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Partial purification of esterases from rabbit serum and their use in regioselective deacylations of sugars

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ABSTRACT

^{14}C-Labelled methyl 2,6-di-*O*-pivaloyl-α-D-glucopyranoside was used as a substrate for the detection of esterase activity in the isolation of esterase II from rabbit serum. On treatment of methyl 2,6-di-*O*-pivaloyl-α-D-glucopyranoside with rabbit serum and esterase II, the 6-*O*-pivaloyl group was removed selectively. Likewise, the 6-*O*-pivaloyl group was removed selectively from methyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-pivaloyl-α-D-glucopyranoside.

INTRODUCTION

Enzymes are well known as selective catalysts in organic chemistry, but carbohydrate molecules represent an especially challenging target for regioselective modifications due to their multiple hydroxyl groups. Regioselective acylations[1–3] and deacylations[4–6] of sugar derivatives have involved enzymes from various sources, ranging from commercially available lipases[5,6] to proteolytic enzymes[2].

The sera of mammals contain esterases that are useful for regioselective deacylations of sugar derivatives[7–10]. Esterases are distributed widely in vertebrate tissues and have been purified from rat, pig, ox, human, and rabbit liver[11], rat and human serum[12], rat intestinal mucosa and kidney[13], mouse kidney[14], and guinea-pig serum[15].

We now report on the partial purification of esterases from rabbit serum, and their use in deacylations of various pivaloylated derivatives of methyl α-D-glucopyranoside and methyl 2-acetamido-2-deoxy-α-D-glucopyranoside.

RESULTS AND DISCUSSION

Mammalian sera possess various enzymic activities and can catalyse the deesterifications of ^{14}C-labelled sugar substrates[8–10] and also cause their intramolecular transfer[9]. The purification of products from sera is complicated especially on a preparative scale. Column chromatography of rabbit serum on DEAE-Sepharose CL-6B gave two peaks that contained esterase activity (Fig. 1), based on the use of methyl 2,6-di-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside (**1**) as substrate. The first peak (esterase

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I) contained ~5.0% of the total activity applied. The rest of the activity associated with esterase I overlapped with serum albumin and was disregarded in further experiments. Esterase I caused non-selective hydrolysis of both pivaloyl groups from **1** and gave a mixture of the 2-pivalate **2**, 6-pivalate **3**, and methyl α-D-glucopyranoside (**4**). Esterase II was eluted with 0.1M NaCl in basic buffer and contained ~75% of the activity applied. Treatment of **1** with esterase II removed the 6-*O*-pivaloyl group and gave the

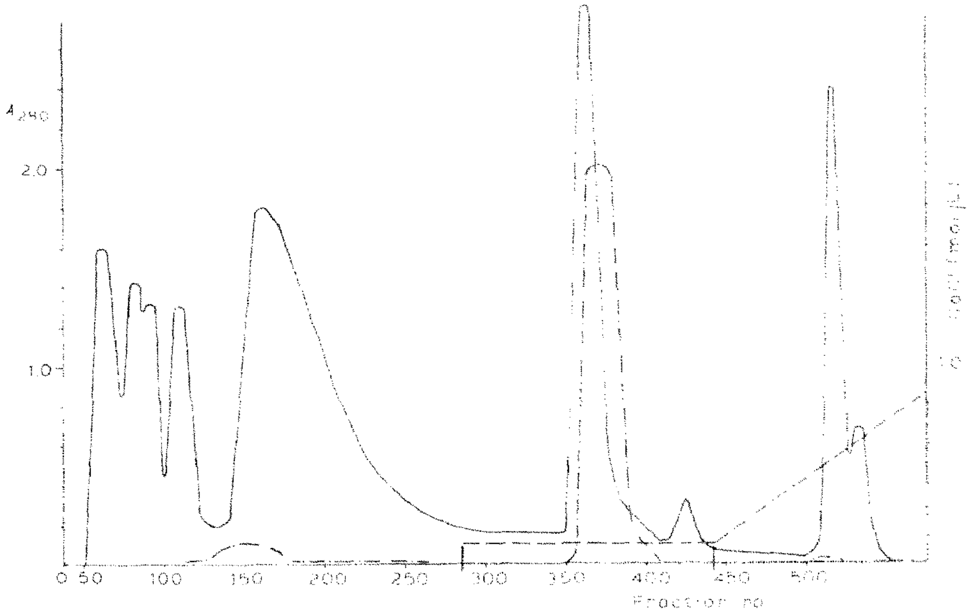
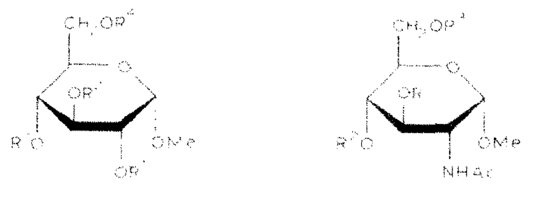


