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Enzymatic hydrolysis of the conjugate of vitamin D and related compounds[1]

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Abstract

The monoglucuronides of vitamin D, 25-hydroxyvitamin D and the corresponding pro-forms were subjected to enzymatic hydrolysis using β-glucuronidase, and substrate specificities were found in the examined enzymes originating from different sources, which were determined using reversed-phase high-performance liquid chromatography with UV detection. The enzymatic hydrolysis of the corresponding monosulfates was also examined using the same system. © 1997 Elsevier Science B.V.

Keywords: Vitamin D; Pro-form; Monoconjugate; Enzymatic hydrolysis; Substrate specificity; Reversed-phase high-performance liquid chromatography

1. Introduction

The so-called vitamin D (D) actually consists of two different compounds, D_3 and D_2, which differ in their side chain structure at the 17β-position of the seco-steroid. Although both D_3 and D_2 are absorbed from the diet, only D_3 is biosynthesized in the skin upon irradiation of provitamin D_3 ($proD_3$). D is 25-hydroxylated in the liver as the first step in its conversion to the 1,25-dihydroxylated compound which is the active metabolite in the intestine and bone [1]. Despite recent intensive investigation of D metabolism, the conjugates of the D metabolites still remain poorly understood. The following are the previously known representative data. Axelson and our group reported that 25-hydroxyvitamin D_3 3-sulfate {25(OH)$D_3$3S} is the major circulating form of D_3 in man [2,3]. We also clarified the existence of 3- or 25-monoglucuronide (G) of 25(OH)D_3 in mammalian bile in comparison with the authentic specimens [4,5] (Fig. 1).

The enzymatic hydrolysis using β-glucuronidase or sulfatase is usually done as the determination method of conjugated metabolites to determine the conjugated form and the structure of the genin. It is important to know the substrate specificities for these conjugates before

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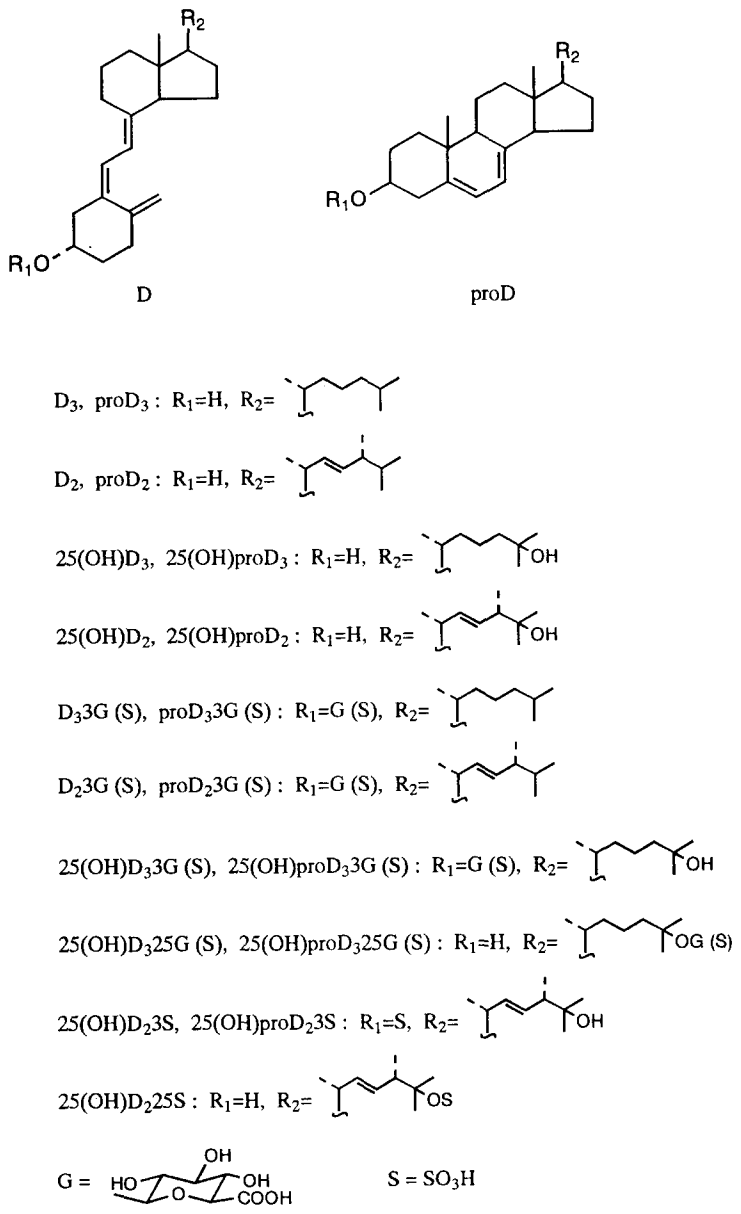

