

## Short communication

## Radical scavenging ability of glycyrrhizin

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## ABSTRACT

Glycyrrhizin (Gly), a major constituent of licorice root, has been used for the treatment of chronic liver diseases in Japan. Reports have been contradictory as to whether Gly scavenges hydroxyl radicals and superoxide anion radicals. We examined the radical scavenging abilities of Gly and glycyrrheic acid (GA), an aglycon of Gly. Gly and GA did not scavenge hydroxyl radicals or superoxide anion radicals, but both scavenged 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, in contrast to previous reports. The scavenging abilities of Gly and GA might play a role in the treatment of chronic liver diseases.

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## 1. Introduction

Glycyrrhizin (Gly), a major constituent of licorice root, is known to have anti-inflammatory,<sup>1,2</sup> antiallergic,<sup>3–6</sup> and antiviral<sup>7,8</sup> activities, and has been used for the treatment of chronic liver diseases by intravenous or oral administration in Japan. Many reports about the biological action of Gly and its mechanisms have been published.<sup>9–11</sup> Gly acts as a radical scavenger in biological systems, such as ischemia–reperfusion injury,<sup>12,13</sup> macrophage-like cells,<sup>14</sup> and gastric epithelial cells.<sup>15</sup> However, conflicting results have been reported regarding whether Gly itself scavenges radicals. Nagai et al reported that Gly had a potent hydroxyl radical trapping action, but did not trap superoxide anion radicals or 1,1-diphenyl-2-picrylhydrazyl (DPPH).<sup>12</sup> Akamatsu et al reported that Gly did not reduce any reactive oxygen species generated in a cell-free xanthine–xanthine oxidase (XOD) system.<sup>16</sup> Kato et al reported that Gly only slightly scavenged superoxide anions generated by hypoxanthine, and that the XOD reaction and did not scavenge DPPH and NO radicals.<sup>14</sup>

Investigating whether Gly scavenges radicals is important to determine the action mechanisms. In the present paper, we describe the ability of Gly to scavenge radicals, including hydroxyl radicals, superoxide anion radicals, and DPPH radicals. Our results showed that Gly scavenged DPPH radicals. Gly administered orally is hydrolyzed to glycyrrheic acid (GA) by  $\beta$ -glucuronidase.<sup>17,18</sup> We also examined whether GA, an aglycon of Gly, scavenged radicals.

## 2. Materials and methods

## 2.1. Materials

Gly (monoammonium salt) and hypoxanthine (Hyp) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). XOD (from cow milk, 20 unit/ml) was obtained from Roche Diagnostics K.K. (Tokyo, Japan), diethylenetriamin pentaacetic acid (DETAPAC) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) from Dojindo Laboratories (Kumamoto, Japan), DPPH from Nacalai Tesque Inc. (Kyoto, Japan), and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals were from commercial sources in the highest available analytical grade.

## 2.2. Preparation of sample solution

Gly was dissolved in 10% ammonia water and diluted to the desired concentration with water.

## 2.3. Hydroxyl radical scavenging assay

Hydroxyl radicals, generated by the Fenton reaction, were trapped by DMPO, and the electron spin resonance (ESR) spectra of DMPO–OH were recorded. The reaction mixture was prepared by combining 50  $\mu$ l of 0.5 mM FeSO<sub>4</sub>–DETAPAC in 100 mM phosphate buffer (pH 7.4), 100  $\mu$ l of various concentrations of Gly, 50  $\mu$ l of 920 mM DMPO, and 50  $\mu$ l of 0.1 mM H<sub>2</sub>O<sub>2</sub> in 100 mM phosphate buffer (pH 7.4) in the order listed for a total volume of 250  $\mu$ l. One minute after the addition of H<sub>2</sub>O<sub>2</sub>, ESR spectra were recorded on a

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JES-TE300 spectrometer with a TE/AT system (JEOL Ltd., Tokyo, Japan). Measurements were carried out at room temperature under the following conditions: magnetic field of  $336.1 \pm 5$  mT, microwave power of 5.00 mW, frequency of 9.42 GHz, modulation amplitude of 0.063 mT, sweep time of 2.0 min, response time of 0.1 s, and received gain of  $\times 400$ . The intensity of the DMPO–OH (DMPO spin adduct of the OH radical) signal was measured as a ratio of the signal intensity at the lowest magnetic field to that of manganese oxide used as an internal standard. A TEMPOL solution was used for the primary standard of ESR absorption, and results are shown as spin concentrations.