Fig. 1. Chromatography of rabbit serum on DEAE-Sepharose CL-6B (see Experimental). The eluate was collected in 5-mL portions, and esterase activity was detected by using ^{14}C-labelled methyl 2,6-di-*O*-pivaloyl-α-D-glucopyranoside as a substrate: —, absorbance at 280 nm; - - -, concentration of NaCl; ······, esterase activity.



1 $R^1 = R^4$ = Piv, $R^2 = R^3$ = H
2 R^1 = Piv, $R^2 = R^3 = R^4$ = H
3 $R^1 = R^2 = R^3$ = H, R^4 = Piv
4 $R^1 = R^2 = R^3 = R^4$ = H

Piv = pivaloyl

5 $R^1 = R^2 = R^3$ = Piv
6 $R^1 = R^3$ = Piv, R^2 = H
7 $R^1 = R^2$ = H, R^3 = Piv
8 R^1 = Piv, $R^2 = R^3$ = H
9 $R^1 = R^2$ = Piv, R^3 = H
10 $R^1 = R^2 = R^3$ = H
11 $R^1 = R^2$ = Piv, R^3 = Ac
12 R^1 = Piv, $R^2 = R^3$ = Ac

2-pivalate **2** as the sole product. Esterase II was sufficiently pure for use in time-dependence studies (using radiolabelled substrates) and in depivaloylations on a preparative scale (using unlabelled substrates). Further purification and classification studies of these esterases will be reported elsewhere.

On treatment of the ^{14}C-labelled **1** with whole rabbit serum, the 6-*O*-pivaloyl group was removed (→**2**) much faster than the 2-*O*-pivaloyl group (→**3**) (Table I). After 5 h, a 6:1 mixture of **2** and **3** was obtained with only 7% of **1** unreacted. Incubation for 24 h gave 55% of the totally deacylated product **4**, which was increased to 86% after 3 days. Similar treatment of unlabelled **1** for 5 h, followed by column chromatography, gave 66.8% of the 2-pivalate **2** and 19.7% of the 6-pivalate **3**. Esterase I caused non-selective deacylation of **1** and the reaction was significantly slower than that with serum (Table I).

TABLE I

Enzymic depivaloylation of **1** by rabbit serum, esterase I, and esterase II[a]

Time (h)	*Serum*				*Esterase I*[f]			*Esterase II*[g]		
	1[b]	**2**[c]	**3**[d]	**4**[e]	**1**[b]	**2**[c]	**3**[d]	**1**[b]	**2**[c]	**4**[e]
0	100				100			100		
1	85	15			92	8		88	12	
5	7	80	13	1	87	10	3	17	83	
6	6	75	15	4	81	11	8	10	90	
7	6	73	17	4	82	11	7	7	93	
24	3	38	4	55	72	20	8	5	95	
72	3	12		86	63	23	14	3	84	13

[a] Analysis based on radioactivity and given as a percentage of the total recovered after t.l.c. [b] Methyl 2,6-di-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside. [c] Methyl 2-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside. [d] Methyl 6-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside. [e] Methyl α-D-[U-^{14}C]glucopyranoside. [f] No **4** was detected at any given reaction time. [g] No **3** was detected at any given reaction time.

TABLE II

Enzymic depivaloylation of **2** and **3** by rabbit serum, esterase I, and esterase II[a]

Time (h)	*Serum*				*Esterase I*				*Esterase II*			
	2[b]	**4**[c]	**3**[d]	**4**[c]	**2**[b]	**4**[c]	**3**[d]	**4**[c]	**2**[b]	**4**[c]	**3**[d]	**4**[c]
0	100		100		100		100		100		100	
1	97	3	81	19							95	5
4	79	21	10	90							79	21
6	65	35	3	97							40	60
24	14	86			90	10	85	15			2	98
72	1	99			75	25	59	41				

[a] Analysis based on radioactivity and given as a percentage of the total recovered after t.l.c. [b] Methyl 2-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside. [c] Methyl α-D-[U-^{14}C]glucopyranoside. [d] Methyl 6-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside.