Fig. 1. Structures of D, related compounds and their conjugates.

applying enzymatic hydrolysis to the specimen obtained from biological fluids. This paper describes the results of the enzymatic hydrolysis of authentic conjugate of D, 25(OH)D and their pro-forms using β-glucuronidase originating from different sources of sulfatase.

2. Experimental

2.1. Instruments and materials

Proton nuclear magnetic resonance spectra (^{1}H-NMR) were measured with a JEOL JNM-EX270

(270 MHz) spectrometer (Tokyo, Japan) using tetramethylsilane as the internal standard (IS). The following abbreviations were used; s = singlet, d = doublet, br = broad, m = multiplet. Fast atom bombardment mass spectra (FAB-MS) were measured with a JEOL JMS-DX303 (Tokyo). Preparative (prep.) thin-layer chromatography (TLC) was conducted with 0.5 mm pre-coated Silica gel $60F_{254}$ (E. Merck, Darmstadt, Germany). The Sep-Pak C_{18} cartridge (packing material was used) was obtained from Waters (Milford, MA, USA). High-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6A chromatograph (Kyoto, Japan) equipped with a Shimadzu SPD-10A or -6AV UV detector (D-form, 265 nm; pro-form, 280 nm) at a flow rate of 1.0 ml min^{-1} under ambient conditions. The following reversed-phase columns were used: Develosil ODS-5 (5 μm; Nomura, Seto, Japan), J'sphere ODS-H80 (4 μm) and YMC-Pack ODS-AM (5 μm) (each YMC, Kyoto) (15 × 0.46 cm i.d.). The pH of the mobile phase containing $NaClO_4$ was adjusted with $HClO_4$. D_2, D_3 and ergosterol ($proD_2$) were obtained from Tokyo Kasei (Tokyo), and 7-dehydrocholesterol ($proD_3$) was obtained from Aldrich (Milwaukee, WI, USA). $25(OH)D_3$ was generously donated by Teikoku Hormone Mfg. (Tokyo). β-Glucuronidase originating from bovine liver (type B-1), *Helix pomatia* (type H-1; containing sulfatase) and *E. coli* (type IX-4) were obtained from Sigma (St. Louis, MO, USA). The enzyme originating from *Helix pomatia* was also used as the sulfatase. $25(OH)D_2$ [6], $25(OH)proD_3$, $25(OH)proD_2$, all monoglucuronides [4] and 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione (MB-OTAD) [7] adduct of $25(OH)proD_3$3G were synthesized in our own laboratory. The other chemicals used were analytical- or guaranteed-reagent grade.

