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HYDROLYSIS OF GLYCYRRHIZIN TO 18β-GLYCYRRHETYL MONOGLUCURONIDE BY LYSOSOMAL β-D-GLUCURONIDASE OF ANIMAL LIVERS

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Abstract—Glycyrrhizin (GL), a main constituent of liquorice, was hydrolysed to 18β-glycyrrhetic acid mono-β-D-glucuronide (GAMG, glycyrrhetyl monoglucuronide) by rat liver homogenate, and the hydrolytic activity was localized in the lysosomes among the same subcellular fractions as acid β-D-glucuronidase activity (*p*-nitrophenyl β-D-glucuronide (pNPG)-hydrolysing activity). Rat liver lysosomes hydrolysed GAMG to 18β-glycyrrhetic acid (GA) at only 30% rate compared with the rate of GL to GAMG. GA was also produced slowly from GL after time lag by the lysosomes. Thus, GL seems to be first hydrolysed to GAMG, which was successively hydrolysed slowly to GA. GL-hydrolysing activity was released together with acid β-D-glucuronidase activity from the lysosomes by sonication. Both activities from the sonicated lysosomes were eluted coincidentally on Sephacryl S-300 and butyl-Toyopearl 650M column chromatography, indicating that both activities are exhibited by the same enzyme. Moreover, GL-hydrolysing activity was inhibited strongly with D-saccharic acid 1,4-lactone, a specific inhibitor of β-D-glucuronidases of various origins. pH optimum of GL-hydrolysing activity was found to be 5.6, different from that (less than 4.0) of pNPG-hydrolysing activity. K_m for GL was found to be 2×10^{-5} M. Although hepatic lysosomes from mouse and cattle hydrolysed GAMG to GA similarly to those from rat, the hydrolysis of GAMG was not detected in lysosomes of human and porcine livers. Accordingly, lysosomal β-D-glucuronidases from human and porcine livers converted GL to GAMG only.

Glycyrrhizin (GL¶, glycyrrhizic acid), a main constituent of liquorice extract (*Glycyrrhiza glabra*), is ingested orally as a sweetener or as a component in oriental medicine. GL shows a steroid-like action [1], and anti-viral [2] and interferon inducing [3] activities. This compound conjugates D-glucuronyl-β-1,2-D-glucuronic acid to the C-3 of 18β-glycyrrhetic acid (GA), which also has potent anti-inflammatory activity [4, 5]. On the other hand, GL seems to cause pseudo-aldosteronism [6], that was suggested to be due to inhibition of renal 11β-hydroxysteroid dehydrogenase by GA [7].

GL is not detected in the sera of human subjects after oral administration of GL, but GA is detected [8]. On the other hand, GL is hydrolysed to GA by human intestinal bacteria [9, 10]. Accordingly, when GL is administered orally, GA, not GL, seems to be absorbed after the hydrolysis of GL to GA in intestine. When GL is injected intravenously to human subjects, GA is also detected in their sera [8]. On the other hand, in rats GA is not detected in the plasma following bolus injection of GL into portal vein, though GA is detected in the plasma following oral administration of GL [11]. Moreover, GL administered intravenously in rats is excreted intact and rapidly into bile [12]. These results suggest that GL administered intravenously is also converted to GA in intestine after bile excretion, though it is reported that GL is hydrolysed to GA by lysosomal β-D-glucuronidases (EC 3.2.1.31) of mouse and rat livers [13]. Moreover, 18β-glycyrrhetic acid mono-β-D-glucuronide (GAMG, 18β-glycyrrhetyl monoglucuronide) is found in the serum of a patient with pseudo-aldosteronism, who has received intravenously large doses of GL for the treatment of liver disease [14]. To elucidate the discrepancy in these results we studied in detail GL hydrolysis with lysosomal β-D-glucuronidases of animal livers.

MATERIALS AND METHODS

Apparatus. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were measured with JEOL GX-270 (^{1}H, 270 MHz) and JEOL GX-400 (^{13}C, 100 MHz) spectrometers using tetramethylsilane as an internal standard. Fast atom bombardment mass spectra (FABMS) were measured with a JEOL JMS DX-300 mass spectrometer under the following conditions: FAB energy, 4 kV; emission current, 10–20 mA; neutral gas, xenon; matrix, glycerol; detection, negative ion.