This esterase, which was unsuitable for syntheses starting from multiacylated substrates, partially de-esterified **2** (25%) and **3** (41%) in 3 days (Table II).

Esterase II hydrolysed **1** with a high degree of regioselectivity (Table I) and gave, after 6 h, 97% of the 2-pivalate **2** as shown by t.l.c., and 85% was isolated by column chromatography. Under similar conditions, esterase II did not affect the 2-pivalate **2**, but hydrolysed completely the 6-pivalate **3** in 24 h. Whole serum hydrolysed **3** in 6 h (Table II) to give 90% of **4**. The disadvantage of whole serum is in its content of small molecules that require removal by column chromatography, a procedure that was unnecessary when esterase II was used.

Similar results were obtained in the methyl 2-acetamido-2-deoxy-α-D-glucopyranoside series. Thus, the 6-*O*-pivaloyl group in the tripivalate **5** was regioselectively hydrolysed by both serum and esterase II, and column chromatography gave 53% and 40%, respectively, of the 3,4-dipivalate **9**. Incubations with radiolabelled substrates gave unreliable results due to their low solubility in the solvent system used. The 3,6-dipivalate **6** was selectively hydrolysed by esterase II to give the 3-pivalate **8** (90% after 24 h) (Table III). In preparative experiments, 91% of **8** was isolated by column chromatography after treatment of **6** with esterase II, as compared to 56% after treatment with serum. As expected, the 6-pivalate **7** was hydrolysed completely by serum and esterase II, but the 3-pivalate **8** was unaffected.

TABLE III

Enzymic depivaloylation of **6** by esterase II[a]

Time (*h*)	*Esterase II*	
	6[b]	**8**[c]
0	100	
1	90	10
3	70	30
5	35	65
7	20	80
24	10	90

[a] Analysis based on radioactivity and given as a percentage of the total recovered after t.l.c. [b] Methyl 2-acetamido-2-deoxy-3,6-di-*O*-pivaloyl-α-D-[1-^{14}C]glucopyranoside. [c] Methyl 2-acetamido-2-deoxy-3-*O*-pivaloyl-α-D-[1-^{14}C]glucopyranoside.

The corresponding compounds in the methyl 2-acetamido-2-deoxy-β-D-glucopyranoside series could not be hydrolysed on a preparative scale due to the low solubility of the substrates. The chemical syntheses of such compounds as the 2-pivalate **2** and the 3-pivalate **8** are complicated[7,16], but they can be produced enzymically in good yields.

The mild conditions involved in the use of purified esterases from rabbit serum should allow wide application in synthetic work.

EXPERIMENTAL

General. — Melting points are uncorrected. Optical rotations were determined for 1% solutions in chloroform or methanol. Column chromatography was performed on silica gel (Kemika, 0.063–0.2 mm), and t.l.c. on Silica Gel 60 (Merck), using *A*, acetonitrile–water (5:1); *B*, ethyl acetate–benzene (2:1); and *C*, ethyl acetate–benzene–ethanol (proportions are given in the text). Detection was effected by charring with sulfuric acid. ^{1}H-N.m.r. spectra (100 MHz, CD_3OD or $CDCl_3$, internal Me_4Si) were recorded with a Jeol JNM FX-100 F.t. spectrometer. Radioactivity was measured by using a Beckman LS-100 C liquid scintillation counter and Aquasol (NEN) as a scintillation cocktail. Protein concentrations were determined by the method of Lowry[17].

Preparation of rabbit sera. — Blood was removed from rabbits (New Zealand white) by heart puncture, transferred to centrifuge tubes, stored for a few hours at room temperature, and then centrifuged at 3000 r.p.m. for 10 min. The supernatant serum was removed by aspiration and stored at −20°.

Assay of esterase activity. — (*a*) The incubation mixtures contained phosphate-buffered saline (PBS, 75 μL, pH 7.2), rabbit serum, dialysed rabbit serum, or concentrated fractions from chromatography columns, methyl sulfoxide (10 μL), and methyl 2,6-di-*O*-pivaloyl-α-D-[U-^{14}C]glucopyranoside (**1**). The resulting mixtures were mM with respect to **1**. After incubation for 20 min at 37°, ethanol (0.6 mL) was added, and proteins were removed by centrifugation (1500*g*, 15 min). Each supernatant solution was concentrated under reduced pressure, and an aliquot (20 μL) of a solution of the residue in aqueous 75% ethanol (100 μL) was subjected to t.l.c. (solvent *C*, 10:2:1.5) with **1**–**4** as references: R_F values: **1**, 0.9; **2**, 0.5; **3**, 0.3; **4**, 0.1. The reference compounds were detected by charring with sulfuric acid. The absorbent that contained each radiolabelled product was suspended in aqueous 75% ethanol (2 mL) and a scintillation cocktail (2 mL), the radioactivity was measured, and the amounts of products were expressed as percentages of the total radioactivity of the plate. Recovery of radioactivity applied to the plates was 96–99%.

