ENZYME-CATALYZED PARTIAL DEACETYLATION OF 1,6-ANHYDRO--2,3,4-TRI-O-ACETYL-β-D-GLUCOPYRANOSE

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Received July 3rd, 1986

Various esterases, lipases, and proteases with esterolytic activity were investigated for their power to catalyse deacetylation of 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose. Out of these, chymotrypsin, esterase *ex* liver, lipase *ex* pancreas, and lipase *ex* wheat-germ have been found to be selective catalysts of deacetylation; chymotrypsin and wheat-germ lipase preferably removed the acetyl at $C_{(3)}$, whereas liver esterase and pancreas lipase the acetyl at $C_{(4)}$. Compared to chemical catalysis, whether by methanolic hydrogen chloride or hydrazine hydrate, the loco-selectivity of the enzyme-catalysed deacetylation appear to be much better.

As is known, the individual acylated hydroxyl groups of monosaccharides differ in their amenability to hydrolysis, catalysed by acids or basic compounds. Excoffier and coworkers[1] studied deesterification of peracylated monosaccharides, catalysed by hydrazine acetate in dimethylformamide, and found that the hydroxyl at $C_{(1)}$ was the first to be liberated. Hydrazinolysis of peracylated methylglycosides in pyridine, or a mixture of pyridine and acetic acid, preferably removed the acyl groups from the $C_{(2)}$-hydroxyl[2].

Our preceding paper[3] describes hydrolysis of per-O-acetyl- and per-O-benzoyl derivatives of 1,6-anhydro-β-D-glucopyranose in the presence of methanolic hydrogen chloride or hydrazine hydrate. The acyl group on the $C_{(3)}$-hydroxyl was found to have a key role in the course of the hydrolysis. This acyl, compared to those at $C_{(2)}$ and $C_{(4)}$, was the stablest in acid hydrolysis by methanolic hydrogen chloride, but the opposite was the case in hydrazinolysis.

Enzyme-catalysed deacetylation of peracetylated D-glucose was studied by Fink and Hay[4], wheat-germ lipase being used (pH 5·6–8·1, 25–37°C); the substrate was in both a homogeneous and a heterogeneous phase. Employing paper chromatography and TLC they observed preferential deacetylaion at $C_{(1)}$ and $C_{(6)}$ which was much faster than at the other positions, the rate decreasing in the order $C_{(4)} > C_{(3)} > C_{(2)}$. The present paper deals with deacetylation of a simple model compound, *viz.* 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose (peracetylated levoglucosan), using various enzymatic systems isolated from groups of esterases, lipases,

and esterolytic proteases. The courses of deacetylation were monitored by gas chromatography; pure monoacetyl and diacetyl derivatives of levoglucosan, used as standards, were isolated chromatographically on silica gel and characterized by methylation analysis, and ^{13}C NMR spectrometry, as previously described[3]. Deacetylation of the starting per-O-acetylated levoglucosan can theoretically produce three monoacetyl and three diacetyl derivatives, and levoglucosan itself. O-Acyl derivatives of levoglucosan can be regarded as cyclic analogues of esterified glycerol. In view of this analogy, acyl derivatives of levoglucosan can be regarded as substrates in reactions catalysed by esterases and lipases. With these derivatives, however, unlike the case of acylated glycerol, it is necessary to bear in mind the steric factors, mainly the fact that the 1,6-anhydro ring may practically shield the hydroxyl group at $C_{(3)}$. Another important circumstance in an enzyme-catalysed hydrolysis is the choice of reaction conditions. The pH's often had to be different from those which would be optimum for the enzymes used, in order to eliminate the contribution by chemical deacetylation in the corresponding buffers. No chemical deacetylation occurred in citrate–phosphate buffers, pH 5·0–5·8. In this respect the acetate buffers were not satisfactory.

Cholinesterase enzymes, cholesterol esterase, trypsin, and carboxypeptidases A and Y did not catalyse deacylation of per-O-acetylated levoglucosan. However, acetylcholinesterases *ex* bovine erythrocytes and *ex Electrophorus electricus*, and pseudocholinesterases *ex* human and equine plasma proved capable of catalysing the deacetylation of 1,6-anhydro-3-O-acetyl-β-D-glucopyranose. Cholesterol esterase did not catalyse even deacetylation of monoacetyl derivatives of levoglucosan in the course of 96 h.