Chemicals. GL monoammonium salt was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and GL dipotassium salt was given by Maruzen Kasei Co. (Onomichi, Japan). GAMG (1-(18β-glycyrrhet-3-yl)-β-D-glucopyranuroic acid) was synthesized as

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¶ Abbreviations used: GL, glycyrrhizin; GAMG, 18β-glycyrrhetic acid mono-β-D-glucuronide; GA, 18β-glycyrrhetic acid; pNPG, *p*-nitrophenyl β-D-glucuronide; MS, mass spectra.

described in the previous paper [14]. GA was purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and purified by repeated crystallization. Above compounds except for GL monoammonium salt were chromatographically pure. β-D-Glucuronidase (EC 3.2.1.31) of *Escherichia coli* (Type VII) was purchased from the Sigma Chemical Co. (MO, U.S.A.). All other reagents were of the best commercial quality available.

Animals and livers. Wistar strains of male and female rats at 4–14 weeks of age and ddy strains of male mice at 4–6 weeks of age were used. One bovine and three porcine livers were purchased from Nippon Ham Co. (Osaka, Japan). Human liver tissues were obtained from non-involved part of the resected specimen of the metastatic liver tumours.

Preparation of subcellular fractions and lysosomes. Fresh livers obtained from rat, mouse and human were used for preparation without storage, and livers from cattle and porcine after storage at −20° for a few months.

Liver homogenates in 0.25 M sucrose were separated into nuclear, mitochondrial, lysosomal, microsomal and soluble fractions with modification of the methods by Imai *et al.* [15]. Hepatic lysosomes were prepared by centrifuging liver homogenates in 0.15 M KCl at 5000 *g* for 10 min and then the supernatant at 10,000 *g* for 20 min. Lysosomes suspended in 50 mM potassium phosphate buffer (pH 7.0) were sonicated and then centrifuged at 100,000 *g* for 90 min to obtain a clear supernatant.

Thin layer chromatography (TLC). TLC for GL, GAMG and GA was performed on silica gel plates (Merck, silica gel 60 F-254, layer thickness 0.25 mm) as follows. The plate was first chromatographed for GA with a solvent system of chloroform/petroleum ether/acetic acid (5:5:1, by vol.), and secondly for GAMG and GL with a solvent system of acetic acid/*n*-butanol/1,2-dichloroethane/H_2O (4:1:4:1, by vol.). The quantity was analysed with a TLC scanner (Shimadzu CS-910) as described in the previous paper [10].

Assay methods of glucuronidase activity. GL- and GAMG-hydrolysing activities were measured as follows. The assay mixture contained 50 nmol of GL or GAMG and 0.1–1 mg of supernatant protein of sonicated lysosomes in a final volume of 0.5 mL of 0.1 M acetate buffer (pH 5.6). The mixture was incubated at 37° for 10–60 min, and the reaction was stopped by the addition of 0.1 M HCl. Immediately, it was extracted twice with 3 mL of ethyl acetate. After evaporating the ethyl acetate phase, the residue was analysed for GL, GAMG and GA by TLC as described above.

Usual β-D-glucuronidase activity was measured using *p*-nitrophenyl β-D-glucuronide (pNPG) as a substrate as described previously [10], except that 0.1 M potassium phosphate buffer (pH 6.3) in the previous assay method [10] was replaced by 0.1 M acetate buffer (pH 4.5).

Measurement of all enzyme activities was performed in duplicate.

Protein was determined by the method of Lowry *et al.* [16].

Isolation of GAMG produced from GL by rat liver lysosomes. The supernatant (230 mg of protein) of the sonicated lysosomes from four male rats (14 weeks old) was incubated with 26 μmol of GL in 100 mL of 0.1 M acetate buffer (pH 5.6). After incubation at 37° for 1 hr, the reaction was stopped by the addition of 1 M HCl. It was extracted twice with an equal volume of ethyl acetate. After evaporating the ethyl acetate phase, GAMG was isolated as powder by preparative TLC, though GA was also detected on the plates.