2.2. Synthesis of monosulfates

The 3-sulfate (3S) were prepared as follows. Freshly prepared sulfur-trioxide pyridine complex was added to a solution of substrate (10 mg) in pyridine (0.5 ml) under ice-cooling, and stirred for *ca.* 10 min. The mixture was neutralized with 5% $NaHCO_3$, saturated with NaCl and extracted with tetrahydrofuran. The organic layer was washed with brine, dried with Na_2SO_4 and the solvent was evaporated in vacuo. The obtained residue was purified by prep. TLC {$CHCl_3$-MeOH-H_2O (80:20:2.5, v/v/v)}. The zone containing the desired compound was extracted with $CHCl_3$-MeOH-H_2O (70:30:4, v/v/v) and the solvent was evaporated in vacuo to yield a colorless semi-solid. The monosulfate in the solid state was slowly decomposed, so the compound was stored as a solution in EtOH. ^{1}H-NMR (CD_3OD) $D_3$3S, δ: 0.57 (3H, *s*, H-18), 0.88 (6H, *d*, J = 6.6 Hz, H-26, 27), 0.94 (3H, *d*, J = 5.9 Hz, H-21), 4.53 (1H, *m*, H-3), 4.78, 5.06 (1H each, *br s*, H-19), 6.04, 6.24 (1H each, *d*, J = 11.2 Hz, H-6, 7); $D_2$3S, δ: 0.57 (3H, *s*, H-18), 0.83, 0.86 (3H each, *d*, J = 6.6 Hz, H-26, 27), 0.93 (3H, *d*, J = 6.9 Hz, H-24^1), 1.03 (3H, *d*, J = 6.6 Hz, H-21), 4.54 (1H, *m*, H-3), 4.78, 5.05 (1H each, *br s*, H-19), 5.20-5.22 (2H, *m*, H-22, 23), 6.03, 6.24 (1H each, *d*, J = 11.2 Hz, H-6, 7); $proD_3$3S, δ: 0.63 (3H, *s*, H-18), 0.87, 0.88 (3H each, *d*, J = 6.3 Hz, H-26, 27), 5.38, 5.56 (1H each, *m*, H-6, 7); $proD_2$3S, δ: 0.63 (3H, *s*, H-18), 0.82, 0.84 (3H each, *d*, J = 6.3 Hz, H-26, 27), 5.37, 5.56 (1H each, *m*, H-6, 7); $25(OH)D_3$3S, δ: 0.56 (3H, *s*, H-18), 0.96 (3H, *d*, J = 5.9 Hz, H-21), 1.18 (6H, *s*, H-26, 27), 4.52 (1H, *m*, H-3), 4.77, 5.05 (1H each, *br s*, H-19), 6.03, 6.24 (1H each, *d*, J = 11.2 Hz, H-6, 7); $25(OH)D_2$3S, δ: 0.57 (3H, *s*, H-18), 0.99 (3H, *d*, J = 6.9 Hz, H-24^1), 1.03 (3H, *d*, J = 6.6 Hz, H-21), 1.10, 1.13 (3H each, *s*, H-26, 27), 4.48 (1H, *m*, H-3), 4.76, 5.04 (1H each, *br s*, H-19), 5.22-5.39 (2H, *m*, H-22, 23), 6.03, 6.25 (1H each, *d*, J = 11.2 Hz, H-6, 7); $25(OH)proD_3$3S, δ: 0.63 (3H, *s*, H-18), 0.95 (3H, *s*, H-19), 0.96 (3H, *d*, J = 8.0 Hz, H-21), 1.20 (6H, *s*, H-26, 27), 4.32 (1H, *m*, H-3), 5.39, 5.57 (1H each, *m*, H-6, 7); $25(OH)proD_2$3S, δ: 0.66 (3H, *s*, H-18), 0.95 (3H, *s*, H-19), 0.99 (3H, *d*, J = 7.0 Hz, H-24^1), 1.06 (3H, *d*, J = 6.6 Hz, H-21), 1.09, 1.14 (3H each, *s*, H-26, 27), 4.24 (1H, *m*, H-3), 5.29, 5.38 (1H each, *m*, H-22, 23), 5.37, 5.55 (1H each, *m*, H-6, 7). FAB-MS: $D_3$3S, m/z: 463 $(M-H)^-$; $D_2$3S, m/z: 475 $(M-H)^-$; $proD_3$3S, m/z:

463 $(M-H)^-$; proD$_2$3S, *m/z*: 475 $(M-H)^-$; 25(OH)D$_3$3S, *m/z*: 479 $(M-H)^-$; 25(OH)D$_2$3S, *m/z*: 491 $(M-H)^-$; 25(OH)proD$_3$3S, *m/z*: 479 $(M-H)^-$; 25(OH)proD$_3$3S, *m/z*: 491 $(M-H)^-$. All the sulfates showed the characteristic ion at *m/z*: 97 (SO_4H).

