Glycyrrhizin β-D-Glucuronidase of Eubacterium sp. from Human Intestinal Flora

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A bacterial strain capable of hydrolyzing glycyrrhizin (GL) to glycyrrhetic acid (GA) was isolated from human feces. This bacterium was identified as Eubacterium sp. The GL-hydrolyzing activity increased in parallel with the growth of this bacterium, which also produced β-D-glucuronidase (EC 3.2.1.31) acting on β-D-glucuronides of phenolic compounds such as phenolphthalein mono-β-D-glucuronide. GL-hydrolyzing activity was recovered in the supernatant fraction after disruption of this bacterium with a French press and was partially purified by means of ammonium sulfate fractionation, and Sephadex G-200 and octyl-Sepharose column chromatographies.

GL-hydrolyzing enzyme was separated from the β-D-glucuronidase which hydrolyzes β-D-glucuronides of phenolic compounds by octyl-Sepharose column chromatography, indicating that the GL-hydrolyzing enzyme is a novel type of β-D-glucuronidase.

Keywords—glycyrrhizin β-D-glucuronidase; Eubacterium sp.; human intestinal bacteria; glycyrrhizin; β-D-glucuronidase

Introduction

Glycyrrhizin (GL), an active principle of liquorice, Glycyrrhiza Glabra L. (Leguminosae), is hydrolyzed to 18β-glycyrrhetic acid (GA), the aglycone, and then transformed to 3-epi-18β-glycyrrhetic acid via 3-dehydro-18β-glycyrrhetic acid by human intestinal flora.1) β-D-Glucuronidase activities (EC 3.2.1.31) occur widely in nature from humans to bacteria, and some of these enzymes have been purified.2–12) However, it is not yet known whether or not these enzymes hydrolyzing monoglucuronide conjugates such as phenolphthalein mono-β-D-glucuronide also show activity towards GL, the diglucuronide of GA, though it has recently been reported by Muro et al.13) that Aspergillus niger produces an enzyme which hydrolyzes GL, but not phenolphthalein mono-β-D-glucuronide.

In the present paper, we report the isolation of a human intestinal bacterium capable of hydrolyzing GL to GA, and the partial purification of GL β-D-glucuronidase.

Materials and Methods

Chemicals—GL monoammonium and GA were purchased from Tokyo Kasei Kogyo Co., Tokyo. Glycyrrhetic acid mono-β-D-glucuronide was a gift from Dr. M. Kanaoka of the Research Institute for Wakan-Yaku (Oriental Medicines), Toyama Medical and Pharmaceutical University. Phenolphthalein mono-β-D-glucuronide (PPG), 4-methylumbelliferyl mono-β-D-glucuronide (MUG), pregnenolone mono-β-D-glucuronide (PNG) and δ-saccharic acid-1,4-lactone were purchased from Sigma Chemical Co., U.S.A. p-Nitrophenol mono-β-D-glucuronide (pNPG) was purchased from Nakarai Chemicals, Ltd., Kyoto. General anaerobic medium (GAM) was a product of Nissui Seiyaku Co., Tokyo. All other reagents were of the best commercial quality available.

Isolation of an Intestinal Bacterium Hydrolyzing GL and Culture of the Bacterium—A suspension of human feces was diluted with GAM medium containing 2 mM GL to prepare a series of 10-fold dilutions. An aliquot of the medium producing GA from GL after cultivation was inoculated on GAM agar plates and then incubated at 37°C for 5 d in an anaerobic box. Well-separated colonies were taken and individually inoculated into about two hundred
tubes of GAM medium containing 2 mm GL. A strain capable of hydrolyzing GL was isolated by repeating these procedures. The bacterium isolated was maintained in GAM semisolid agar.

**Thin-Layer Chromatography (TLC)**—TLC for GA and GA mono-β-D-glucuronide was performed on silica gel plates (Merck, Silica gel 60 F-254, layer thickness 0.25 mm) with the solvent system of chloroform–petroleum ether–acetic acid (5:5:1, v/v). GA and GA mono-β-D-glucuronide were detected on TLC plates under ultraviolet (UV) light. The quantity was analyzed with a TLC scanner (λs = 250 nm, λh = 400 nm) by using calibration lines obtained with authentic samples.