1-(18β-Glycyrrhet-3-yl)-β-D-glucopyranuroic acid (GAMG). GAMG had the following spectral properties; FABMS *m/z* (negative ion): 691(M + 2Na-1)$^-$, 668(M + Na-1)$^-$, 651(668-OH)$^-$, 623(668-COOH)$^-$, 469(Aglycone-1)$^-$, 451(469-H_2O)$^-$. ^{1}H-NMR(270 MHz, CD_3OD): δ0.82, 0.87, 1.08, 1.12, 1.14, 1.15, 1.43 (each 3H, s, Me), 4.37(1H, d, J = 7.3 Hz, H-1′), 5.67(1H, s, H-12). ^{13}C-NMR(100 MHz, pyridine-d_5): aglycone moiety, δ39.6(C-1), 26.9(C-2), 88.8(C-3), 40.0(C-4), 55.4(C-5), 17.7(C-6), 33.0(C-7), 43.5(C-8), 62.2(C-9), 37.3(C-10), 199.3(C-11), 128.7(C-12), 169.5(C-13), 45.6(C-14), 26.6(C-15), 26.7(C-16), 32.2(C-17), 48.7(C-18), 41.8(C-19), 44.1(C-20), 31.7(C-21), 38.5(C-22), 28.3(C-23), 16.8(C-24), 17.0(C-25), 18.9(C-26), 23.7(C-27), 28.76(C-28), 28.81(C-29), 179.1(C-30); sugar moiety, 107.0(C-1′), 78.2(C-2′), 75.5(C-3′), 73.5(C-4′), 73.4(C-5′), 150.4(C-6′). On methylation with diazomethane in MeOH, the compound yielded a monomethyl ester, methyl 1-(18β-glycyrrhet-30-yl)-β-D-glucopyranuronate: FABMS *m/z* (negative ion): 682(M + Na-1)$^-$, 667(682-Me)$^-$, 659(M-1)$^-$, 469(Aglycone-1)$^-$, 451(469-H_2O)$^-$.

RESULTS

Lysosomal localization of the hydrolysing activity from GL to GAMG

Rat liver homogenates hydrolysed GL to GAMG, and this hydrolysing activity was localized in the same lysosomal fraction as β-D-glucuronidase, pNPG-hydrolysing activity, judging from their subcellular distributions as shown in Fig. 1. The ratio of GL-hydrolysing activity to pNPG-hydrolysing activity was almost the same, that is 0.024–0.029, in all the fractions. Most of both activities were recovered in the supernatant fraction after centrifugation of the sonicated lysosomes. Although pH optimum of pNPG-hydrolysing activity was found to be less than 4.0 in acetate buffer, that of GL-hydrolysing activity was 5.6 in acetate buffer, and GL-hydrolysing activity in phosphate buffer was much lower than that in acetate buffer.

Figure 2 shows that GL was hydrolysed to GAMG, but not to GA, by the supernatant of the sonicated lysosomes. However, the supernatant hydrolysed slightly GAMG to GA, and the optimal pH of the GAMG-hydrolysing activity was less than 4.0 similar to that of pNPG-hydrolysing activity. On the other hand, β-D-glucuronidase of *Escherichia coli* did not hydrolyse GL at all but did rapidly GAMG to GA (Fig. 2). When the supernatant of the sonicated lysosomes and β-D-glucuronidase of *E. coli* were mixed, GL was hydrolysed to GA. Using the supernatant of the sonicated lysosomes time course of GL-hydrolysis was measured as shown in Fig. 3. GL was converted to GAMG and then a little amount of GA

