

## Anti-Allergic Activity of 18 $\beta$ -Glycyrrhetic acid-3-O- $\beta$ -D-glucuronide

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Glycyrrhizin (18 $\beta$ -glycyrrhetic acid-3-O- $\beta$ -D-glucuronopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucuronide, GL) was transformed to 18 $\beta$ -glycyrrhetic acid-3-O- $\beta$ -D-glucuronide (GAMG) by *Streptococcus* LJ-22. The anti-allergic activities of GL and GAMG was measured using a RBL cell assay system and contact hypersensitivity model mice. GAMG exhibited anti-allergic activity with IC<sub>50</sub> values of 0.28 mM. GAMG, which is sweeter than GL, and 18 $\beta$ -glycyrrhetic acid, which is a GAMG metabolite by human intestinal bacteria, also inhibited the passive cutaneous anaphylaxis and skin contact inflammation. In conclusion, GAMG may be useful as a new sweet food additive and an anti-allergic agent.

**Key words:** 18 $\beta$ -Glycyrrhetic acid-3-O- $\beta$ -D-glucuronide, Glycyrrhizin, *Streptococcus* LJ-22, Anti-allergic activity

### INTRODUCTION

Glycyrrhizin (18 $\beta$ -glycyrrhetic acid-3-O- $\beta$ -D-glucuronopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucuronide, GL), which is the main component of a licorice extract (*Glycyrrhiza glabra*), is used both orally as a sweetener and a component in Oriental medicine. GL has steroid-like action, antiviral activity and anti-inflammatory activity (Kumagai *et al.*, 1957; Finney and Sommers, 1958; Pompeo *et al.*, 1979; Abe *et al.*, 1982; Hirabayashi *et al.*, 1991).

Brieskorn and Lang (1978) and Mizutani *et al.* (1994) synthesized 18 $\beta$ -glycyrrhetic acid-3-O- $\beta$ -D-glucuronide (GAMG), which is sweeter than GL. Mizutani and Yuki-yoshi (1995) reported that GAMG exhibited potent chemopreventive activity for a tumor. Recently Kuramoto *et al.* (1994), and Kim *et al.* (1999) isolated GAMG by the microbial transformation of GL. They used *Cryptococcus magnus* MG-27, which is a eukaryote, and *Streptococcus* LJ-22 as the microbes, respectively. Kim *et al.* (2000) also reported that GAMG exhibited antirotaviral and antitumor activities. However, there is a paucity of reports on the anti-allergic activity of GAMG.

Therefore, this study transformed GL to GAMG by

*Streptococcus* LJ-22, isolated GAMG and then measured its anti-allergic activity.

### MATERIALS AND METHODS

#### Materials and microorganisms

The GL, GA, human serum albumin (HSA), *p*-nitrophenyl- $\beta$ -D-glucopyranoside, anti-dinitrophenol (DNP)-IgE, DNP-HAS, disodium cromoglycate (DSCG), oxazolone were purchased from Sigma Chemical Co. (U.S.A). Griess reagent was purchased from Promega Co. (USA).

The general anaerobic medium (GAM) was purchased from Nissui Pharm. Co., Ltd. (Japan). *Streptococcus* LJ-22 was isolated in our previous study (Kim *et al.*, 1999).

#### Biotransformation of GL and Isoaltion of GAMG

GL was transformed to GAMG by *Streptococcus* LJ-22 according to a previously reported method (Kim *et al.*, 1999). *Streptococcus* LJ-22, which was isolated from human intestinal bacteria, was cultured in 5 L of a tryptic soy (TS) broth with 1 g GL. After cultivation at 37°C for 24 h, it was adjusted to pH 2 with 1 N HCl and extracted twice with ethyl acetate. After evaporating the ethyl acetate fraction, the resulting powder was applied to a silica gel column (2.5 $\times$ 40 cm) using CHCl<sub>3</sub>/methanol (10:1 $\rightarrow$ 5:1) as the elution solvent. The isolated GAMG was crystallized

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with  $\text{CHCl}_3/\text{MeOH}$  (5:1).

