Localization of Enzymes Involved in Metabolism of Glycyrrhizin in Contents of Rat Gastrointestinal Tract

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Most digested food 2 h after overnight feeding in rat remained in the stomach, duodenum, upper small intestine, lower small intestine, cecum and colon, all of which indicated pH between 4 and 7 and had glycyrrhizin (GL) hydrolizing activity. This enzyme activity was highest in the cecal and colonic contents among all gastrointestinal contents. Also, 3α-hydroxyglycyrrhetinic acid (3α-hydroxyGA) and 3β-hydroxyglycyrrhetic acid (3β-hydroxyGA) oxidizing enzymes were localized in the same cecal content. Namely, rat gastrointestinal bacteria had the ability to hydrolyze GL to 3β-hydroxyGA by glycyrrhetin β-δ-glucuronidase and to oxode 3β-hydroxyGA and 3α-hydroxyGA to 3α-oxoGA by 3β-hydroxyglycyrrhetinase and 3α-hydroxyglycyrrhetinase, respectively. In medium of pH 1 to pH 10, metabolites 3β-hydroxyGA, 3-oxoGA and 3α-hydroxyGA obtained from the metabolism of GL were the highest in pH 8. The intestinal contents of pH 6 or pH 7 were able to produce metabolites 3β-hydroxyGA in the metabolism of GL. However, the stomach content at pH 4.2 was lowest in metabolite 3β-hydroxyGA. It is unknown whether or not GL is metabolized to 3β-hydroxyGA by the stomach content in vivo.

Key words: glycyrrhizin; glycyrrhetin β-δ-glucuronidase; 3β-hydroxyglycyrrhetic acid; intestinal bacteria

3β-Hydroxyglycyrrhetic acid (3β-hydroxyGA), an aglycone of glycyrrhizin (GL, a main component of liquorice, Glycyrrhiza glabra L.), has a triterpenoid structure and a pronounced anti-inflammatory property. GL has a steroid-like action. From metabolites obtained in the metabolism of GL by human intestinal flora, GL is hydrolyzed to 3β-hydroxyGA, which is converted to 3α-hydroxyglycyrrhetic acid (3α-hydroxyGA) through 3-oxo-hydroxyglycyrrhetic acid (3α-oxoGA). The enzymes involved in the metabolism of GL are GL β-δ-glucuronidase, which hydrolyzes GL to 3β-hydroxyGA in Eubacterium sp. GLH, 3β-hydroxysteroid dehydrogenase, oxido-reducing between 3β-hydroxyGA and 3α-oxoGA in Ruminococcus sp. PO1, and 3α-hydroxyglycyrrhetinase (3α-hydroxyGA) dehydrogenase, oxido-reducing between 3α-hydroxyGA and 3α-oxoGA in Clostridium innocuum isolated from human fecal flora. After oral administration of GL to the rat, metabolite 3β-hydroxyGA was detected in the plasma. This suggested that GL was hydrolyzed to 3β-hydroxyGA by bacteria in the digestive tract, internal organs like the liver or both. Data on the enzymes involved in the metabolism of GL by the contents of various regions in the gastrointestinal tract are difficult to obtain from examinations on humans. This paper reports a survey of metabolites and enzymes involved in the metabolism of GL based on contents of the rat digestive tract, and the effect on medium of pH 1 to 10 of rat intestinal bacteria.

MATERIALS AND METHODS

Chemicals GL monoammonium and 3β-hydroxyGA were purchased from Tokyo Kasei Kogyo Co. (Tokyo) and Nacalai Tesque Inc. (Kyoto), respectively. 3-OxoGA and 3α-hydroxyGA were kindly supplied by Dr. M. Hattori, Toyama Medical and Pharmaceutical University, Japan. GAM broth was a product of Nissui Seiyaku Co., Tokyo. All other reagents were of the best quality commercially available.

Gastrointestinal Flora Under anaerobic conditions, the contents of the stomach, duodenum, small intestine, cecum and colon obtained from the Wistar strain of male rats (7 weeks old) were used for the preparation of bacteria without storage. The rat was given no food at all for 2 h after consuming diet. The stomach, duodenum, small intestine, cecum and colon were dissected from one another. The duodenum was cut at 7 cm from the stomach. The small intestine was cut in two parts of equal length, the upper small intestine and lower small intestine. The gastrointestinal contents were separately transferred to a glass tube and to GAM medium. In the tube, measurement was made of pH of the gastrointestinal contents by adding distilled water (g/ml); treatment was also made of suspension containing 20 mM potassium phosphate buffer (pH 7.2, g/ml) by a sonicator. Sonicated bacteria were centrifuged at 6000 g × 10 min at 4°C, and the supernatant fluid was used to measure the enzyme activities. The enzyme activities did not occur in control animals because the examination used a suspension of the gastrointestinal contents. In the GAM medium, a mixture of the gastrointestinal contents and the medium (g/10 ml) was precultured at 37°C overnight in anaerobic box. The preculture was then added to 9 volumes of fresh GAM medium in the presence and absence of 1 mM GL. That in the absence of 1 mM GL was cultivated for measurement of the enzyme activities involved in the metabolism of GL, and that in the presence of 1 mM GL was used for the quantitative analysis of metabolites in the metabolism of GL. GL hydrolyzing and 3α-hydroxyGA and 3β-hydroxyGA oxidizing activities were recovered in the precipitated bacterial fraction of the cultured medium after centrifugation, but not in the supernatant fluid.