The 25-sulfate (25S) was prepared as follows. The 3-acetate of the corresponding free compound was sulfated as already described. The solution of KOH in MeOH (0.1 M, 1 ml) was added to the solution of the obtained residue in MeOH (1 ml), and then stirred in the dark under argon at room temperature for 3.5 h. The mixture was diluted with H_2O (7 ml), neutralized with 5% HCl and applied to a Sep-Pak C_{18} column (5.5 × 1.0 cm i.d.). After washing with H_2O (27 ml), the 25S was eluted with MeOH (15 ml) as a mixture containing free compound (*ca.* 25%), which was determined by HPLC: Develosil ODS-5, MeOH-$NaClO_4$ (pH 3.0; 163 mM) (6:1, v/v), retention time (t_R) 5.4 min {25(OH)D$_3$25S}, 12.5 min {25(OH)D$_3$}; MeOH-$NaClO_4$ (pH 3.0; 163 mM) (4:1, v/v), t_R 12.7 min {25(OH)D$_2$25S}, 34.8 min {25(OH)D$_2$}. The 25S was easily hydrolyzed even with evaporation of the solvent to give the corresponding genin, so the compound was stored as a solution in EtOH.

2.3. Enzymatic hydrolysis of monoglucuronide

The monoglucuronide {0.25 nmol in EtOH (20 μl)} dissolved in acetate buffer {AcONa-AcOH (pH 5.0; 100 mM)} (0.88 ml) and β-glucuronidase (500 Fishman units) in acetate buffer (0.1 ml) were separately pre-incubated at 37°C for 15 min. The two solutions were then mixed and incubated at 37°C for 2 h. The reaction was stopped by the addition of AcOEt (1 ml). After the addition of IS {0.25 nmol in EtOH (20 μl)}, the reaction mixture was extracted with AcOEt, the organic layer was evaporated in vacuo and the residue obtained was submitted to the following HPLC to determine the liberated genin and remaining substrate: J'sphere ODS-H80; for D$_2$3G: MeOH-$NaClO_4$ (pH 3.0; 163 mM) (19:1, v/v), t_R 9.5 min (D$_2$3G), 21.0 min (D$_2$), 12.2 min (IS = proD$_3$3G); for D$_3$3G: MeOH-$NaClO_4$ (pH 3.0; 163 mM) (14:1, v/v), t_R 12.4 min (D$_3$3G), 28.1 min (D$_3$), 26.3 min (IS = D$_2$); for proD3G: MeOH-$NaClO_4$ (pH 3.0; 163 mM) (39:1, v/v), t_R 7.9 min (proD$_2$3G), 21.6 min (proD$_2$), 7.2 min (proD$_3$3G), 22.7 min (proD$_3$), 13.3 min (IS = D$_2$); for 25(OH)D$_3$G: MeOH-$NaClO_4$ (pH 3.0; 163 mM) (5:1, v/v), t_R 8.8 min {25(OH)D$_3$25G}, 8.9 min {25(OH)D$_3$3G}, 20.8 min {25(OH)D$_3$}, 17.5 min {IS = 25(OH)proD$_3$3G MBOTAD adduct}; for 25(OH)proD$_3$G: MeOH-$NaClO_4$ (pH 3.0; 163 mM) (11:2, v/v), t_R 6.4 min {25(OH)proD$_3$3G}, 7.2 min {25(OH)proD$_3$25G}, 20.5 min {25(OH)proD$_3$}, 16.4 min {IS = 25(OH)D$_3$}. The calibration graphs were constructed by the addi-

