ENZYME-CATALYSED PARTIAL DEACYLATION OF 1,6-ANHYDRO--2,3,4-TRI-O-BUTYRYL-β-D-GLUCOPYRANOSE IN THE PRESENCE OF METHANOL

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Lipase ex pancreas (EC 3.1.1.3), lipase ex wheat-germ (EC 3.1.1.3), and chymotrypsin (EC 3.4.4.5) are compared in their power to catalyse deacylation of 1,6-anhydro-2,3,4-tri-O-butyryl-β--D-glucopyranose. The action of the three enzymes has been found locoselective. In the use of a hydrolase the selectivity of the reaction was affected by composition of the reaction mixture. Lipase ex porcine pancreas in a solution containing 20% of methanol (v/v) catalysed deacylation preferentially from C-2, but on raising the methanol concentration to 50% (v/v) deacylation from C-4 prevailed over that from C-2. Analogously, at the lower methanol concentration deacylation effected by wheat-germ lipase occurred to a greater extent on C-2, whereas at the higher concentration on C-3. With chymotrypsin ex hog pancreas at the lower concentration of methanol the prevailing deacylation was on C-4, at the higher on C-3. Compared to a chemically catalysed hydrolysis, whether by methanolic hydrogen chloride or hydrazine hydrate, the locoselectivity of an enzyme-catalysed deacylation of 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose is substantially higher.

The preceding papers deal with partial hydrolysis of acetyl and benzoyl derivatives of 1,6-anhydro-β-D-glucopyranose in acid and alkaline media. The course of hydrolysis was found to depend on the conformation of 1,6-anhydro-β-D-glucopyranose, reaction conditions and the nature of the acyl groups (the benzoyl derivatives were more stable than the acetyl derivatives)[1]. Next we studied the effects of polar and steric properties of the residue bound to the carbonyl group of the ester after hydrolysis of per-O-butyryl-and per-O-palmitoyl derivatives of 1,6-anhydro-β-D-glucopyranose. The stabilities and electron structures of 1,6-anhydro-β-D-glucopyranose and of the neutral and protonized forms of its acyl derivatives were calculated by the PCILO method. Both the experimental and the calculated data suggested that the differences in reactivity of the ester group at C-3 in relation to the reaction conditions (least and most reactive in acid and alkaline hydrolysis, respectively) are due to the possibility of stabilization of the intermediate (oxonium cation) in the acid hydrolysis and of the activated complex in the alkaline hydrolysis. The selectivity of hydrolysis

increased with increasing +I-effect and decreased with growing bulkiness of the moiety on the carbonyl group of the ester[2].

In studying the enzyme-catalysed deacetylation of 1,6-anhydro-2,3,4-tri-O-acetyl--β-D-glucopyranose we observed[3] that chymotrypsin, liver esterase and pancreatic and wheat-germ lipases were locoselective in their action. With chymotrypsin and/or wheat-germ lipase the preferential deacetylation was that from the hydroxyl at C-3, whereas with liver esterase or pancreatic lipase from the hydroxyl at C-4. In the use of cells from the yeast-like microorganism *Aureobasidium pullulans* CCY 27-1-14a, free or immobilized, the preferential deacetylation was at C-2. Compared with a chemically catalysed deacetylation, whether by methanolic hydrogen chloride or hydrazine hydrate, the locoselectivity of biocatalysis proved much better.