#### 2.4. Superoxide anion radical scavenging assay

Superoxide anion radicals, generated by the Hyp–XOD system, were trapped by DMPO, and the ESR spectra of DMPO–OOH were recorded. The reaction mixture was prepared by combining 50  $\mu$ l of 2 mM Hyp in 100 mM phosphate buffer (pH 7.4), 35  $\mu$ l of 5.5 mM DETAPAC in 100 mM phosphate buffer (pH 7.4), 25  $\mu$ l of various concentrations of Gly, 25  $\mu$ l of 8.0 M dimethyl sulfoxide (DMSO), 15  $\mu$ l of 9.2 M DMPO, and 50  $\mu$ l of 0.2 unit/ml XOD in 100 mM phosphate buffer (pH 7.4) in the order listed for a total volume of 200  $\mu$ l. One minute after the addition of XOD, ESR spectra were recorded, as in the hydroxyl radical scavenging assay, under the following conditions: magnetic field of  $336.4 \pm 5$  mT, microwave power of 8.00 mW, frequency of 9.43 GHz, modulation amplitude of 0.063 mT, sweep time of 2.0 min, response time of 0.1 s, and received gain of  $\times 320$ . The intensity of the DMPO–OOH (DMPO spin adduct of the  $O_2^-$  radical) signal was measured as described above.

#### 2.5. DPPH radical scavenging assay

A mixture was prepared by adding 100  $\mu$ l of various concentrations of Gly to 100  $\mu$ l of 0.25 mM DPPH in ethanol. The mixture was allowed to stand at room temperature for 30 min, and ESR spectra were recorded under the following conditions: magnetic field of  $336.0 \pm 5$  mT, microwave power of 5.00 mW, frequency of 9.42 GHz, modulation amplitude of 0.079 mT, sweep time of 2.0 min, response time of 0.1 s, and received gain of  $\times 100$ . The intensity of the DPPH signal was measured as described above.

#### 2.6. Statistical analysis

Data were analyzed using Student's *t* test, and *p* values of less than 0.05 were considered significant.

### 3. Results

The ESR signal of DMPO–OH, the hydroxyl radical adduct, was observed in the reaction system, and the signal disappeared upon addition of DMSO, a hydroxyl radical scavenger (data not shown). The concentration of DMPO–OH did not decrease upon addition of Gly, even at the final concentration 10 mM (Fig. 1). The spin concentration of DMPO–OH increased with increasing GA concentration.

The concentration of DMPO–OOH did not decrease upon addition of Gly, and similar results were obtained with GA addition (Fig. 2).

The DPPH radical spectrum showed a pentad signal (Fig. 3). The signal intensity was attenuated with increasing Gly concentration, and nearly complete reduction of DPPH was observed at a Gly concentration of 12.5 mM. GA showed a greater reduction of DPPH than Gly, resulting in complete reduction at 2.5 mM (Fig. 4). The concentrations corresponding to a 50% reduction in the signal intensity of DPPH ( $EC_{50}$ ) were 2.18 mM Gly and 0.38 mM GA.