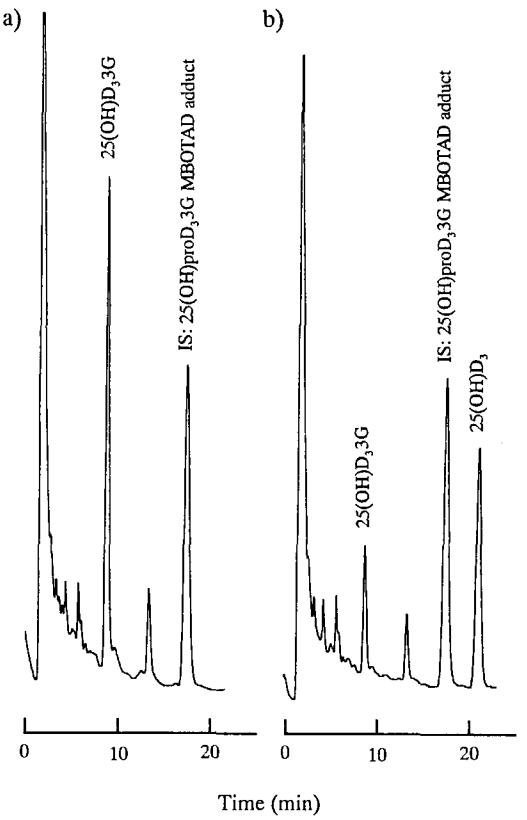

Fig. 2. Chromatograms of 25(OH)D$_3$3G and its hydrolyzed product. (a) Extract from incubation medium containing inhibitor. (b) Extract from incubation medium containing β-glucuronidase originating from bovine liver. Conditions: J'sphere ODS-H80, MeOH-$NaClO_4$ (pH 3.0; 163 mM) (5:1, v/v), 1 ml min^{-1}, UV 265 nm.

Table 1
Calibration graphs for D, related compounds and their conjugates

	Compounds	Regression equation	Regression (r)
(a)	25(OH)$D_3$3G	$y = 5.81x + 0.055$	0.999
	25(OH)$D_3$25G	$y = 8.83x - 0.074$	0.999
	25(OH)pro$D_3$3G	$y = 3.52x - 0.022$	0.993
	25(OH) pro$D_3$25G	$y = 3.92x$	1.000
	$D_3$3G	$y = 2.90x - 0.003$	0.999
	$D_2$3G	$y = 6.55x + 0.165$	0.990
	pro$D_3$3G	$y = 2.27x - 0.005$	0.999
	pro$D_2$3G	$y = 1.12x - 0.025$	0.994
	25(OH)D_3	$y = 4.87x - 0.029$	0.999
	25(OH)proD_3	$y = 2.83x - 0.015$	0.995
	D_3	$y = 3.17x - 0.008$	0.995
	D_2	$y = 8.23x + 0.060$	0.996
	proD_3	$y = 1.62x + 0.014$	0.993
	proD_2	$y = 1.66x - 0.048$	0.997
(b)	25(OH)$D_3$3S	$y = 2.30x - 0.135$	1.000
	25(OH)$D_2$3S	$y = 2.34x - 0.101$	0.999
	25(OH)pro$D_3$3S	$y = 2.39x - 0.029$	0.999
	25(OH)pro$D_2$3S	$y = 1.90x - 0.057$	0.999
	$D_3$3S	$y = 4.11x + 0.015$	0.999
	$D_2$3S	$y = 5.79x - 0.420$	0.997
	pro$D_3$3S	$y = 1.90x + 0.001$	0.999
	pro$D_2$3S	$y = 2.13x - 0.080$	0.992
	25(OH)D_3	$y = 2.56x - 0.150$	1.000
	25(OH)D_2	$y = 1.00x - 0.013$	0.997
	25(OH)proD_3	$y = 1.26x - 0.028$	1.000
	25(OH)proD_2	$y = 0.91x - 0.003$	0.998
	D_3	$y = 3.56x - 0.358$	0.998
	D_2	$y = 3.13x - 0.083$	0.996
	proD_3	$y = 0.98x + 0.029$	0.998
	proD_2	$y = 1.08x - 0.009$	0.999

y, Peak height ratio (substrate or genin *vs.* IS).
x, nmol per tube.
(a) For β-glucuronidase (range 0.03–0.25 nmol per tube).
(b) For sulfatase (range 0.25–1.0 nmol per tube).

tion of an authentic sample {0.03, 0.06, 0.13, 0.25 nmol in EtOH (20 μl)} to the incubation medium containing glucarolactone (570 nmol) as an inhibitor followed by the treatment as already described, and the peak height ratio method was used.