In the use of lipase *ex* bovine pancreas the enzymic deacetylation occurred at the $C_{(4)}$ hydroxyl group, giving rise to 1,6-anhydro-2,3-di-O-acetyl-β-D-glucopyranose as the predominant product. The degrees of deacetylation from the $C_{(2)}$ and $C_{(3)}$ positions were about one-tenth of that from $C_{(4)}$.

The formation of monoacetyl derivatives was negligible in the first 24 h (Table I). Deacetylation catalysed by esterase *ex* bovine liver took a similar course, the principal product being 1,6-anhydro-2,3-di-O-acetyl-β-D-glucopyranose. Hydrolysis to monoacetyl derivatives occurred to a very limited extent (Table II). These results resemble the effect of esterase to fully esterified glycerol, in which an acyl split off first from one of the outer hydroxyl groups[5].

Chymotrypsin attacked almost exclusively the acetyl on $C_{(3)}$; the $C_{(2)}$ position was not affected at all, the $C_{(4)}$ hydroxyl released the acetyl group at a very slow rate. The 3-O-acetyl and 4-O-acetyl derivatives were formed in quantities 50 to a hundred times smaller than the 2,4-di-O-acetyl derivative. The 2-O-acetyl derivative was not formed at all (Table III). The very tardy formation of monoacetyl derivatives in the use of the given enzymes virtually precludes exhaustive deacetylation to 1,6-anhydro-β-D-glucopyranose (Tables I–III). Like with chymotrypsin, hydrolysis

TABLE I

Hydrolysis of 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose with pancreatic lipase

Time h	Position of the acetyl group[a]							
	$C_{(0)}$	$C_{(2)}$	$C_{(3)}$	$C_{(4)}$	$C_{(2,3)}$	$C_{(2,4)}$	$C_{(3,4)}$	$C_{(2,3,4)}$
2	0·0	0·6	0·1	0·1	17·1	1·7	0·9	79·5
4	0·0	0·0	0·6	2·4	26·4	2·5	1·2	66·9
6	0·0	0·0	0·6	2·1	29·6	2·8	1·2	63·7
24	0·0	2·0	0·4	4·5	42·1	4·9	1·9	44·2

[a] Percentage of the individual acetyl derivatives is given.

TABLE II

Hydrolysis of 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose with liver esterase

Time h	Position of the acetyl group[a]							
	$C_{(0)}$	$C_{(2)}$	$C_{(3)}$	$C_{(4)}$	$C_{(2,3)}$	$C_{(2,4)}$	$C_{(3,4)}$	$C_{(2,3,4)}$
3	0·0	0·3	0·2	0·5	5·0	2·8	4·0	87·2
5	0·0	0·4	0·4	0·6	7·6	2·7	2·9	85·4
17	0·0	0·7	1·0	1·3	18·1	3·1	3·8	72·0
25	0·1	1·0	1·4	1·7	20·0	3·8	4·1	67·9
48	0·3	2·0	4·2	3·4	36·4	4·9	3·4	45·4

[a] Percentage of the individual acetyl derivatives is given.

TABLE III

Hydrolysis of 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose with chymotrypsin from porcine pancreas

Time h	Position of the acetyl group[a]							
	$C_{(0)}$	$C_{(2)}$	$C_{(3)}$	$C_{(4)}$	$C_{(2,3)}$	$C_{(2,4)}$	$C_{(3,4)}$	$C_{(2,3,4)}$
41	0·0	0·0	0·0	0·0	1·9	21·0	0·0	77·1
66	0·0	0·0	0·2	0·5	2·6	35·7	0·0	61·0
90	0·0	0·0	0·4	0·8	3·2	41·8	0·0	53·8

[a] Percentage of the individual acetyl derivatives is given.

