Substrate Specificity of Glycyrrhizinic Acid Hydrolase

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Glycyrrhizinic acid hydrolase produced by *Aspergillus niger* selectively hydrolyzed the 3-*O*- β -D-glucuronide linkage of glycyrrhizinic acid. The substrate specificity of this enzyme was investigated for synthetic glucuronides of aliphatic alcohols as well as natural glucuronide saponins. It was revealed that the glucuronide linkage with low molecular weight alcohols was not cleaved by this enzyme, while the 3-*O*- β -D-glucuronide linkage of saponins of oleanolic acid was selectively hydrolyzed. It was also disclosed that both the 4-hydroxyl and carboxyl groups of the glucuronide moiety must be unsubstituted for hydrolysis by this enzyme.

Glycyrrhizinic acid (glycyrrhizin, **1**) is a wellknown pharmacologically active sweet saponin of Licorice roots. Recently, Muro *et al.* isolated an enzyme named "glycyrrhizinic acid hydrolase" (GH) from *Aspergillus niger* GRM3, which was cultivated in a medium containing flowers of *Sophora japonica* Linne.¹⁾ This enzyme selectively hydrolyzed the 3-*O*- β -D-glucuronide linkage of **1** to give the aglycone, glycyrrhetic acid (**2**) and 2-*O*- β -D-glucuronyl-D-glucuronic acid (**4**) in a high yield. It is noteworthy that this hydrolytic action was quite different from that of the general β -glucuronidases,^{2~4)} which hydrolyze **1** to give **2** and 2 mol of D-glucuronic acid. Recently, Kanaoka *et al.* reported that the 3-*O*- β -D-glucuronide of **2** was hydrolyzed by GH, while the 3*O*- β -D-glucuronide ester of **2** was not hydrolyzed.⁵⁾ In order to confirm the scope and limitation of the usefulness of GH as a novel reagent in the chemistry of natural glycosides, its substrate specificity was investigated.

EXPERIMENTAL

Synthesis of β -D-glucuronides (5-8) of methanol, 1-propanol, 2-propanol and cyclohexanol. A mixture of methyl (tri-*O*-acetyl- α -D-glucopyranosyl bromide)uronate (**13**)⁶⁾ (5.3 g), Hg (CN)₂ (5.3 g), an anhydrous aglycone alcohol (10 ml) and anhydrous CH₃CN (20 ml) was stirred at room temperature for 4 hr. The mixture was concentrated to dryness and the residue was dissolved in CHCl₃. After removing the insoluble material by filtration, the filtrate was concentrated to dryness, and to the residue was added 5% methanolic KOH (100 ml). The mixture was stirred at room temperature overnight and filtered. The filtrate was deionized with Amberlite MB-3 and then concentrated to dryness. The product was purified by chromatography on silica gel using a solvent of CHCl₃-MeOH-H₂O (6:4:1, homogeneous) and then on Sephadex LH-20 with a MeOH solvent.

5: a white powder, $[\alpha]_D^{25} - 54.4^\circ$ ($c = 1.10$, H₂O), 50% yield from **13**, ¹H-NMR (in D₂O): δ 4.25 (1H, d, $J = 7.3$ Hz, anomeric proton). *Anal.* Found: C, 40.28; H, 5.90%. *Calcd.* for C₇H₁₂O₇: C, 40.39; H, 5.81%.

6: a white powder, $[\alpha]_D^{25} - 72.6^\circ$ ($c = 1.00$, H₂O), 22% yield from **13**, ¹H-NMR (in D₂O): δ 4.37 (1H d, $J = 7.3$ Hz, anomeric proton). *Anal.* Found: C, 45.72; H, 6.88%. *Calcd.* for C₉H₁₆O₇: C, 45.76; H, 6.83%.

7: a white powder, $[\alpha]_D^{25} - 46.4^\circ$ ($c = 1.09$, H₂O), 38% yield from **13**, ¹H-NMR (in D₂O): δ 4.40 (1H d, $J = 7.8$ Hz, anomeric proton). *Anal.* Found: C, 45.17; H, 7.20%. *Calcd.* for C₉H₁₆O₇·1/2H₂O: C, 44.08; H, 7.40%.