Each enzymic deacylation was performed at least twice and the results were 96% reproducible. T.l.c. and determinations of radioactivity were repeated 2–3 times, the reproducibility being 96–98%.

(*b*) A screening assay of esterase activity during the purification procedure was performed on diluted fractions. Incubation mixtures contained individual fractions (100–300 μL, depending on the expected activity), methyl sulfoxide (10 μL), radiolabelled **1**, and PBS (pH 7.2, 200–400 μL). Each resulting mixture had a volume of 500 μL. After incubation for 12 h, the work-up procedure in (*a*) was applied.

Preparation of partially purified enzyme. — All procedures were carried out at 4°. Unless stated otherwise, 0.067M phosphate buffer (pH 6.3) was used for equilibration of the gel and elution. After each step, the fractions that contained esterase activity were collected and concentrated by ultrafiltration using an Amicon Diaflo UM-10 membrane. Esterase activities in the concentrated fractions were determined as described above.

Rabbit serum (60 mL) was dialysed twice against phosphate buffer (2 L) and centrifuged at 100 000*g* for 1 h, in order to remove the precipitate that did not contain esterase activity.

The supernatant serum (62 mL) was applied to a column (2.5 × 100 cm) of DEAE-Sepharose CL-6B and eluted with the phosphate buffer (1.2 L). Elution was continued with 0.1M NaCl phosphate buffer (600 mL) followed by a linear gradient of NaCl (0.1–1.5M) in the same buffer. The eluate was collected in 5-mL portions and the esterase activity was determined as described above. Two peaks (I and II) with esterase activity were detected (Fig. 1). The materials in these peaks were concentrated by ultrafiltration, and the concentrates were freeze-dried. No loss of activity was detected due to freeze-drying.

Preparative enzymic deacylations. A suspension (or solution) of the *O*-pivaloyl derivative (0.124 mmol each) in methyl sulfoxide (500 μL) and PBS (10 mL) was incubated with rabbit serum (1 mL), or esterase I or esterase II (freeze-dried, protein content of 8 mg), at 37°. The pH was adjusted periodically to 7.2 by the addition of 0.1M NaOH, and each reaction was monitored by t.l.c. Each reaction was stopped by adding ethanol (10 mL), the precipitated proteins were removed by centrifugation, the solvent was evaporated, and the residue was subjected to column chromatography.

(*a*) Treatment of the 2,6-dipivalate **1** (ref. 7) (45 mg) with rabbit serum for 5 h, followed by column chromatography (solvent *C*, 10:2:1.5), produced methyl 2-*O*-pivaloyl-α-D-glucopyranoside[7] (**2**; 23 mg, 66%) and methyl 6-*O*-pivaloyl-α-D-glucopyranoside[7] (**3**; 7 mg, 19%).

Incubation for 24 h, followed by column chromatography (solvent *A*), gave methyl α-D-glucopyranoside (**4**; 18 mg, 75%).

Treatment of **1** with esterase II for 6 h, followed by a rapid filtration through a short column of silica gel (solvent *C*, 10:2:1.5), gave **2** (29 mg, 85%) as the sole product.

^{14}C-Labelled **1** (a mixture of 42.5 mg of unlabelled and 2.5 mg of the ^{14}C-labelled product with specific activity of 28 MBq/mmol) was treated with rabbit serum or esterase II, and the reactions were monitored by t.l.c. (solvent *C*, 10:2:1.5). The results are presented in Table I.

(*b*) Treatment of the 2-pivalate **2** (34 mg) with rabbit serum for 3 days, followed by column chromatography (solvent *A*), gave methyl α-D-glucopyranoside (**4**; 23 mg, 95%).

^{14}C-Labelled **2** (a mixture of 32 mg of unlabelled and 2.5 mg of the ^{14}C-labelled product with specific activity of 34 MBq/mmol) was treated with rabbit serum, esterase I, or esterase II, and each reaction was monitored by t.l.c. (solvent *A*). The results are presented in Table II.