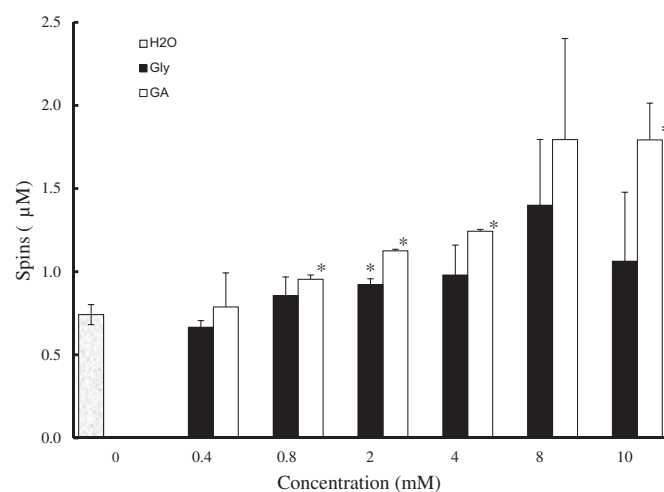


Fig. 1. OH radical scavenging ability of Gly and GA. Data represent the mean with SD ( $n = 2-3$ ). \* $p < 0.05$ .

### 4. Discussion

Reports have been contradictory as to whether Gly reduces radicals. Nagai et al reported that Gly reduced OH radicals, but not  $O_2^-$  or DPPH radicals, based on ESR measurements.<sup>12</sup> Akamatsu et al reported that Gly did not reduce  $O_2^-$  or OH radicals in a cell-free system.<sup>16</sup> Kato et al reported that Gly reduced  $O_2^-$  radicals slightly, but did not reduce DPPH radicals.<sup>14</sup>

Our results showed that neither Gly nor GA reduced OH radicals; instead, the concentration of radicals increased, in contrast to the report of Nagai et al. They reported that 1 mM Gly trapped about 90% of DMPO–OH, but detailed experimental conditions were not described. The amount of OH radicals generated by the Fenton reaction would be much greater in our experiments than in the work of Nagai et al. Our results showed that Gly or GA might activate the Fenton reaction or produce OH radicals. Akamatsu et al reported an assay of  $O_2^-$  formation for the reduction of cytochrome *c* by measuring absorbance at 550 nm; OH radicals were analyzed with a gas chromatograph to quantitate the amount of ethylene formed from  $\alpha$ -keto-methylbutyric acid.<sup>16</sup> We estimated the amount of  $O_2^-$  and OH radicals as the DMPO adduct with ESR. Results showed that Gly did not scavenge  $O_2^-$  and OH radicals, consistent with the report by Akamatsu et al. Thus, the differences between our results and those of Nagai et al were likely due to experimental conditions, not the assay method.

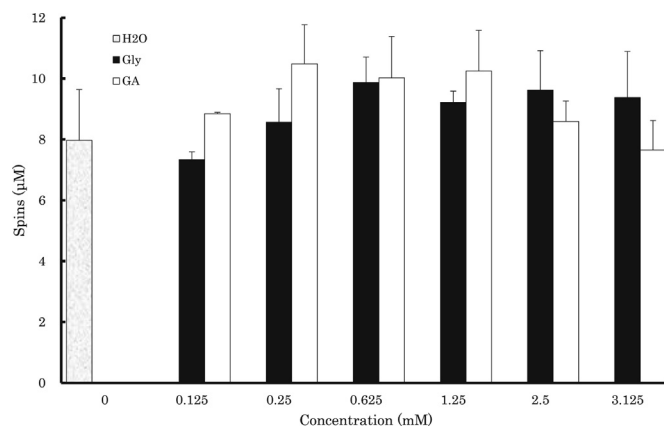


Fig. 2.  $O_2^-$  radical scavenging ability of Gly and GA. Data represent the mean with SD ( $n = 2-3$ ).