8: a white powder, $[\alpha]_D^{25} - 56.9^\circ$ ($c=0.96$, H_2O), 44% yield from **13**, 1H -NMR (in D_2O): δ 4.44 (1H d, $J=7.8$ Hz, anomeric proton). *Anal.* Found: C, 52.15; H, 7.33%. Calcd. for $C_{12}H_{20}O_7$: C, 52.17; H, 7.30%.

Synthesis of β -D-glucuronides (9, 10 and 11) of l- and d-menthols and 5 α -cholestan-3 β -ol. A mixture of l-menthol (2 g), **13** (2.5 g) and $CdCO_3$ (2.5 g) in anhydrous toluene, after removing moisture by co-distillation with toluene, was refluxed for 2 hr. After concentration to dryness, the residue was treated with $CHCl_3$ to remove the insoluble material by filtration, and the filtrate was concentrated to dryness, yielding a methyl ester triacetate of **9** as colorless needles, mp 125~126.5°C from $MeOH-H_2O$, $[\alpha]_D^{19} - 75.0^\circ$ ($c=0.95$, $CHCl_3$), 46% yield from **13**, 1H -NMR (in $CDCl_3$): δ 4.61 (1H d, $J=8.1$ Hz, anomeric proton). *Anal.* Found: C, 58.02; H, 7.70%. Calcd. for $C_{23}H_{36}O_{10}$: C, 58.46; H, 7.68%. This acetate (600 mg) was saponified with 5% methanolic KOH (50 ml) by stirring overnight at room temperature and followed by neutralization with Dowex 50W-X8 to give **9** as a white powder, $[\alpha]_D^{19} - 85.3^\circ$ ($c=1.09$, $MeOH$), 1H -NMR (in C_5D_5N): δ 5.06 (1H d, $J=7.3$ Hz, anomeric proton).

10 and **11** were synthesized in the same way as with **9**, and elemental analyses were also carried out for the respective crystalline methyl ester triacetates. Methyl ester triacetate of **10**: colorless needles, mp 182~182.5°C from $MeOH-H_2O$, $[\alpha]_D^{19} + 2.3^\circ$ ($c=0.99$, $CHCl_3$), 50% yield from **13**, 1H -NMR (in $CDCl_3$): δ 4.61 (1H d, $J=7.6$ Hz, anomeric proton). *Anal.* Found: C, 58.52; H, 8.04%. Calcd. for $C_{23}H_{36}O_{10}$: C, 58.46; H, 7.68%.

10: a white powder, $[\alpha]_D^{19} - 8.5^\circ$ ($c=1.05$, $MeOH$), 1H -NMR (in C_5D_5N): δ 5.02 (1H d, $J=7.1$ Hz, anomeric proton). Methyl ester triacetate of **11**: colorless needles, mp 177~180°C from $EtOH-H_2O$, $[\alpha]_D^{19} - 6.4^\circ$ ($c=1.19$, $CHCl_3$), 54% yield from **13**, 1H -NMR (in $CDCl_3$): δ 4.65 (1H d, $J=7.8$ Hz, anomeric proton). *Anal.* Found: C, 67.71; H, 9.24%. Calcd. for $C_{40}H_{64}O_{10}$: C, 68.15; H, 9.15%.

11: a white powder, 1H -NMR (in C_5D_5N): δ 5.99 (1H d, $J=7.5$ Hz, anomeric proton). Optical rotation could not be determined due to the low solubility. In this case, a methyl ester triacetate of the 5 α -cholestan-3 β -yl α -D-glucuronide was obtained from the mother liquor of recrystallization of the methyl ester triacetate of **11** as colorless plates, mp 157~161°C from CH_3CN-H_2O , $[\alpha]_D^{19} + 90.3^\circ$ ($c=1.03$, $CHCl_3$), 2% yield from **13**, 1H -NMR (in $CDCl_3$): δ 5.30 (1H d, $J=3.6$ Hz, anomeric proton).