Enzyme Assay and Bacterial Growth The enzyme activities for hydrolysis of GL and oxidation of 3α-hydroxyGA and 3β-hydroxyGA were measured as follows: The assay mixture for hydrolysis consisted of 0.1 mmol GL, 50—100 µl of the enzyme solution and
50 mm acetate buffer (pH 5.6) in a final volume of 0.5 ml. The assay mixture for oxidation consisted of 20 μmol 3α-hydroxyGA or 0.1 mmol 3β-hydroxyGA, 0.8 mmol NADP+, 50—100 μl of the enzyme solution and 50 mm potassium phosphate buffer (pH 8) in a final volume of 0.5 ml. Both mixtures were incubated at 37°C for 15 min and the reaction was stopped by the addition of 50 μl of 1 M HCl, then extracted with ethyl acetate. The products were detected on thin layer chromatography (TLC) plate as described below. The bacterial growth was monitored by measuring the turbidity at 650 nm. The assay of four rats was performed and the turbidity value was obtained.

**Determination of Metabolites** The cultured medium (100 μl) containing GL was acidified by the addition of 50 μl of 1 M HCl. Metabolites were extracted twice with 2 ml of ethyl acetate. 3α-HydroxyGA, 3β-hydroxyGA and 3-oxoGA were separated on TLC plates (Merck, Silica gel 60F-254, layer thickness 0.25 mm) by a solvent system of chloroform–petroleum ether–acetic acid (5 : 5 : 1, v/v). 3α-HydroxyGA, 3β-hydroxyGA and 3-oxoGA as metabolites and authentic compounds were detected on TLC plates under UV light. The quantity was measured with a TLC scanner (λ = 250 nm, λ = 400 nm) using calibration lines obtained with authentic samples.

**RESULTS**

**GL Hydrolyzing and 3α-HydroxyGA and 3β-HydroxyGA Oxidizing Activities in Rat Gastrointestinal Contents** Rat gastrointestinal contents at 2h after an overnight feed indicated between pH 4 and 7 with the stomach content being pH 4.2. Among the enzymes involved in the metabolism of GL, the GL hydrolyzing enzyme was localized in the stomach, duodenal, upper small intestinal, lower small intestinal, cecal and colonic contents (Table 1). Activity of this enzyme was highest, however, contents of the cecum and colon. Also, 3α-hydroxyGA and 3β-hydroxyGA oxidizing enzymes were localized in the cecal content. These results indicated that gastrointestinal bacteria in all regions of the rat digestive tract had GL hydrolyzing enzyme and the ability to metabolize 3β-hydroxyGA from GL. It is unknown whether bacteria which are a target by cultivation of the gastrointestinal contents can multiply or not. Stomach contents possessing weak GL hydrolyzing activity and the gastrointestinal contents except that from the cecum which showed 3α-hydroxyGA and 3β-hydroxyGA oxidizing activities were cultivated in GAM medium under anaerobic conditions.

**GL Hydrolyzing and 3α-HydroxyGA and 3β-HydroxyGA Oxidizing Activities by Cultivation of Rat Gastrointestinal Contents** The stomach, duodenal, cecal and colonic contents had all of the enzymes involved in the metabolism of GL, indicating bacterial growth (Fig. 1). GL hydrolyzing activity was the lowest in the stomach content and highest in contents of the duodenum and cecum. These results indicated that bacteria possessing GL hydrolyzing enzyme were not grown by cultivation of the stomach contents. Namely, other bacteria except those possessing GL hydrolyzing enzyme were predominant. Figure 1 also shows two peaks of GL hydrolyzing activity; one occurred in earlier periods (12 to 36 h) of cultivation and the other in later periods (48 to 60 h).

3α-HydroxyGA and 3β-hydroxyGA oxidizing activities were found only in the cecal content (Table 1). By cultivation of the gastrointestinal contents, these enzyme activities then occurred in the stomach, duodenal, cecal and colonic contents indicating growth of bacteria.

**Metabolic Time Course of GL by Rat Gastrointestinal Flora** GL was metabolized to 3β-hydroxyGA and 3-oxoGA by the contents in all regions of the digestive tract. Metabolite 3β-hydroxyGA in the duodenal, cecal and colonic contents was higher than in those of the stomach, upper small intestine and lower small intestine. 3β-HydroxyGA increased linearly and reached a maximal stage by cultivation for 48 to 60 h; metabolite 3α-hydroxyGA was also found in the stomach, cecal and colonic contents (Fig. 2). Bacteria possessing GL hydrolyzing and 3α-hydroxyGA and 3β-hydroxyGA oxidizing enzymes are thus confirmed to exist in the contents of the rat gastrointestinal tract. These results indicated that rat gastrointestinal bacteria have the same metabolic GL pathway as human intestinal bacteria.