**Enzyme Assay**—The enzyme activity for hydrolysis of GL was measured as follows. The assay mixture for hydrolysis contained 50 nmol of GL monoammonium salt, 0.1 M acetate buffer (pH 5.6), and 5—50 μl of enzyme solution in a final volume of 0.2 ml. The mixture was incubated at 37 °C for 10—20 min and the reaction was stopped by adding 0.1 ml of 1 N HCl. Then, the mixture was extracted twice with 2 ml of ethyl acetate. The ethyl acetate solution was concentrated to a small volume and the amount of GA was determined by TLC as described above.

The enzyme activities for hydrolysis of PPG and pNPG were measured by the following method: The assay mixture contained 0.5 μmol of substrate, 50 mM potassium phosphate buffer (pH 6.3), and 5—50 μl of enzyme solution in a final volume of 1.0 ml. The mixture was incubated at 37 °C for 10—30 min and the reaction was stopped by adding 0.25 ml of 5% Na2CO3. The enzyme activities were measured at 565 and 405 nm for hydrolysis of PPG and pNPG, respectively. The reaction rates were calculated by using calibration lines for phenolphthalein and p-nitrophenol, respectively.

**Determination of Acetic Acid**—*Eubacterium* sp. precultured at 37 °C for 48 h was inoculated into 10 ml of peptone yeast extract Fildes broth containing glucose and cultured at 37 °C for 4 d. An aliquot of the culture broth was acidified with 1/10 volume of 1 N HCl and then extracted with a half volume of ether. An aliquot (3 μl) of the extract treated with Na2SO4 was injected into a GC column (4 mm i.d. × 2 m) of Diasolid L-1 (Nippon Chromat. Co., 60—80 mesh) coated with 10% SP-1200 and 1% H3PO4. GC was carried out with a linear increase of column temperature from 100 to 160 °C (8 °C/min).

**Identification of β-D-Glucuronic Acid by High Performance Liquid Chromatography (HPLC)**—Enzyme solution from the octyl-Sepharose column was mixed with 60 μg of glycyrrhizin in 80 μl of 10 mM Tris–HCl buffer (pH 7.4) and then incubated at 37 °C for 1 h. A 50 μl aliquot of the supernatant fraction obtained by centrifugation at 6000 rpm for 20 min was applied to a Synchronpack AX-300 column (250×4.1 i.d.) on a Gilson HPLC system. The column was eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM Tris–HCl buffer (pH 7.5) at a flow rate of 1 ml/min and monitored at 225 nm.

**Determination of Molecular Weight of GL β-D-Glucuronidase**—The molecular weight of the enzyme was estimated by Sephadex G-200 column chromatography according to the method of Andrews14 using phenol red, ovalbumin, bovine serum albumin, rabbit muscle fructose-1,6-bisphosphate aldolase, and blue dextran as molecular weight markers.

## Results

### Isolation and Characterization of a Human Intestinal Bacterium Capable of Hydrolyzing GL into GA

From several hundred colonies, which were formed on GAM agar inoculated with a diluted suspension of feces, no GL-hydrolyzing bacteria were isolated. However, a bacterium hydrolyzing GL, strain A-1, was isolated by repeated colonization of a bacterial suspension capable of hydrolyzing GL. This bacterium in GAM medium converted GL completely to GA. The strain was identified as the genus *Eubacterium*; it was strictly anaerobic, gram-
positive and rod-shaped, showed sugar fermentation activities as listed in Table I and produced acetic acid from glucose. The strain showed similar characteristics to Eubacterium contorium, but the species was not identified.

Culture and GL-Hydrolyzing Activity of the Isolated Bacterium

The bacterial culture reached the stationary phase at 24 h after inoculation as shown in Fig. 1, indicating that this bacterium grow very slowly. GL-hydrolyzing activity increased with cell growth, continued to increase even after the stationary phase, reached the maximal activity 36 h after inoculation, and then decreased gradually.