catalysed by wheat-germ lipase affected mainly the acetyl on the $C_{(3)}$ hydroxyl. However, the formed 2,4-di-O-acetyl derivative hydrolysed further, predominantly at $C_{(2)}$, giving thus rise to the 4-O-acetyl derivative as the main product. Unlike the other enzymes, however, the wheat-germ lipase catalysed even hydrolysis of mono-acetyl derivatives to 1,6-anhydro-β-D-glucopyranose, but a side reaction gave the 3-O-acetyl derivative to an extent of 25 to 50% in respect to the 4-O-acetyl derivative. This fact can be explained by a transfer of acetyl in the presence of the wheat-germ lipase, preferably from the $C_{(4)}$ position, because after the starting 2,3,4-tri-O-acetyl derivative and the di-O-acetyl derivatives with one acetyl at $C_{(3)}$ had been practically eliminated by the reaction, the amount of the 3-O-acetyl derivative kept increasing (Table IV)[6,7]. Using peracetylated levoglucosan as a simplified model, in contrast to peracetylated D-glucose, employed by Fink and Hay[4], we did not observe preferential deacetylation from $C_{(4)}$ (as prevailing over that from $C_{(2)}$ and $C_{(3)}$), in the use of the wheat-germ lipase. We explain this fact by the difference in steric arrangement between the molecules of D-glucose and levoglucosan, or by a difference in sensitivity of the analytical methods used. However, a preferential release of acetyl from $C_{(4)}$ was observed in the use of the liver esterase, when 1,6-anhydro-2,3-di-O--acetyl-β-D-glucopyranose was the dominant product.

By comparing the results of the enzymic and the chemical hydrolyses of 1,6--anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose, the enzymic hydrolysis had several advantages over the chemical hydrolysis. Thus in the use of lipase *ex* hog pancreas, or esterase *ex* hog liver, like in the use of chymotrypsin, the deacetylation was selective and led to one product. Besides, the enzyme-catalysed hydrolyses gave much higher yields from the starting compound. Hydrolysis of peracetylated levoglucosan catalysed by the pancreatic lipase afforded 42% of 1,6-anhydro-2,3-di-O-acetyl-β-D--glucopyranose, with the esterase the yield was 36%, but in an acid hydrolysis it was

TABLE IV

Hydrolysis of 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose with lipase from wheat-germ

Time h	Position of the acetyl group[a]							
	$C_{(0)}$	$C_{(2)}$	$C_{(3)}$	$C_{(4)}$	$C_{(2,3)}$	$C_{(2,4)}$	$C_{(3,4)}$	$C_{(2,3,4)}$
1	1·2	3·2	5·3	22·2	5·5	50·6	8·0	4·0
2	3·8	5·6	8·4	37·2	4·7	35·0	4·6	0·7
4	16·5	8·5	11·8	47·2	1·0	15·0	0·0	0·0
6	37·2	4·9	18·6	33·6	0·2	5·5	0·0	0·0
24	52·3	6·4	10·4	29·6	0·0	1·3	0·0	0·0

[a] Percentage of the individual acetyl derivatives is given.

only 27% and a number of by-products was formed[5]. To prepare 1,6-anhydro-2,4--di-O-acetyl-β-D-glucopyranose it is also preferable to use chymotrypsin, since the product is obtained in a dominating yield. A disadvantage of this hydrolysis is a rather long reaction time. With the wheat-germ lipase the 2,4-di-O-acetyl derivative was obtained in a yield of about 50%. The latter enzyme also proved useful for the preparation of the 3-O-acetyl derivative. In contrast to chemical hydrolysis, the enzyme-catalysed preparation of the 2,4-di-O-acetyl derivative was by far the more rewarding, the yield being much higher and the contents of by-products lower.

EXPERIMENTAL

The starting materials, viz. 1,6-anhydro-β-D-glucopyranose, 1,6-anhydro-2,3,4-tri-O-acetyl-1,6-anhydro-2,3-di-O-acetyl-, 1,6-anhydro-2,4-di-O-acetyl-, 1,6-anhydro-3,4-di-O-acetyl-, 1,6--anhydro-2-O-acetyl-, 1,6-anhydro-3-O-acetyl-, and 1,6-anhydro-4-O-acetyl-β-D-glucopyranose, were prepared and tested as described previously[3].

Enzymes: Acetylcholinesterases from bovine erythrocytes (EC 3.1.1.7) and from *Electrophorous electricus* were Serva products (Heidelberg, F.R.G.), pseudocholinesterase (EC 3.1.1.8) from human plasma, specific activity 4 ncat mg^{-1}, was obtained as described in ref.[8]. Cholesterol esterase and esterase from porcine liver (EC 3.1.1.1) were products from the firm Boehringer & Sons (Mannheim, F.R.G.). Lipase from porcine pancreas was isolated as described in ref.[9]. Wheat-germ lipase (EC 3.1.1.3) and carboxypeptidases A and Y (EC 3.4.2.1) were Koch–Light products (Colnbrook, England). Crystalline chymotrypsin (EC 3.4.4.5) and trypsin (EC 3.4.4.4) from porcine and bovine pancreas were Sigma products (St. Louis, U.S.A.). The other constituents of the reaction mixtures and buffers, A.R. grade, were mostly Lachema chemicals, Nat. Corp., Brno, Czechoslovakia.