2.4. Enzymatic hydrolysis of monosulfate

The monosulfate {1.0 nmol in EtOH (20 μl)} dissolved in acetate buffer (0.88 ml) and sulfatase (2.3 units, from *Helix pomatia*) in acetate buffer (0.1 ml) were separately pre-incubated at 37°C for 15 min. The two solutions were then mixed and incubated at 37°C for 2 h. The reaction was stopped by the addition of AcOEt (1 ml). After the addition of IS {1.0 nmol in EtOH (20 μl)}, the reaction mixture was extracted with AcOEt and the organic layer was evaporated *in vacuo*. The residue was submitted to the following HPLC to determine the liberated genin and remaining substrate: YMC-Pack ODS-AM; for D3S: MeOH-$NaClO_4$ (pH 3.0; 163 mM) (19:1, v/v), t_R 8.0 min ($D_2$3S), 17.0 min (D_2), 8.0 min ($D_3$3S), 17.3 min (D_3), 26.6 min (IS = proD_3); for proD3S: MeOH–$NaClO_4$ (pH 3.0; 163 mM) (19:1, v/v), t_R 8.4 min (pro$D_2$3S), 23.1 min (proD_2), 9.4 min (pro$D_3$3S), 26.6 min (proD_3), 17.3 min (IS = D_3); for 25(OH)$D_3$3S: MeOH-$NaClO_4$ (pH 3.0; 163 mM) (5:1, v/v), t_R 7.5 min {25(OH)$D_3$3S}, 17.7 min {25(OH)D_3}, 22.5 min {IS = 25(OH)proD_3}; for 25(OH)$D_2$3S or 25(OH)proD3S: MeOH-$NaClO_4$ (pH 3.0; 163 mM) (5:1, v/v), t_R 9.3 min {25(OH)$D_2$3S}, 20.9 min {25(OH)D_2}, 7.5 min {25(OH)pro$D_2$3S}, 23.7 min {25(OH)proD_2}, 7.6 min {25(OH)pro$D_3$3S}, 22.5 min {25(OH)proD_3}, 17.7 min {IS = 25(OH)D_3}. The calibration graphs were constructed by the addition of an authentic sample {0.25, 0.50, 1.0 nmol in EtOH (20 μl)} to the incubation medium containing the solution of K_2HPO_4 (350 mM) (pH 5.0, adjusted with H_3PO_4) (0.1 ml) as an inhibitor followed by the treatment as described above, and the peak height ratio method was used.

3. Results and discussion

During the characterization and determination of the conjugated metabolites using the enzymatic hydrolysis, it is important to examine the substrate specificity of the enzyme before applying it to the specimen obtained from biological fluids. In a previous paper of this series, we isolated and characterized the positionally isomeric pair of monglucuronides (3G and 25G) of 25(OH)D_3 from rat bile [5]. These positional isomers were subjected to the enzymatic hydrolysis

Table 2
Enzymatic hydrolysis of monoglucuronides of D and related compounds using β-glucuronidase

Enzyme source	Substrate	Liberated genin[a]	Remaining substrate[a]
Bovine liver	25(OH)$D_3$3G	67.7 ± 3.1	24.5 ± 3.2
	25(OH)$D_3$25G	29.4 ± 4.1	67.0 ± 4.9
	25(OH)pro$D_3$3G	77.8 ± 6.2	n.d.[b]
	25(OH)pro$D_3$25G	58.6 ± 6.0	37.6 ± 5.6
	$D_3$3G	10.8 ± 1.9	85.1 ± 3.9
	$D_2$3G	15.6 ± 2.4	99.3 ± 7.3
	pro$D_3$3G	57.1 ± 3.2	37.6 ± 8.2
	pro$D_2$3G	30.9 ± 1.3	47.9 ± 6.9
Helix pomatia	25(OH)$D_3$3G	12.8 ± 0.9	85.8 ± 3.5
	25(OH)$D_3$25G	52.8 ± 10.8	49.4 ± 4.2
	25(OH)pro$D_3$3G	81.7 ± 2.8	n.d.
	25(OH)pro$D_3$25G	25.9 ± 3.0	57.3 ± 4.6
	$D_3$3G	n.d.	92.7 ± 5.6
	pro$D_3$3G	13.0 ± 2.0	84.0 ± 6.8
E. coli	25(OH)$D_3$3G	69.2 ± 5.2[c]	24.7 ± 5.1[c]
	25(OH)$D_3$25G	69.2 ± 5.2[c]	n.d.[c]
	25(OH)pro$D_3$3G	77.5 ± 2.6	n.d.
	25(OH)pro$D_3$25G	84.6 ± 6.7	n.d.
	$D_3$3G	48.5 ± 3.3	41.4 ± 5.1