Purification of GL β-D-Glucuronidase

Eubacterium grown for 37 h in 5 l of GAM medium under anaerobic conditions was used as a starting material for enzyme purification. All fractionation steps were carried out at 0—4 °C. Bacteria collected were disrupted with a French press (Ohtake Factory Co.) and then centrifuged at 12000 × g for 20 min. Most of the GL-hydrolyzing activity was recovered in the supernatant fraction. It was fractionated with ammonium sulfate of 35—60% saturation. The precipitate obtained was dissolved in 20 mM potassium phosphate buffer (pH 7.2) and passed through a column of Sephadex G-200 (2.2 × 80 cm, Pharmacia Fine Chemicals) equilibrated with 10 mM glycine—NaOH buffer (pH 8.5). β-D-Glucuronidase activities toward GL and PPG were eluted in the same fractions as two peaks. The latter peak showed a molecular weight of about 65 kilodaltons (Fig. 2). When the diluted enzyme solution
was applied to the column, the former peak of these hydrolyzing activities decreased or disappeared, suggesting the presence of monomer and dimer of the enzyme(s).

Fractions containing these glucuronidase activities were collected and applied to a column of octyl-Sepharose (1.5 × 7.5 cm, Pharmacia Fine Chemicals) equilibrated with 10 mM glycine–NaOH buffer (pH 8.5) containing 50 mM NaCl. A large amount of PPG-hydrolyzing activity passed through the column with the same buffer containing 50 mM NaCl without absorption on the gel, though a little GL-hydrolyzing activity remained. By rechromatography of this void fraction, PPG-hydrolyzing activity was obtained virtually without contamination by GL-hydrolyzing activity. Most of the GL-hydrolyzing activity was eluted with the same buffer, together with a small amount of PPG-hydrolyzing activity (Fig. 3). The PPG-hydrolyzing activity and GL-β-D-glucuronidase were eluted as separate but partly mutually contaminated peaks in other preparations. pNPG-hydrolyzing activity showed almost the same elution profile as PPG-hydrolyzing activity. The result showed that GL- and PPG-hydrolyzing activities are due to different enzymes, and Eubacterium produced two kinds of β-D-glucuronidases having a similar molecular weight, GL-β-D-glucuronidase and β-D-glucuronidase (EC 3.2.1.31). In these steps of purification, GL-β-D-glucuronidase was purified 6-fold with a yield of 16% (specific activity of the enzyme, 12 nmol/min·mg of protein).

Properties of GL-β-D-Glucuronidase

As shown in Fig. 4, GL- and PPG-β-D-glucuronidases had the same pI value of 4.1, though another shoulder of PPG-hydrolyzing activity was observed at a lower pI region.
Accordingly, these two enzymes were not separable by diethylaminoethyl (DEAE)-cellulose column chromatography.

The pH optimum of GL β-d-glucuronidase was determined to be 5.6 and $K_m$ for GL was 0.11 mm under the standard assay conditions. After hydrolysis of GL with the enzyme, GA was detected on a thin layer plate, but GA mono-β-d-glucuronide was not detected. β-D-Glucuronic acid was detected by HPLC using an anion-exchange column, but diglucuronic acid was not detected. Therefore, it was not clear whether GL was converted to GA and β-d-glucuronic acid via GA and diglucuronic acid or via GA monoglucuronide and glucuronic acid.

The enzyme was able to hydrolyze GA mono-β-d-glucuronide twice as fast as GL. It could not be concluded that GL β-d-glucuronidase does not hydrolyze PPG and pNPG, because the enzyme preparation still contained β-d-glucuronidase activity (EC 3.2.1.31).