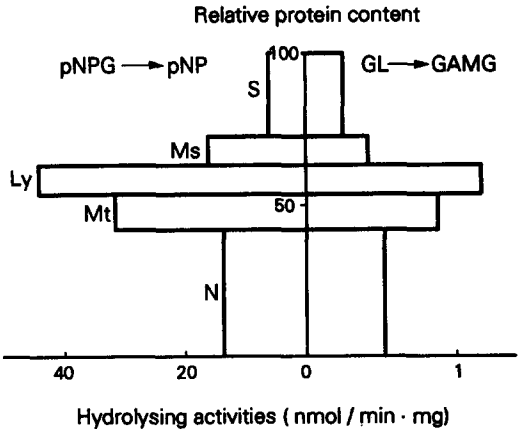

Fig. 1. Subcellular distribution of the activities hydrolysing GL to GAMG and pNPG to pNP in rat liver. The abscissa indicates the specific activity of GL hydrolysis on the right side and that of pNPG hydrolysis on the left side. The subcellular fractions are shown on the ordinate by their relative protein contents (%). N, Mt, Ly, Ms and S represent the nuclear, mitochondrial, lysosomal, microsomal and soluble fractions, respectively.

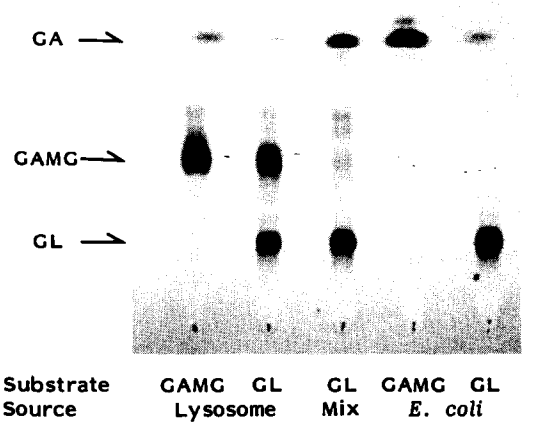

Fig. 2. Hydrolysis of GL and GAMG by rat liver lysosomes and β-D-glucuronidase of *Escherichia coli*. GL was treated with the supernatant (0.57 mg of protein) of the sonicated lysosomes (a second lane from the left side), β-D-glucuronidase (0.5 mg of protein) of *E. coli* (a right lane) and mixture of both (a center lane). GAMG was also treated with the supernatant of the sonicated lysosomes (a left lane) and β-D-glucuronidase of *E. coli* (a second lane from the right side). The reaction was carried out at 37° for 30 min as described in Materials and Methods. A photograph of a silica-gel plate containing fluorescent indicator after development was taken under ultraviolet light. Faint spots of GA at the second lane from the left side and at the right lane were due to the contamination involved in GL used as the substrate.

was detected after long time of incubation. Moreover, the major product from GL with the supernatant of the sonicated lysosomes was identified as GAMG by MS and NMR analyses.

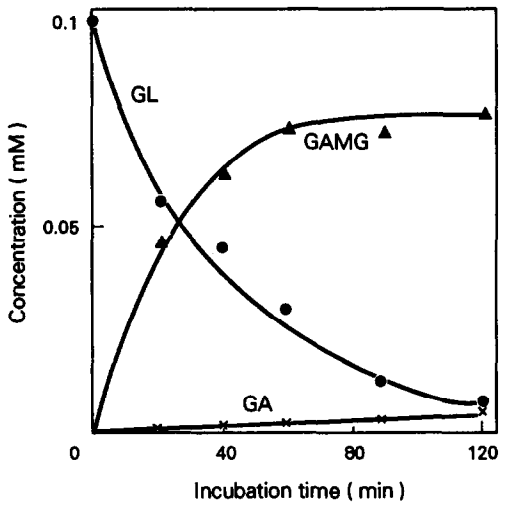

Fig. 3. Time course of GL-hydrolysis with rat liver lysosomes. The supernatant (0.57 mg of protein) of the sonicated lysosomes was incubated with 0.1 mM GL in 0.5 mL of 0.1 M acetate buffer (pH 5.6). Metabolites were analysed at the indicated period of time as described in Materials and Methods.