$^{13}\text{C}$ -NMR (125 MHz,  $\text{CDCl}_3$ ) of GAMG  $\delta$ : 40.21 (C-1), 27.61 (C-2), 90.74 (C-3), 40.53 (C-4), 56.43 (C-5), 18.44 (C-6), 33.82 (C-7), 44.64 (C-8), 63.13 (C-9), 38.08 (C-10), 202.65 (C-11), 128.96 (C-12), 172.77 (C-13), 46.76 (C-14), 26.97 (C-15), 27.41 (C-16), 32.98 (C-17), 49.93 (C-18), 42.43 (C-19), 44.90 (C-20), 32.02 (C-21), 39.02 (C-22), 28.41 (C-23), 16.94 (C-24), 16.96 (C-25), 19.31 (C-26), 23.81 (C-27), 28.75 (C-28), 29.19 (C-29), 180.38 (C-30), 106.95 (C-1), 77.71 (C-2'), 76.54 (C-3), 75.31 (C-4), 73.18 (C-5), 172.7 (C-6).

### Assay of anti-allergic activity in RBL-2H3 cell line

The inhibitory activity of GAMG against the release of  $\beta$ -hexosaminidase from RBL-2H3 cells was evaluated according to Ryu *et al.* (2000). The RBL-2H3 cells were grown in DMEM supplemented with 10% fetal bovine serum and L-glutamine. Before the experiment, the cells were dispensed into 24 well plates at a concentration of  $5 \times 10^5$  cells per well, using a medium containing 0.5  $\mu\text{g}/\text{mL}$  of the mouse monoclonal IgE, and were incubated overnight at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  to sensitize the cells. The cells were washed with 500  $\mu\text{L}$  of a siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM  $\text{MgCl}_2$ , 25 mM PIPES and 40 mM NaOH) and incubated in 160  $\mu\text{L}$  of a siraganian buffer (5.6 mM glucose, 1 mM  $\text{CaCl}_2$  and 0.1% BSA were added) for an additional 10 min at  $37^\circ\text{C}$ . The cells were then exposed to 40  $\mu\text{L}$  of the test materials for 20 min, followed by a treatment with 20  $\mu\text{L}$  of the antigen (DNP-HSA, 1  $\mu\text{g}/\text{mL}$ ) for 10 min at  $37^\circ\text{C}$  to force the cells to evoke allergic reactions (degranulations). The reaction was quenched by cooling the cells in an ice bath for 10 min. The reaction mixture was centrifuged and 25  $\mu\text{L}$  aliquots of the supernatant were transferred to 96 well plates and incubated with 20  $\mu\text{L}$  of the substrate (1 mM *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide) for 1 h at  $37^\circ\text{C}$ . The reaction was quenched by adding 0.2 mL NaOH. The absorbance was measured by ELISA at 405 nm.

### Nitric oxide assay

The nitric oxide concentration was determined by measuring the nitrite concentration from the cell culture supernatant using the Griess reagent (Promega, USA) according to the manufactures protocol (Ryu *et al.*, 2000). The RAW 264.7 cells were stimulated with LPS (1  $\mu\text{g}/\text{mL}$ ) and the test compounds for 24 h. The cells were briefly centrifuged, and 150  $\mu\text{L}$  of the cell culture supernatant was mixed with 150  $\mu\text{L}$  of a Griess reagent, and then incubated 5-10 min at room temperature (light protected). The absorbance was measured using an ELISA reader at 540 nm against a calibration curve using sodium nitrate as the standard.

### Passive cutaneous anaphylaxis (PCA) reactions

An IgE-dependent cutaneous reaction was measured according to the method reported by Katayama *et al.* (1978). The male ICR mice (25-30 g) were injected intradermally with 10  $\mu\text{g}$  of the anti-DNP IgE into each of two dorsal skin sites that had been shaved 48h earlier. The sites were outlined using a water-insoluble red marker. Forty-eight hours later each mouse was administered 200  $\mu\text{L}$  of 3% Evans blue PBS containing 200  $\mu\text{g}$  of DNP-HSA intravenously *via* the tail vein. The test compounds were administered 1 h prior to the DNP-HSA injection. Thirty minutes after the DNP-HSA injection, the mice were sacrificed and their dorsal skins were removed in order to measure the pigment area. After extraction with 1 mL of 1.0 N KOH and 4 mL of a mixture of acetone and 0.6 N phosphoric acid (13:5), the amount of dye was determined colorimetrically at 620 nm.