The present paper deals with deacylation of a more complex model, viz. 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose (perbutyrylated levoglucosan), isolated hog-pancreas chymotrypsin, hog-pancreas lipase and wheat-germ lipase being employed. Since the butyryl derivatives of levoglucosan are less soluble than the acetyl derivatives it was necessary to use a reaction mixture containing a greater portion of an organic solvent miscible with water. For this reason we also investigated the effect of concentration of the organic solvent (methanol) on locoselectivity of the reaction. The course of deacylation was monitored by gas chromatography; pure monobutyryl and dibutyryl derivatives of levoglucosan as standards were isolated by chromatography on silica gel and characterized by methylation analysis and ^{1}H NMR spectra, as previously described[2]. Deacylation of per-O-butyryl levoglucosan can theoretically produce a mixture of three mono-, three di-O-butyryl derivatives and the completely deesterified levoglucosan. O-Acyl-levoglucosans are regarded here as cyclic analogues of esterified glycerol, shielded by the 1,3-dioxolane ring. This steric effect is most marked on the axial hydroxyl group at C-3 (ref.[3]). In contrast to 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose, where chemical lability of the acetyl groups made it necessary to carry out the enzymic deacetylation[3,4] away from the pH optima of the enzymes, 1,6-anhydro-2,3,4-tri-O-butyryl--β-D-glucopyranose binds its bulkier butyryl groups more firmly, so that the enzymic deacetylation could be conducted in this case under conditions closer to the optimum pH's of the enzymes used. Besides, the butyryl groups, in contrast to the acetyl groups in 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose[3], did not exhibit any intramolecular rearrangement.

In the use of hog-liver pancreas in the system $V(CH_3OH)$: $V(H_2O) = 1 : 4$ the deacylation proceeded preferentially from the hydroxyl on C-2, the main reaction product being 1,6-anhydro-3,4-di-O-butyryl-β-D-glucopyranose. Furher deacylation prevailed at C-4, giving rise to 1,6-anhydro-3-O-butyryl-β-D-glucopyranose, but the portion of the latter was much smaller than that of the di-O-butyryl derivative (Table I). However, on increasing the volume concentration of methanol to 50%, deacylation occurred almost exclusively from the hydroxyl on C-4, affording 1,6-

-anhydro-2,3-di-O-butyryl-β-D-glucopyranose and traces of mono-O-butyryl derivatives (Table I). Hydrolysis of 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose with wheat-germ lipase in a medium of 20% (v/v) methanol took a similar course (Table II). However, in 50% (v/v) methanol the locoselectivity of deacylation proved shifted to C-3, 1,6-anhydro-2,4-di-O-butyryl-β-D-glucopyranose being the only substantial product (Table II). With hog-pancreas chymotrypsin in 20% (v/v) methanol the reaction affected mainly the C-4 hydroxyl, yielding 1,6-anhydro-2,3-di-O-butyryl-β-D-glucopyranose as the predominant product (Table III), whereas in 50% (v/v) methanol the chief product was 1,6-anhydro-2,4-di-O-butyryl-β-D-glucopyranose (Table III). Comparison of our results obtained in chemical[1,2] and in enzymic[3,4] hydrolysis of per-O-acyl derivatives of levoglucosan reveals that in the chemical hydrolysis the selectivity decreased with increasing size of the acyl, whereas in the enzymic hydrolysis rather the opposite was the case. As for the enzymic hydrolysis of peracylated levoglucosans it was of interest to compare the deacylation of 1,6-anhydro-2,3,4-tri-O-acetyl-β-D-glucopyranose in 10% (v/v) methanol, and the deacylation of 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose in 50% (v/v) methanol, the same enzyme being used; the positions of preferential hydrolysis were the same in the two cases. This fact can be ascribed to the solvation effect of methanol, directed against intramolecular interactions, which results in different conformations of the starting per-O-butyryllevoglucosan and the corresponding di-O-butyryl – and mono-O-butyryl derivatives, depending on the character of the reaction medium.

TABLE I

Hydrolysis of 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose with pancreatic lipase. The results are expressed in percentage of the individual butyryl derivatives

Time h	Position of the butyryl group							
	C(0)	C(2)	C(3)	C(4)	C(2, 3)	C(2, 4)	C(3, 4)	C(2, 3, 4)
			$V(CH_3OH) : V(H_2O) = 0{\cdot}25$					
3	0·0	6·1	17·5	1·2	7·3	0·0	50·0	17·9
6	0·9	9·3	27·2	2·3	4·7	0·0	48·3	7·3
24	1·1	10·4	32·1	2·2	1·8	0·0	47·6	4·8
			$V(CH_3OH) : V(H_2O) = 1{\cdot}0$					
3	0·0	1·1	0·6	0·0	21·7	0·0	0·5	76·1
7	0·0	1·3	2·3	0·4	39·5	0·0	0·6	55·9
24	0·0	1·4	4·9	0·9	68·0	0·0	0·0	24·8
48	0·3	1·7	3·0	1·9	77·8	0·0	0·0	15·3
72	0·4	1·6	3·4	2·5	86·4	0·0	0·0	5·7