Dimethyl ester (3) of 1. A solution of **1** (517 mg) in 2% methanolic HCl (35 ml) was stirred at room temperature for 2 hr. To the solution was added Ag_2CO_3 , and the resulting precipitate was removed by filtration. The filtrate was concentrated to dryness and the residue was chromatographed on silica gel with a solvent of $CHCl_3$ - $MeOH$ - H_2O (40:15:2, homogeneous) to give **3** in a yield of 75%

as a white powder, $[\alpha]_D^{19} + 55.5^\circ$ ($c=1.03$, $MeOH$). 1H -NMR (in C_5D_5N): δ 3.71 and 3.81 (3H each, s, OCH_3), ^{13}C -NMR (in C_5D_5N): δ 51.9 and 52.0 (1C each, OCH_3). *Anal.* Found: C, 58.30; H, 7.79%. Calcd. for $C_{44}H_{66}O_{16} \cdot 3H_2O$: C, 58.39; H, 8.02%.

Other substrates. The following natural glucuronide saponins of oleanolic acid (**14**), which had already been isolated by our research group, were also used as substrates chikusetsusaponins-IV (**16**), -IVa (**17**) and -V (ginsenoside-Ro, **18**)⁷⁻¹⁰ from *Panax japonicus* C. A. Meyer; pseudo-ginsenoside-RT₁ (**19**) from *P. pseudo-ginseng* Wall. subsp. *himalaicus* Hara;¹¹ hemslosides Mal (**20**) and Ma2 (**21**) from *Hemsleya macrocarpa* C. Y. Wu;¹² stipuleanosides R₁ (**22**) and R₂ (**23**) from *P. stipuleanatus* H. T. Tsai and K. M. Feng,¹³ which were kindly supplied by Dr. T. Yang, Kunming Institute of Botany, Academia Sinica, China.

The ethyl ester (**24**) of **17** was obtained from an ethanolic extraction of rhizomes of *Hemsleya chinensis* C. Y. Wu, and the methyl ester (**25**) of **18** was obtained from a methanolic extraction of rhizomes of *Panax japonicus*, both of which may have been artifacts formed during the extraction.

The 3-O- β -D-glucuronide (**12**) of chenodeoxycholic acid¹⁴ was kindly supplied by Dr. Toshifumi Akizawa, Tokyo Medical and Dental University.

Enzyme preparation. An aqueous solution of **GH** (1U/ml) was prepared (the activity being determined as described previously¹¹). Commercial β -glucuronidase [Sigma No. G-0876 (Lot. No. 73F-0553), 95400 Fishman Units/ml] was used for comparing the activity with that of **GH**.

Conditions for hydrolysis with GH. To a solution of a substrate (10 mg) in an acetate buffer (pH 4.4, 0.5 ml) was added **GH** (0.25 ml), and the mixture was incubated at 45°C. The reaction process was followed by thin-layer chromatography (TLC) on silica gel ($CHCl_3$ - $MeOH$ - H_2O solvent (6:4:1, homogeneous), H_2SO_4 detection) every 15 min.

In the case of **17**~**21**, a solution (or suspension) of a substrate (100 mg) and **GH** (0.5 ml) in an acetate buffer (pH 4.3, 5.0 ml) was incubated at 45°C, and the reaction process was followed by similar TLC. When the hydrolysis had been completed, the resulting precipitate was collected by filtration to give a prosapogenin, β -D-glucopyranosyl oleanolate (**15**), which was identified by comparing the optical rotation and ^{13}C -NMR spectrum with those of an authentic sample. The filtrate was chromatographed on DIAION HP-20, successively eluting with H_2O and $MeOH$. From the $MeOH$ eluate, a small amount of **15** was isolated. The H_2O eluate was deionized by chromatography on Sephadex LH-20, to afford a mono- or oligosaccharide, which was identified by ^{13}C -NMR spectroscopy (Table I).

FIG. 1. Glycyrrhizin and Synthetic Glucuronides.

FIG. 2. Natural Glucuronide Saponins.