### Contact hypersensitivity

An oxazolone-induced dermatitis was measured according to the method reported by Yasutomo *et al.* (2002). The ICR mice (female, 25-28 g) were sensitized by applying 100  $\mu\text{L}$  of 1.5% oxazolone in ethanol to the abdomen. Subsequently, a total of 20  $\mu\text{L}$  of 1% oxazolone in a mixture of acetone and olive oil (4:1) was applied to both sides of the ear every 3 days beginning from 7 days after sensitization. The ear thickness was measured using a Digimatic Micrometer (Mitsutoyo, Japan) 72 h after each application of the oxazolone. The GL, GAMG or GA was applied in a total volume of 20  $\mu\text{L}$  to both sides of the ear 30 min before and 3 h after each oxazolone application.

### Statistical analysis

All the data from the *in vivo* experiments are expressed as a mean  $\pm$  S.D. and the statistical significance was determined using a Student's *t*-test.

## RESULTS AND DISCUSSION

When GL is orally administered to humans, GL is mainly metabolized to GA by intestinal bacteria and to a minor extent to GA via GAMG. GAMG is quickly metabolized to GA instead of GL. Therefore, it suggests that GAMG may better express its pharmacological action (anti-inflammatory and antiviral activities) than GL. In addition, GAMG is valuable as a sweetener, compared to GL. However, its anti-allergic activity has not been studied, although the activity of GL and GA has been reported. Therefore, this study evaluated the anti-allergic activity of GAMG via its inhibitory activity upon  $\beta$ -hexosaminidase release from the RBL-2H3 cells (Table I). GAMG, GL and GA all inhibited the release of  $\beta$ -hexosaminidase with  $\text{IC}_{50}$  values of 0.28, 0.37 and 0.23 mM, respectively. They

**Table I.** Inhibitory effect of GAMG on b-hexosaminidase release from the RBL 2H3 cells induced by IgE

Compound	IC <sub>50</sub> (mM)
GL	0.37
GAMG	0.28
GA	0.23
Azelastine	0.10
DSCG	0.50

GL, glycyrrhizin; GAMG, 18 $\beta$ -glycyrrhetic acid-3-O- $\beta$ -D-glucuronide; GA, 18 $\beta$ -glycyrrhetic acid; DSCG, disodium cromoglycate

were more potent than that of DSCG, which is clinically used (IC<sub>50</sub> was 0.5 mM).

This study also investigated the inhibitory activity of GAMG on the PCA reaction after being administered either orally or intraperitoneally 60 min prior to antigen challenge in mice (Table II). The intraperitoneally administered GAMG potently inhibited the PCA reaction on mice. Meanwhile, the orally administered GAMG slightly exhibited the inhibitory activity. In addition, the intraperitoneally administered GL and GA also significantly inhibited the PCA reaction. These results suggest that GL, GAMG and GA all have anti-allergic activity.

The inhibitory effect of GAMG on contact hypersensitivity induced by oxazolone was also investigated (Fig. 1). Dexamethasone, which was used as a standard agent, exhibited potent inhibitory activity. GA exhibited weak inhibitory activity. However, GAMG and GL did not inhibit contact hypersensitivity. Among these compounds, the most potent anti-allergic agent appears to be GA.

In addition, to understand the anti-allergic mechanism of GAMG, this study investigated the murine macrophage cell line, RAW 264.7, induced by LPS for 24 h. The nitrite concentration in the induced cells increased 4.5-fold. However, when the cells were treated with 1.0 to 200  $\mu$ M of GL, GAMG and GA for 24 h, the nitrite concentration

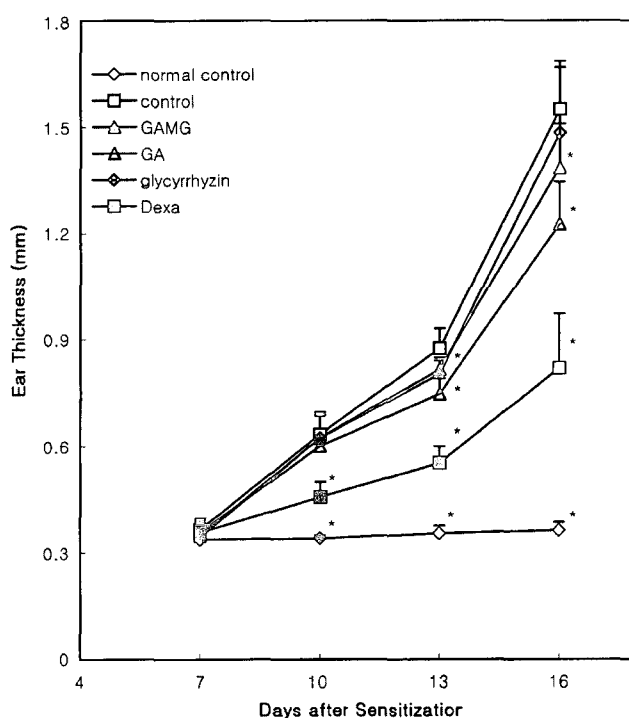
**Table II.** Inhibitory effect of GL and GAMG on the PCA reaction