Effects of Various Reagents on GL β-D-Glucuronidase

The enzyme was inactivated by trypsin digestion (1 mg/ml, a 25 °C for 30 min) and by heating (at 100 °C for 1 min). Powerful inhibition was obtained with D-saccharic acid 1,4-lactone ($2.5 \times 10^{-3}$ M). Sulfhydryl reagents such as p-chloromercuribenzoic acid ($1.0 \times 10^{-4}$ M) and 5,5'-dithio-bis(2-nitrobenzoic acid) ($1.0 \times 10^{-3}$ M) were found to inhibit completely the enzyme activity. On the other hand, diisopropylfluorophosphate had no effect on the enzyme activity. These results suggest that GL β-d-glucuronidase has sulfhydryl group(s) but not reactive hydroxyl group(s) in its active site.

Discussion

Eubacterium sp. isolated from human feces contained two kinds of β-d-glucuronidases as shown in the elution profile of octyl-Sepharose column chromatography (Fig. 3). One was β-d-glucuronidase (EC 3.2.1.31) showing a substrate specificity similar to that reported hitherto. Namely, the enzyme hydrolyzed PPG, pNPG, MUG and PNG, but not GL. The other was a new type of β-d-glucuronidase, GL β-d-glucuronidase. This enzyme could hydrolyzed GL and GA mono-β-d-glucuronide to GA. However, it was not clear whether or not the enzyme was able to hydrolyze PPG, pNPG, MUG and PNG. These two glucuronidases showed similar physical properties, such as isoelectric point (4.1) and molecular weight (65000), and had the same susceptibility to various reagents, except for the affinity to octyl-Sepharose gel. However, the molecular weight of these enzymes was different from those of β-d-glucuronidases from rat liver lysosomes (280000, 310000),7,10) rat liver microsomes (310000, 290000),10,11) female rat preputial gland (320000),9) bovine liver (290000),8) human placenta (310000)12) and GL hydrolase from Aspergillus niger (150000).13) Further, the isoelectric point of the present enzymes was more acidic than those of β-d-glucuronidases from rat liver lysosomes (5.8—6.6),10) rat liver microsomes (6.9—7.6, 6.7),10,11) female rat preputial gland (6.15, 6.7 and 6.8),6,9) bovine liver (5.1)8) and GL hydrolase from Aspergillus niger (about 6).13)

Human intestinal flora metabolized GL to GA and 3-epi-GA.1) Twelve strains isolated from human intestine did not hydrolyze GL except for a weak hydrolysis of GL by Peptostreptococcus intermedius.15) We found that the newly isolated Eubacterium sp. could convert GL to GA. Ruminococcus sp. isolated from human feces was also capable to hydrolyzing GL to GA.15) However, there seem to be very few strains capable of hydrolyzing GL in human intestinal flora in comparison with the numerous strains capable of hydrolyzing monoglucuronides of phenolic compounds. Other bacterial strains hydrolyzing GL were not isolated from human feces although we surveyed more than five hundred colonies. Accordingly, GL glucuronidase may be rare, whereas β-d-glucuronidase (EC 3.2.1.31) seems to be abundant in intestinal bacteria. In fact, β-d-glucuronidases of Escherichia coli purchased
from Sigma Chemical Co. and of *Bacteroides clostridiiformis* isolated from rat feces in our laboratory could hydrolyze PPG and *p*NPG but not GL (data not shown). *Ruminococcus* sp. capable of hydrolyzing GL also had GL β-D-glucuronidase and β-D-glucuronidase (EC 3.2.1.31) activities, like *Eubacterium* sp. (unpublished data).

Although β-D-glucuronidase of bovine liver purchased from Sigma Chemical Co. could hydrolyzed GL to GA at less than one-hundredth of the rate of hydrolysis of *p*NPG (data not shown), it was not clear whether the enzyme preparation contained GL β-D-glucuronidase in addition to β-D-glucuronidase (EC 3.2.1.31). GL hydrolase purified from *Aspergillus niger* could hydrolyze GL but not PPG or *p*NPG. That enzyme is different from our enzyme, judging from the molecular weight and the isoelectric point.

The absence of GL-hydrolyzing activity with rat liver, and the high activity in rat intestinal flora (unpublished data), means that intestinal bacteria play an important role in GL metabolism, suggesting that intestinal flora may contribute greatly to GL metabolism in humans, too.

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