(*c*) Treatment of the 6-pivalate **3** (34 mg) with rabbit serum for 6 h, followed by column chromatography (solvent *A*), gave methyl α-D-glucopyranoside (**4**; 20 mg, 83%).

Hydrolysis of **3** with esterase II for 24 h, followed by a rapid filtration through a short column of silica gel, gave methyl α-D-glucopyranoside (**4**; 22 mg, 91%).

^{14}C-Labelled **3** (mixture of 32 mg of unlabelled and 2.5 mg of ^{14}C-labelled product

with specific activity of 30 MBq/mmol) was treated with rabbit serum, esterase I, or esterase II, and each reaction was monitored by t.l.c. (solvent *A*). The results are presented in Table II.

(*d*) Treatment of the tripivalate **5**[16] (60 mg) with rabbit serum for 4 days, followed by column chromatography (solvent *B*), gave methyl 2-acetamido-2-deoxy-3,4-di-*O*-pivaloyl-α-D-glucopyranoside (**9**; 27 mg, 53%), m.p. 165–168° (from ethyl acetate–light petroleum), as needles, $[\alpha]_D$ +57° (chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 5.74 (d, *J* 9.38 Hz, NH), 5.34 (t, $J_{2,3}$ 9.67, $J_{3,4}$ 10.25 Hz, H-3), 5.06 (t, 9.38 Hz, H-4), 4.73 (d, $J_{1,2}$ 3.52 Hz, H-1), 3.42 (s, OMe), 1.93 (s, NAc), 1.17 (s, PivO-4), and 1.13 (s, PivO-3).

Anal. Calc. for $C_{19}H_{33}NO_8$: C, 56.56; H, 8.24; N, 3.47. Found: C, 56.31; H, 8.10; N, 3.38.

Treatment of **9** (44 mg) with acetic anhydride–pyridine (1:1) for 16 h at room temperature gave the 6-acetate **11** as a solid (45 mg, 93%), $[\alpha]_D$ +62° (chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 5.69 (d, *J* 9.38 Hz, NH), 4.72 (d, $J_{1,2}$ 3.52 Hz, H-1), 3.41 (s, OMe), 2.11 (s, AcO-6), 1.93 (s, NAc), 1.15 (s, PivO-4), and 1.12 (s, PivO-3).

Anal. Calc. for $C_{21}H_{35}NO_9$: C, 56.61; H, 7.92; N, 3.14. Found: C, 56.84; H, 7.94; N, 3.23.

Treatment of **5** with esterase II for 7 days, followed by column chromatography (solvent *B*), gave **9** (20 mg, 40%).

(*e*) Treatment of the 3,6-dipivalate **6** (ref. 16) (50 mg) with rabbit serum for 24 h, followed by column chromatography (solvent *C*, 2:2:1), afforded methyl 2-acetamido-2-deoxy-3-*O*-pivaloyl-α-D-glucopyranoside as a solid (**8**; 33 mg, 91%), $[\alpha]_D$ +64° (methanol). ^{1}H-N.m.r. data (CD_3OD): δ 4.72 (d, $J_{1,2}$ 3.91 Hz, H-1), 3.51 (s, OMe), 1.26 (s, PivO-3).

Anal. Calc. for $C_{14}H_{25}NO_7$: C, 52.65; H, 7.89; N, 4.39. Found: C, 52.83; H, 7.68; N, 4.25.

Conventional treatment of **8** (20 mg) with acetic anhydride–pyridine (1:1) for 16 h at room temperature gave the known[16] 4,6-diacetate **12** (22 mg, 88%).

Treatment of **6** with purified esterase II, followed by column chromatography (solvent *C*, 2:2:1), gave **8** (22 mg, 56%).

^{14}C-Labelled **6** (a mixture of 35.5 mg of unlabelled and 15.5 mg of the ^{14}C-labelled product with specific activity of 5.8 MBq/mmol) was treated with esterase II, and the reaction was monitored by t.l.c. (solvent *C*, 2:2:1). The results are presented in Table III.

(*f*) Treatment of the 6-pivalate **7** (ref. 16) (40 mg) with rabbit serum for 24 h, followed by column chromatography (solvent *C*, 2:2:1), gave methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**4**; 19 mg, 66%).

Treatment of **7** with esterase II for 24 h gave (t.l.c.) **4** (not isolated).

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