Group	Dose (mg/kg)	Inhibition (%)	
		p.o.	i.p.
GL	5	16.2 $\pm$ 4.2	50.9 $\pm$ 1.2
GAMG	5	28.5 $\pm$ 3.1	42.7 $\pm$ 1.2
GA	5	— <sup>a</sup>	61.0 $\pm$ 1.5
DSCG	50	38 $\pm$ 0.2	—
Azelastine	5	71.5 $\pm$ 4.3	70.0 $\pm$ 5.6

All agents were administered *p.o.* or *i.p.* prior to challenge with antigen. GL, glycyrrhizin; GAMG, 18 $\beta$ -glycyrrhetic acid-3-O- $\beta$ -D-glucuronide; GA, 18 $\beta$ -glycyrrhetic acid; DSCG, disodium cromoglycate.

Values are expressed as means $\pm$ S.D. (n=5).

<sup>a</sup>not detectable



**Fig. 1.** Effects of GL, GAMG, GA on the contact hypersensitivity of mice induced by the repeated application of oxazolone. One percent 1% of the agents tested was applied to the mouse ear. GL, glycyrrhizin; GAMG, 18 $\beta$ -glycyrrhetic acid-3-O- $\beta$ -D-glucuronide; GA, 18 $\beta$ -glycyrrhetic acid; Dexa, dexamethasone. Values represent means $\pm$ S.D. for eight mice. \* $P$ <0.05, \*\* $P$ <0.001; significantly different from the control group.

was significantly reduced in a dose dependent manner with IC<sub>50</sub> values of 90, 120 and 50  $\mu$ M, respectively. In addition, the IC<sub>50</sub> values of dexamethasone was observed to be 10  $\mu$ M.

GL, GAMG and GA inhibited the release of  $\beta$ -hexosaminidase from the RBL-2H3 cells. These compounds also inhibited nitric oxide production in the LPS-induced RAW 264.7 cells, although Kondo and Takano (1994) reported that glycyrrhizin induced NO production in the macrophages. These results suggest that the anti-allergic activity of GAMG and GA, which is a metabolite of GL by human intestinal bacteria, is more potent than that of GL. However, these compounds did not show any antioxidant activity on the DPPH free radical and superoxide radical generation system. Therefore, the inhibitory mechanism of GAMG on nitric oxide production in the RAW264.7 cells and the  $\beta$ -hexosaminidase release from the RBL-2H3 cells appears to be different from those of antioxidants, such as quercetin (Data not shown). Thus far, it is unclear if the anti-inflammatory effect of GAMG is associated with the anti-allergic effect observed by the inhibitory action on the mast cell degranulation process *in vitro*. Nevertheless, it will be valuable to further examine the anti-allergic and anti-inflammatory effect of GAMG since this compound

**Table III.** Inhibitory effect of GAMG on the nitric oxide production of LPS-induced RAW264.7 cells

Compound	IC <sub>50</sub> (mM) NO
GL	0.09
GAMG	0.12
GA	0.05
Dexamethasone	0.01

GAMG, 18 $\beta$ -glycyrrhetic acid-3-O- $\beta$ -D-glucuronide; GA, 18 $\beta$ -glycyrrhetic acid.

GL, glycyrrhizin; GAMG, 18 $\beta$ -glycyrrhetic acid-3-O- $\beta$ -D-glucuronide; GA, 18 $\beta$ -glycyrrhetic acid

possesses anti-allergic activity.

In addition, Mizutani *et al.* (1994) reported that the intensity of the sweetness of GAMG was 940 times that of sucrose, although GL was 170 times the sweetness of sucrose. The intensity of the sweetness of GAMG was found to be much higher than that of GL.

Based on these findings, GAMG may be useful as a new food additive as well as an anti-allergic agent.

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