TABLE II

Hydrolysis of 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucose with lipase from wheat-germ. The results are expressed in percentage of the individual butyryl derivatives

Time h	Position of the butyryl group							
	C(0)	C(2)	C(3)	C(4)	C(2, 3)	C(2, 4)	C(3, 4)	C(2, 3, 4)
				$V(CH_3OH) : V(H_2O) = 0{\cdot}25$				
3	0·0	5·0	18·4	0·8	6·6	0·0	54·1	15·1
6	0·4	7·1	29·7	1·5	4·0	0·0	57·1	0·2
24	1·0	10·8	32·3	1·9	4·4	0·0	49·6	0·0
				$V(CH_3OH) : V(H_2O) = 1{\cdot}0$				
3	0·0	0·8	0·0	1·5	0·8	24·4	0·0	72·5
7	0·0	1·2	0·0	2·3	1·3	33·5	0·0	61·7
24	0·0	1·5	0·0	2·5	1·6	46·8	0·0	47·6
48	0·5	1·9	0·0	4·1	3·1	62·9	0·0	27·5
72	0·8	2·5	0·0	4·5	3·4	78·6	0·0	10·2

TABLE III

Hydrolysis of 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose with chymotrypsin. The results are expressed in percentage of the individual butyryl derivatives

Time h	Position of the butyryl group							
	C(0)	C(2)	C(3)	C(4)	C(2, 3)	C(2, 4)	C(3, 4)	C(2, 3, 4)
				$V(CH_3OH) : V(H_2O) = 0{\cdot}25$				
3	0·0	1·0	1·0	1·3	51·5	0·0	0·0	45·2
24	0·4	1·0	1·5	1·5	75·3	0·0	0·0	20·3
48	1·0	1·3	0·0	2·4	82·2	0·0	0·0	13·1
				$V(CH_3OH) : V(H_2O) = 1$				
3	0·0	0·0	0·0	0·0	0·9	3·5	0·0	95·6
7	0·0	0·0	0·0	0·0	0·9	6·6	0·0	92·5
24	0·0	0·0	0·0	0·0	1·3	13·9	0·0	84·8
48	0·0	0·0	0·0	0·0	2·0	24·8	0·0	73·2
72	0·0	0·0	0·3	0·2	2·4	40·1	0·0	57·0
96	0·0	0·0	0·3	0·2	2·5	57·5	0·0	39·5

At the lower concentration of methanol the greater stability of the ester bound at C-3 of the butyryl derivative of levoglucosan, compared to the acetyl derivative, is due not only to steric reasons, but also to the increasing +I-effect of the alkyl on the carbonyl carbon atom. This strengthens the hydrogen bound between C(3)—O and O(6), thus weakening the solvation effect of methanol.

As can be seen from our preceding paper[2], giving PCILO charges on selected atoms in the region around the reaction site of hydrolysis for C(3)- substituted derivatives, an increase in size of the alkyl on the ester groups at C-3 diminishes the positive charge on the carbon and increases the negative charge on the oxygen of the carbonyl group. The greatest change was observed between the acetyl and the propionyl derivatives. Further elongation of the alkyl no longer brought about any significant changes. Our results obtained in the enzymic deacylation of per-O-butyryl derivatives of levoglucosan suggest two locoselective modes of deacylation, depending on concentration of methanol in the reaction mixture. How the locoselectivity of deacylation will change in a progressive increase of methanol concentration from 20 to 50% (v/v) will be the subject of further study.