[a]% (mean ± S.D.), $n = 5$.
[b]Not detectable.
[c]Data in reference [4].

using β-glucuronidase originating from different sources and the results showed that, in general, the enzyme preferably hydrolyzed the 25G isomer [4]. These data prompted us to precisely examine the substrate specificities of the enzyme for monoglucuronides of D and related compounds including the pro-form and the enzymatic hydrolysis of the corresponding monosulfates was also examined.

3.1. Substrate specificity of β-glucuronidase

In a previous paper, we separately determined the remaining substrate (monoglucuronide) and liberated genin in the incubation medium using different HPLC conditions [4]. The method is tedious and time-consuming, therefore, we developed a simultaneous determination method of these compounds resulting from enzymatic hydrolysis.

The separation of monoglucuronide, its genin and IS was examined using reversed-phase HPLC with an ODS column and MeOH- or MeCN-NaClO_4 (pH 3.0; 163 mM) as a mobile phase. Both organic modifiers gave good separation, but MeOH was superior to MeCN in its shorter retention time (< 30 min) of genin. The representative chromatograms are shown in Fig. 2.

Authentic monglucuronide and genin at two levels (0.03 or 0.25 nmol per tube) were incubated with incubation medium containing an inhibitor, extracted with AcOEt and the reasonable absolute recovery rates (> 88.1%; mean, $n = 2$) were obtained from both compounds. No interference from the incubation medium was detected at the t_R of these compounds, and the calibration graphs constructed using the IS method showed good linearity ($r > 0.99$) from 0.03 to 0.25 nmol per tube (Table 1).

Each monoglucuronide (0.25 nmol) was subjected to enzymatic hydrolysis using β-glucuronidase (500 Fishman units) originating from bovine liver, *Helix pomatia* and *E. coli*, and the rates of liberating genin and remaining substrate were examined (Table 2).

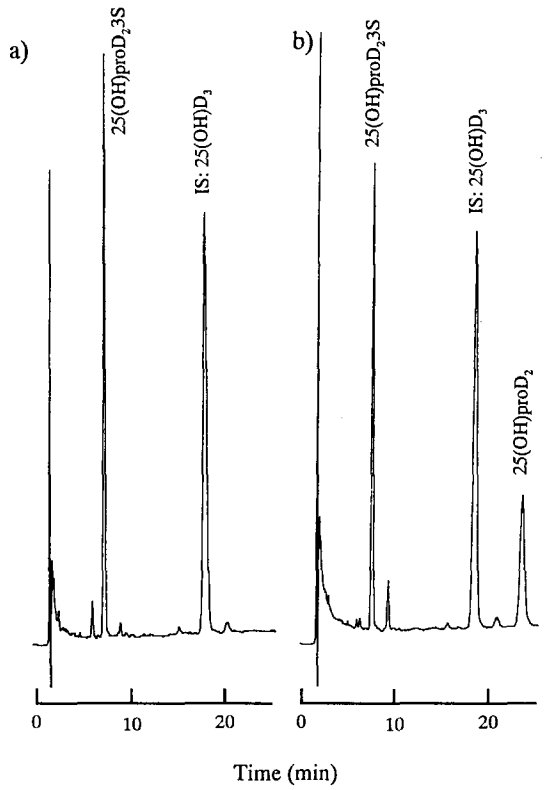

Fig. 3. Chromatograms of 25(OH)proD$_2$3S and its hydrolyzed product. (a) Extract from incubation medium containing inhibitor. (b) Extract from incubation medium. Conditions: YMC-Pack ODS-AM, MeOH-NaClO$_4$ (pH 3.0; 163 mM) (5:1, v/v), 1 ml min^{-1}, UV 280 nm.