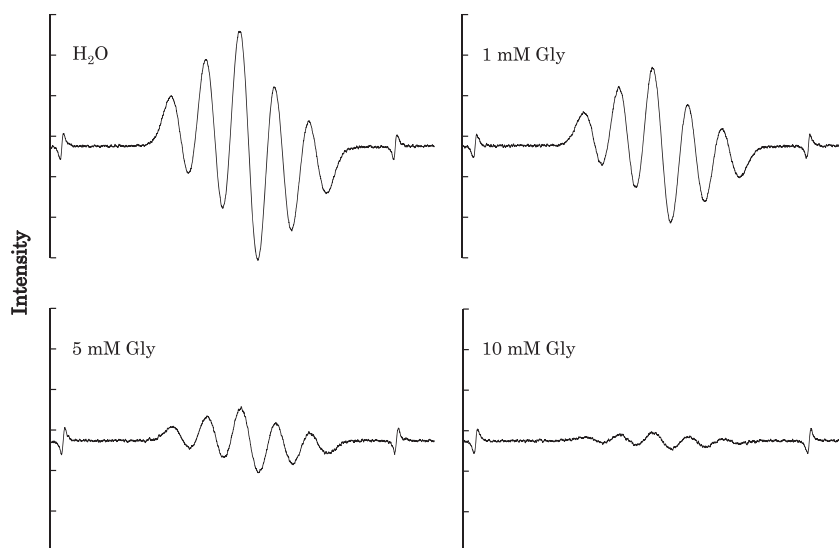


Fig. 3. ESR spectra of DPPH radicals in the absence or presence of Gly.

Previous studies demonstrated that Gly did not reduce DPPH radicals based on ESR and absorbance measurements.<sup>10–12</sup> However, in our experiments, Gly reduced DPPH radicals at increased Gly concentrations. Racková et al<sup>11</sup> used a low Gly concentration of 0.6 mg/ml (714  $\mu$ M). Nagai et al<sup>12</sup> and Kato et al<sup>14</sup> used Gly at 1 mM and 12.2 mM, respectively. Although they did not describe the detailed assay conditions, they may have measured DPPH immediately after the reaction mixture was prepared. The reaction of DPPH and Gly was not fast. Many investigators have measured the reduction of DPPH 30 min after mixing the test sample and DPPH solutions. The reaction of DPPH by Gly or GA began immediately after mixing, reached about 50% after 1 min, and reached a plateau after 30 min at room temperature (data not shown). Thus, we recorded the ESR spectrum 30 min after mixing DPPH with Gly or GA.

DPPH reduction required Gly or GA at concentrations that were 100 times and 20 times, respectively, that of DPPH. One mole of ascorbic acid or  $\alpha$ -tocopherol, well-known reducing agents of DPPH, reduces 2 mol of DPPH.<sup>19</sup> Hence, the effects of Gly and GA on DPPH reduction were not strong.

Whether the reducing abilities of Gly or GA play a role in biological systems is not clear. Further studies are currently under way in our laboratory.

### Conflicts of interest

All authors have none to declare.

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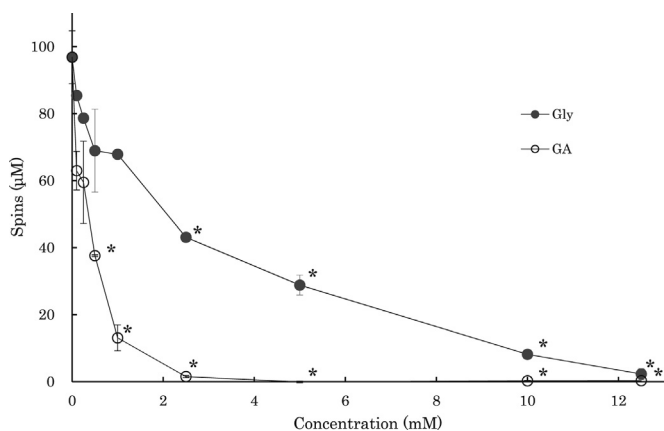


Fig. 4. DPPH radical scavenging ability of Gly and GA. Each point represents the mean with SD ( $n = 2–3$ ). \* $p < 0.05$ .