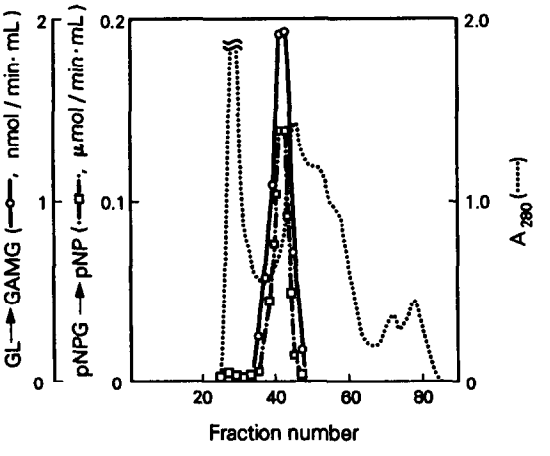

Fig. 4. Sephacryl S-300 column chromatography of GL- and pNPG-hydrolysing activities. The supernatant (60 mg of protein) of the sonicated lysosomes was applied to the Sephacryl S-300 column (1.8 × 78 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.2). The column was eluted with the same buffer and fractions (3 mL per tube) were collected.

These results indicate that GL is hydrolysed to GAMG first and then GAMG is hydrolysed to GA very slowly by rat liver lysosomes.

Hydrolysis of GL to GAMG by lysosomal β-D-glucuronidase

The supernatant of the sonicated lysosomes of rat liver was gel-filtrated on a Sephacryl S-300 (Pharmacia LKB, Biotechnology) column. pNPG- and GL-hydrolysing activities were eluted in the same fractions with the same profile (Fig. 4) and the peak

Table 1. GL-, GAMG- and pNPG-hydrolysing activities of animal lysosomes.

Source of hepatic* lysosomes	Hydrolysing activities† (nmol/min/mg) GL → GAMG	GAMG → GA	pNPG → pNP	GL → GAMG/ pNPG → pNP
Mouse	0.439 ± 0.028‡	0.055 ± 0.009‡	5.12 ± 0.42‡	0.086
Rat	0.607 ± 0.068‡	0.225 ± 0.032‡	24.3 ± 3.99‡	0.025
Cattle	0.075	0.055	5.05	0.015
Pig	0.863 ± 0.119‡	ND	1.20 ± 0.09‡	0.68
Human				
Male 1	1.52	ND	7.30	0.21
Male 2	1.54	ND	8.88	0.17
Female 1	0.96	ND	4.16	0.23

* Livers of three male mice (5 weeks old), six male rats (10 weeks old) and three male pigs (6 months old) and a liver of one adult cattle were homogenated individually, and each lysosomal fraction was prepared.
† The supernatant of the sonicated lysosomes was used.
‡ Means ± SD.
ND, not detected.

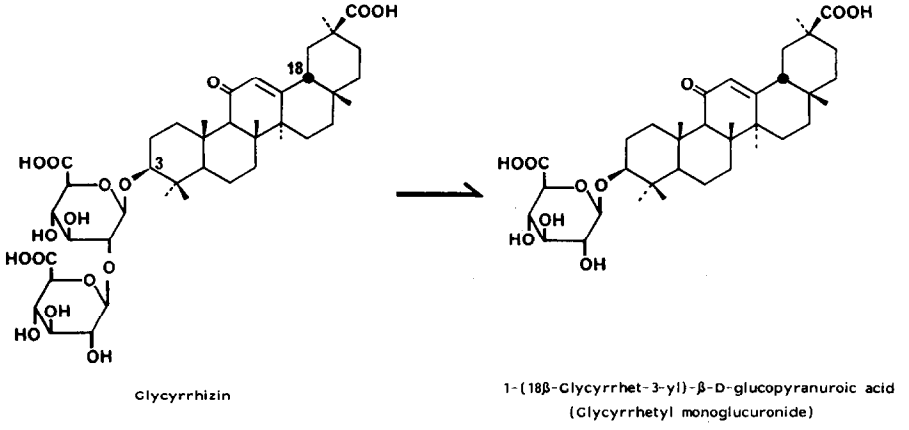

Fig. 5. Hydrolysis of GL to GAMG by lysosomal β-D-glucuronidase of mammalian liver.

was estimated to be around 250 kD of apparent molecular weight from the elution volume. Moreover, a hydrophobic chromatography of the supernatant using a butyl-Toyopearl 650 M (Tosoh Co., Tokyo) column showed the same elution profile of both activities, though a few peaks of the activities were observed. During all the purification procedures the ratios of GL-hydrolysing activity to pNPG-hydrolysing activity were almost constant.