Silufol plates (Kavalier, Sázava, Czechoslovakia) were used for chromatography of the acyl derivatives and to monitor the courses of the enzymic hydrolyses; the spots were developed by spraying with 5% H_2SO_4 in methanol, followed by heating to 100°C.

Products of enzymic hydrolysis were determined quantitatively by gas chromatography, employing an apparatus Hewlett–Packard, model 5 830 A, with a flame-ionization detector. A stainless steel column, i.d. 2 mm, length 300 cm, was packed with 3% OV 225 on Supercorporate, particle size 0·135—0·150 mm. Heating of the column was programmed with a temperature gradient of 1°C min^{-1}, starting from 135°C (6-min isotherm) to 220°C (30-min isotherm). The temperature of the feed was 220°C, the temperature of the detector 300°C; the flow velocity of nitrogen as carrier was 15 m min^{-1}. The results were recalculated to normalized percentage, based on calibration with authentic substances.

Enzymic hydrolysis of 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose. A solution of the substance (1 g) in methanol (10 ml) was added to a citrate–phosphate buffer (50 ml, 0·05 mol l^{-1}, pH 5·4), followed by addition of the enzyme chosen (*a*) porcine liver esterase, total activity 2·6 ncat, specific activity 2 ncat mg^{-1}; *b*) porcine pancreatic lipase, total activity 2 ncat and specific activity 1 ncat mg^{-1}; *c*) porcine pancreatic chymotrypsin, total activity 1·5 ncat and specific activity 2·8 ncat mg^{-1}; *d*) wheat-germ lipase, total activity 1·9 ncat and specific activity 1·8 ncat mg^{-1}). The enzymic hydrolyses were allowed to proceed at 40°C; at selected time intervals 2-ml samples were withdrawn from the reaction mixture, taken to dryness, and analysed by gas chromatography to determine the contents of the individual acetyl derivatives and levoglucosan. To work up a larger quantity, the reaction mixture was resolved chromatographically

on a column of silica gel (4.0 × 90 cm) in a system ethyl acetate–benzene–2-propanol (8 : 4 : 1, v/v) (ref.[10]). The results show (Tables I and II) that porcine liver esterase and porcine pancreatic lipase enhance the formation of 1,6-anhydro-2,3-di-O-acetyl-β-D-glucopyranose[11]. Hydrolysis of peracetylated levoglucosan catalysed by pancreatic chymotrypsin (porcine or bovine) or wheat-germ lipase gave rise preferentially to 1,6-anhydro-2,4-di-O-acetyl-β-D-glucopyranose (Tables III and IV, *cf.*[12]). The other enzymes, *viz.* trypsin, cholesterol esterase, carboxypeptidases A and Y, and cholinesterases failed to induce hydrolysis under the given conditions. The enzymes of the cholinesterase type, both acetyl- and pseudocholinesterases (1 ncat, specific activity ~4 ncat . . mg^{-1}) catalysed under these conditions hydrolysis of 1,6-anhydro-3-O-acetyl-β-D-glucopyranose to 1,6-anhydro-β-D-glucopyranose. In the use of trypsin, carboxypeptidases and cholesterol esterases no hydrolysis of the monoacetyl derivatives occurred.

The isolated reaction products (1,6-anhydro-2,3-di-O-acetyl-, 1,6-anhydro-2,4-di-O-acetyl-, 1,6-anhydro-3,4-di-O-acetyl-, 1,6-anhydro-2-O-acetyl-, 1,6-anhydro-3-O-acetyl-, and 1,6-anhydro-4-O-acetyl-β-D-glucopyranoses) were characterized by their melting points, elemental analysis, optical rotation, methylation analysis, ^{13}C NMR spectra, and by comparison of their chromatographic mobilities with those of authentic substances. All the data were in accordance with our previous results[3].

Acknowledgement for technical assistance is due to Miss E. Jablonczióvá.

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Translated by J. Salák.