EXPERIMENTAL

Materials: 1,6-anhydro-β-D-glucopyranose[1,5], m.p. 178—180°C, α_D −67·2° (*c* 1, H_2O), 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose[6], syrup, α_D −37·5°C (*c* 1, $CHCl_3$), 1,6-anhydro-2,3-di-O-butyryl-β-D-glucopyranose, 1,6-anhydro-2,4-di-O-butyryl-β-D-glucopyranose, 1,6-anhydro-3,4-di-O-butyryl-β-D-glucopyranose, 1,6-anhydro-2-O-butyryl-β-D-glucopyranose, 1,6-anhydro-3-O-butyryl-β-D-glucopyranose, and 1,6-anhydro-4-O-butyryl-β-D-glucopyranose were obtained and characterized as previously described[2].

Enzymes. Hog-pancreas lipase was isolaed from a pancreatic extract by a method described in ref.[7]. Wheat-germ lipase (EC 3.1.1.3) was a Koch-Light product (Colnbrook, England). Crystalline chymotrypsin ex hog pancreas (EC 3.4.4.5) was a Sigma product, St. Louis, U.S.A.

The other materials as constituents of the reaction mixtures and buffer solutions were analytical grade Lachema chemicals (Brno, Czechoslovakia). Silufol foils (Kavalier, Sázava, Czechoslovakia) were used for chromatographic separation of butyryl derivatives of levoglucosan and to monitor the course of enzymic hydrolysis; the spots were developed with 5% sulfuric acid in methanol on heating the foils to 100°C.

Enzymic Hydrolysis of 1,6-Anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose

A. To 100 mg of 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose dissolved in 1 ml of absolute methanol was added 4 ml of a phosphate buffer (pH 7·0, 0·05 mol dm^{-3}), containing hog-pancreas lipase (total activity 2 ncat and specific activity 1 ncat mg^{-1}), or wheat-germ lipase, (total activity 1·9 ncat, specific activity 1·8 ncat mg^{-1}), or chymotrypsin (total activity 1·3 ncat, specific activity 2·8 ncat mg^{-1}). The enzymic reaction proceeded in a temperature-controlled oven at 37°C, 0·5 ml samples being withdrawn at selected time intervals. The samples were mixed with 5 ml of absolute methanol and filtered; the filtrates were taken to dryness in a rotary vacuum evaporator. The remaining water was removed by repeated evaporation with anhydrous methanol. The liberated hydroxyl groups in the hydrolytic products of 1,6-anhydro-2,3,4-tri-O-

-butyryl-β-D-glucopyranose were converted to trifluoroacetyl groups by adding 0·2 ml of trifluoroacetanhydride. In this form the samples were analysed by gas chromatography.

B. To 100 mg of 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose in 2·5 ml of absolute methanol was added 2·5 ml of the phosphate buffer (pH 7·0, 0·05 mol dm^{-3}) containing hog-pancreas lipase, or wheat-germ lipase or chymotrypsin, the total and the specific activities being the same as in procedure *A*. Samples of the hydrolysate were also worked up as in the foregoing procedure.

Gas chromatography ran in a Hewlett–Packard chromatograph, model 5830 A, equipped with a flame-ionization detector. A stainless steel column was used, i.d. 2 mm, length 300 cm, packed with 3% OV 225 on Supercorporate (particle size 0·135—0·150 mm). The heating of the column was programmed with a temperature gradient of 1°C/min, starting from 135°C (6-min. isotherm). The temperature of the inlet was 220°C, the detector temperature was 300°C. The carrier gas was nitrogen at a flow rate of 15 ml/min. The results were evaluated by a computer in normalized percentage after calibration with authentic standards.

In a separate experiment, starting from 1,6-anhydro-2,3,4-tri-O-butyryl-β-D-glucopyranose (1 g), the reaction products were isolated by chromatography on a column of silica gel (Silikagel L-100/250, Lachema, Czechoslovakia), using a procedure previously described[2]. They were characterized by melting points, optical rotation, elemental analysis and ^{1}H NMR spectra. The R_F values of the compounds in the analysed mixture were found to agree with those of the authentic standards[2].

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