**Effect on Medium of pH 1 to 10 on Gastrointestinal Bacteria** The cecal content was cultivated into medium adjusted to pH 1 to 10 containing 1 mm GL. Metabolite 3β-hydroxyGA was the highest in medium of pH 8 and was not detected in medium of pH 1 to 3. As shown in Table 1, the stomach content was pH 4.2 and the contents of other regions were between pH 6 and 7. These results indicated that GL is difficult to metabolize in an acidic environment. It is therefore unknown whether GL is metabolized in the contents of the stomach or not, although it is definitely metabolized in contents of the intestinal tract at between pH 6 and 7.

<table>
<thead>
<tr>
<th>Region</th>
<th>pH</th>
<th>GL hydrolyzing activity (nmol/min/mg content)</th>
<th>3β-HydroxyGA oxidizing activity</th>
<th>3α-HydroxyGA oxidizing activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>4.2</td>
<td>3.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Duodenum</td>
<td>6.2</td>
<td>28.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Upper small intestine</td>
<td>6.6</td>
<td>18.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lower small intestine</td>
<td>6.6</td>
<td>18.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cecum</td>
<td>6.2</td>
<td>86.9</td>
<td>9.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Colon</td>
<td>6.6</td>
<td>69.8</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The value of the enzyme activities was shown by wet weight of rat gastrointestinal contents and means of four male rats. ND: not detected.
DISCUSSION

Hattori et al.\(^3\) reported the metabolism of GL by human intestinal bacteria; however, they did not detect 3-oxoGA as a metabolite in this metabolism. 3-OxoGA was obtained as a metabolite in the metabolism of GL by cultivation of rat gastrointestinal contents (Fig. 2). Thus, the metabolism of GL was demonstrated to be included in rat gastrointestinal flora. GL was hydrolyzed to 3β-hydroxyGA by GL β-D-glucuronidase and 3α-hydroxyGA and 3β-hydroxyGA were oxidized to 3-oxoGA by 3α-hydroxyGA dehydrogenase and 3β-hydroxyGA dehydrogenase, respectively (Fig. 4).

No information exists about the enzymes involved in
Fig. 3. Effect on the Metabolism of GL and Bacterial Growth in Medium of pH 1 to 10
Rat cecal content (10 ml/mg) was anaerobically precultured in GAM medium at overnight. This precultured medium was cultivated by addition to GAM medium adjusted to pH 1 to 10 containing 1 mM GL for 3d. Metabolites were measured as described in Materials and Methods. ■, 3β-hydroxyGA; □, 3-oxoGA; □, 3α-hydroxyGA.

Fig. 4. Metabolism of GL by Rat Gastrointestinal Flora

the metabolism of GL by rat gastrointestinal bacteria. GL hydrolyzing activity indicated two peaks of growth at 12 to 36 h and 48 to 60 h by cultivation (Fig. 1), suggesting that bacteria possessing this enzyme are of two types. These bacteria are identical with time of growth in the culture of Ruminococcus sp. POI-3 (6 to 12 h) and Eubacterium sp. GLH (24 to 48 h) possessing GL β-D-glucuronidase isolated from human fecal flora. Wang et al. reported that no degradation of GL was observed in the small intestinal contents of rats within 3 h. As a condition of their cultivation, a saline solution of 1 mM GL warmed to 37 °C was added to each portion of gastrointestinal contents (5 ml/g wet weight). However, the present paper describes that GAM medium contain-
ing 1 mM GL was cultivated by the addition of the gastrointestinal contents, so GL was metabolized to 3β-hydroxyGA in all these contents including those of the upper small intestine and lower small intestine (Fig. 2). Disagreement in these results may be due to the difference in cultural methods.

3β-HydroxyGA in the plasma after oral administration of GL to the rat appeared within 1 to 24 h. After one hour, a considerable amount of unchanged GL was recovered from the epithelial cells of the stomach and small intestine and a significant amount of metabolite 3β-hydroxyGA was found only in the epithelial cells of the stomach. Early appearance of 3β-hydroxyGA in the plasma may be due to the condition of bacterial cultivation in the digestive tract, the internal organ possessing GL hydrolyzing enzyme (the author's unpublished observations) or both. The metabolism of GL was not accomplished by bacterial cultivation using the general method in acidic medium within 3 d (Fig. 3). Thus, 3β-hydroxyGA accumulated in the epithelial cells of the stomach may be a product degraded from GL by these cells which possess GL hydrolyzing enzyme (unpublished observations). Late and continuous appearance of 3β-hydroxyGA in the plasma well coincided with the metabolism of GL by cultivation of gastrointestinal flora (Fig. 2). GL and its metabolites may be circulated enterohepatically and metabolized continually by gastrointestinal bacteria.

REFERENCES