The enzyme originating from *E. coli* showed the highest hydrolysis rate in all the examined enzymes. For instance, although the other enzymes barely hydrolyze D$_3$3G, the hydrolysis rate using this enzyme was 48.5%.

The hydrolysis rates of D-form monoglucuronides were relatively lower than those of the corresponding pro-form, such as D$_3$3G (10.8%) *vs.* proD$_3$3G (57.1%) using the enzyme originating from bovine liver. These data mean the enzyme preferably hydrolyzed the conjugate having a steroid structure in comparison with a seco-steroid conjugate. But an exception has been observed for the hydrolysis of 25(OH)proD$_3$25G (25.9%) in comparison with that of 25(OH)D$_3$25G (52.8%) using the enzyme originating from *Helix pomatia*.

Table 3
Enzymatic hydrolysis of monosulfates of D and related compounds using sulfatase originating from *Helix pomatia*

Substrate	Liberated genin[a]	Remaining substrate[a]
25(OH)D$_3$3S	n.d.[b]	107.1 ± 21.6
25(OH)D$_2$3S	6.4 ± 0.3	98.1 ± 4.6
25(OH)proD$_3$3S	31.7 ± 1.8	45.6 ± 6.1
25(OH)proD$_2$3S	28.9 ± 5.4	65.9 ± 5.4
D$_3$3S	0.1 ± 0.05	102.6 ± 4.4
D$_2$3S	n.d.	97.4 ± 8.4
proD$_3$3S	0.9 ± 0.4	87.3 ± 5.1
proD$_2$3S	3.5 ± 0.2	107.2 ± 5.5

[a]% (mean ± S.D.), $n = 5$.
[b]Not detectable.

The conjugates having a 25-hydroxy group were preferably hydrolyzed more than the corresponding conjugates of the D or pro-D form, such as 25(OH)D$_3$3G (67.7%) *vs.* D$_3$3G (10.8%) using the enzyme originating from bovine liver.

The enzymes originating from bovine liver and *E. coli* preferably hydrolyzed the 3-conjugate and 25-conjugate, respectively. On the contrary, that originating from *Helix pomatia* preferably hydrolyzed the 25-conjugate of 25(OH)D$_3$G and 3-conjugate of 25(OH)proD$_3$G more than those of the respective isomers.

3.2. Substrate specificity of sulfatase

We also examined the enzymatic hydrolysis of the monosulfates of D and related compounds. Although we prepared the 25S, the compound was easily hydrolyzed even with evaporation of the solvent to give the corresponding genin which was confirmed by HPLC. These data prompted us to see only the 3S as a substrate. The remaining substrate and liberating genin was analyzed by HPLC using YMC-Pack ODS-AM column and MeOH-NaClO$_4$ (pH 3.0; 163 mM) as a mobile phase (Fig. 3). The recovery rates of the monosulfates and genins (each 1.0 nmol per tube) extracted with AcOEt from incubation medium were more than 57% (mean, $n = 2$), and the calibration graphs constructed using the IS method showed good linearity ($r > 0.99$) from 0.25 to 1.0 nmol per tube (Table 1). Each monosulfate (1.0

nmol) was subjected to enzymatic hydrolysis using sulfatase originating from *Helix pomatia*, however, only a slight hydrolysis (< 10%) has been observed on all the examined monosulfates except for 25(OH)proD3S (31.7, 28.9%) (Table 3).

4. Conclusions

The proposed determination method using AcOEt extraction is convenient and gives the satisfactory separation of monoglucuronides or monosulfates and the corresponding genins without interference from the incubation medium in its UV/HPLC chromatogram. The enzyme originating from *E. coli* is suitable for the enzymatic hydrolysis of the monoglucuronides of D and related compounds in its high hydrolysis rates. Although the other incubation conditions (pH, buffer, incubation time, the purification of the enzyme) have not been examined, the substrate specificity was found in β-glucuronidase originating from different sources.

The 25S was easily hydrolyzed even without enzyme, but the 3S was not satisfactorily hydrolyzed using the enzyme originating from *Helix pomatia*. These conjugates should be subjected to acidic solvolysis to give the corresponding genin as previously reported [3].

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