrin (1 μM), shifting the IC₅₀ to 3.85±0.08 μM (data not shown), whereas it was increased significantly by pretreatment with glycyccoumarin (1 μM), shifting the IC₅₀ to 1.44±0.35 μM. Moreover, isoquercitrin did not increase the content of cAMP or cGMP in mouse jejunum (data not shown). These results suggest that the relaxant action of isoquercitrin on the mouse jejunum might be not related to its PDE inhibition.

**Effects of Related Constituents in Licorice on Contractile Responses in Mouse Jejunum**  Although isoquercitrin-genin showed potent concentration-dependent inhibition of CCh-induced contraction of mouse jejunum, the effect of liquiritigenin (a major flavanone of licorice, Fig. 1) was considerably weak. Glycosides such as liquiritin, liquiritin apioside and isoliquiritin (major constituents of licorice, Fig. 1) were inactive. However, davidigenin, a metabolite of liquiritigenin,14 having a chalcone structure like that of isoquercitrin-genin, showed potent inhibition with an IC₅₀ value of 5.07±1.63 μM (Fig. 5).

**Enhancement of the Relaxant Action of Licorice Extract by Naringinase**  HPLC analysis of the aqueous extract of licorice did not show any peak of isoquercitrin-genin, suggesting only a trace amount of this compound in the extract (Fig. 6), though its glycosides, isoliquiritin and isoquercitrin apioside, were present abundantly.15) When the aqueous extract of licorice was treated with naringinase, the amounts of glycosides such as liquiritin and isoliquiritin were decreased, while those of their aglycones such as liquiritin and isoliquiritin were increased (Fig. 6). Furthermore, the inhibition of CCh-induced contraction of mouse jejunum with the aqueous extract of licorice was significantly increased by treatment with naringinase, shifting the IC₅₀ from 358±104 to 150±38 μg/ml (Fig. 7). In addition to the
through inhibition of PDEs. We have also reported that paverine is related to accumulation of intracellular cAMP and papaverine. It is generally accepted that the action of licorice, but not an aqueous extract of Paeoniae Radix, have been reported to inhibit the contraction induced by ACh in guinea pig ileum, and in mouse jejunum and ileum. Recently, glycycomarin has been isolated from the aqueous extract of licorice and shown to have an IC50 value of 4.96 μM, in addition to glycycomarin is potent inhibitors of PDEs, the former (IC50 180 μM) is much weaker, by two orders of magnitude, than the latter (IC50 7 μM). Accordingly, the relaxant effect of glycycomarin seemed to have no relationship to its weak PDE inhibition.

The content of glycycomarin in the aqueous extract of liquorice is trace (Fig. 6), but its glycosides such as isoliquiritin and isoliquiritin apioside are present in abundance, although they showed no relaxant effect (Fig. 5). When Shakuyakukanzoto is administered orally, the glycosides are not absorbed and carried into the lower part of the intestine, where intestinal bacteria producing β-D-glicosidases are present. There, isoliquiritigenin is produced from its glyco- sides, supported by isoliquiritigenin production from isoliquiritin with naringinase (β-D-glicosidase) (Fig. 6). The inhibitory effect of the aqueous extract of licorice on CCh-induced contraction was significantly increased after treatment with naringinase (Fig. 7), shifting the IC50 from 358 μg/ml to 150 μg/ml. As the content of isoliquiritigenin in the aqueous extract of licorice after treatment with naringinase was 0.246% (w/w), as determined by HPLC, isoliquiritigenin at 208 μg/ml obtained by subtraction of the two IC50 values was assumed to be about 2 μM. This concentration was close to the IC50 of isoliquiritigenin, suggesting that enhancement of the inhibitory effect by treatment with naringinase was caused mainly by isoliquiritigenin produced. Furthermore, among the various parts of the intestine isoliquiritigenin exerted the most potent inhibition against rectal contraction (Fig. 8), suggesting that isoliquiritigenin is one of the genuine principles of Shakuyakukanzoto that relieves pain in the lower intestine. In fact, the aqueous extract of licorice exerts a relaxant effect on contraction in the lower part of the rat intestine induced by Rhei Rhizoma. On the other hand, the antispasmodic effect of liquiritigenin, another of the major flavonoids in liquorice, was much weaker than that of isoliquiritigenin. However, liquiritigenin is not found in licorice root but is transformed from liquiritigenin by human intestinal flora, inhibited the CCh-evoked contraction (Fig. 5) with almost the same potency as isoliquiritigenin. As davidigenin also has a chalcone structure in its fundamental skeleton, similar to that of isoliquiritigenin, whereas liquiritigenin does not, the chalcone structure may be important for the relaxant effect.

In conclusion, this study has clarified for the first time that isoliquiritigenin acts as a potent antispasmodic agent on mouse intestine, especially the lower intestine by transformation from its glycosides.

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