The hydrolysis of GL to GAMG was strongly inhibited with D-saccharic acid 1,4-lactone, a specific inhibitor of β-D-glucuronidases, and its I_{50} was about 2×10^{-9} M. Apparent K_m value for GL was measured to be about 2×10^{-5} M.

These results indicate that the hydrolysis of GL to GAMG is catalysed by β-D-glucuronidase of rat liver lysosomes.

Hydrolysis of GL to GAMG by hepatic lysosomes of other animals

Hepatic lysosomes of mouse, cattle, pig and human also hydrolysed GL to GAMG as shown in Table 1. The lysosomes of mouse and bovine livers hydrolysed GAMG to GA in the same way as that of rat, but with lower than rat in specific activity. However, the hydrolysis of GAMG to GA was not detected at all in lysosomes of porcine and human livers. The specific activity hydrolysing GL to GAMG was the highest in human and the lowest in cattle. On the other hand, the pNPG-hydrolysing activity was the highest in rat and the lowest in pig. Accordingly, the ratios of the GL to GAMG hydrolysing activity to pNPG-hydrolysing activity were less than 10% in mouse, rat and cattle, in which GAMG was hydrolysed to GA, but the ratios were more than 20% in human and porcine, in which GAMG seems not to be hydrolysed.

On butyl-Toyopearl 650 M column chromatography of the supernatant of sonicated porcine lysosomes, GL- and pNPG-hydrolysing activities showed the same elution profile as was the case of rat lysosomes. Thus, it seems that β-D-glucuronidases of porcine and human livers hydrolyse GL to GAMG only, but not to GA (Fig. 5).

DISCUSSION

Various β-D-glucuronidases (EC 3.2.1.31) from

bacteria up to mammals are known. Although researchers have used β-D-glucuronidases on the market, derived from shellfish and helix, for the purpose of the determination of GL by measuring GA after hydrolysis of GL into GA, only a few kinetic studies on the hydrolysis of GL with β-D-glucuronidases have been reported [10, 13]. β-D-Glucuronidase from *Eubacterium* sp. GLH hydrolyses GL and GAMG to GA [10], though the enzyme from *Escherichia coli* hydrolyses GAMG but not GL (Fig. 2). β-D-Glucuronidases from shellfish and helix hydrolysed both GL and GAMG to GA (unpublished data). In the present study lysosomal β-D-glucuronidases of mammals, especially human and pig, converted GL to GAMG (Fig. 5), but did not hydrolyse GAMG further (Table 1). Accordingly, β-D-glucuronidases relating to GL metabolism may be divided into three types from distinction of GL- and GAMG-hydrolysing capacities. The first one has both GL- and GAMG-hydrolysing capacities such as the enzymes from *Eubacterium* sp. GLH, shellfish and helix. The second has GL-hydrolysing activity only such as human and porcine enzymes. The third has GAMG-hydrolysing activity only such as *Escherichia coli* enzyme.

When GL was injected intravenously to human subjects, GA was observed in their sera [8]. However, serum concentrations of GA were lower and its appearance time was later after the intravenous injection of GL than those of GA after oral administration of GL [8]. Moreover, GL in serum seems to be excreted rapidly into intestinal tract through bile, suggested by fast disappearance of GL from sera [8] and by bile excretion of GL in rat [12]. The present study suggests that β-D-glucuronidase of human liver does not convert GL to GA. Accordingly, GA in serum after the intravenous injection of GL may be derived from GA produced by the bacterial hydrolysis of GL in intestine, in a similar way to GA in serum after oral administration of GL.

On the other hand, GAMG was found in the serum of a patient with pseudo-aldosteronism after intravenous administration of large doses of GL [14]. A large amount of GAMG, compared with GA, was present in the serum [14] and little GAMG was produced from GL by intestinal bacteria [9, 10]. In this case GAMG seems to be produced from GL by lysosomal β-D-glucuronidase of liver.

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