Glc, β -D-glucopyranosyl; Xyl, β -D-xylopyranosyl; Ara(p or f), α -L-arabino(pyranosyl or furanosyl).

Conditions for hydrolysis with commercial β -glucuronidase. To a solution of a substrate (10 mg) in an acetate buffer (pH 5.0, 0.5 ml) was added a solution of the β -glucuronidase (0.25 ml), and the mixture was incubated at 37°C. The reaction was followed in the way already mentioned.

Due to the very low solubility in the buffer, **11** was not hydrolyzed by either the commercial β -glucuronidase or GH.

RESULTS AND DISCUSSION

It was found that the commercial β -glucuro-

FIG. 3. Oligosaccharides from Natural Glucuronide Saponins.

TABLE I. ^{13}C -NMR CHEMICAL SHIFTS OF OLIGOSACCHARIDES

	26	27	28	29
GlcUA	α	β	α	β
1	92.6	95.3	92.6	95.4
2	81.4	81.9	81.3	82.5
3	72.6*	74.2	72.4*	74.3
4	72.4*	72.6*	72.4*	72.7*
5	72.2*	76.8	72.7*	76.3
6	177.6	182.2	73.0	74.6
	Glc	Xyl	Ara(p)	Glc
1	104.7	103.5	105.6	104.5
2	74.0	74.0	72.0	74.1
3	76.5	76.3	73.0	76.7
4	70.2	70.0	69.1	70.2
5	76.3	65.8	67.0	76.7
6	61.3	—	—	61.3
	Ara(p)			
1	104.2			
2	72.1			
3	73.3			
4	69.2			
5	67.0			

Chemical shifts are in ppm from TMS (in D_2O).

* May be exchanged.

nidase non-selectively hydrolyzed a glucuronide linkage and other glycoside linkages, but not glycosyl ester linkages; **5**~**10** and **12** gave glucuronic acid, **16**~**21** and **23** afforded **15** and the respective component monosaccharides, and **22** afforded **14** and the component monosaccharides.

It has already been reported that *p*-nitrophenyl β -D-glucuronide and phenolphthalein β -D-glucuronide, which have been used as the standard substrates of general β -glucuronidase, were not hydrolyzed by **GH**.¹⁾ In the present study, it was revealed that **GH** does not cleave glucuronide linkages with small-size aliphatic alcohols; no hydrolysis by **GH** was observed with **5**~**10** even after 24 hr of incubation, while by incubating with **GH**, **12** was slowly hydrolyzed to give chenodeoxycholic acid and glucuronic acid.

On the other hand, **18** was rapidly hydrolyzed by **GH** to give a prosapogenin (**15**) and 2-*O*- β -D-glucopyranosyl-D-glucuronic acid (**26**) quantitatively within 30 min. Selective hydrolysis of the glucuronide linkage by **GH** was also observed with **17** and **19**~**21**, affording the common prosapogenin **15** together with D-glucuronic acid from **17**, 2-*O*- β -D-xyopyranosyl-D-glucuronic acid (**27**) from **19**, 3-*O*- α -L-arabinopyranosyl-D-glucuronic acid (**28**) from **20**, and 2-*O*- β -D-glucopyranosyl-(3-*O*- α -L-arabinopyranosyl)- α -D-glucuronic acid (**29**) from **21**. It is notable that in **17**~**21**, the 4-hydroxyl group of the glucuronide moiety is unsubstituted.

In contrast to these glucuronides, **16**, **22** and **23** (in which the 4-hydroxyl group of the glucuronide moiety is glycosylated) were not hydrolyzed by **GH**. When the carboxyl group of the glucuronide moiety was esterified, **GH** could not hydrolyze the glucuronide linkage; **24**, **25** and **3** were recovered unchanged by incubating with **GH**.

These results indicate that for the hydrolysis

of a β -D-glucuronide linkage by **GH**, both the 4-hydroxyl group and the carboxyl groups in a glucuronide moiety must be free. This high substrate specificity observed in the present study is significant in the application of this enzyme to chemical studies on natural glucuronide saponins.

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