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PERDEUTERIOACETYLATION WITH COMBINED NMR AND MS ANALYSIS AS A METHOD FOR DETERMINING THE EVOLUTION OF INDIVIDUAL HYDROLYSIS PRODUCTS DURING REGIOSELECTIVE ENZYMATIC HYDROLYSIS OF GLUCOSE PENTAACETATE.

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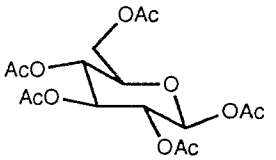
A new procedure is described that is based on NMR and MS analysis of the products of perdeuterioacetylation of the products of partial hydrolysis of β-D-glucose pentaacetate by the lipase from *Aspergillus niger*. This procedure eliminates the need for separation and estimation of each individual product and is of general applicability.

Keywords: Lipase, hydrolysis, β-D-glucose pentaacetate, perdeuterioacetylation

1. Introduction

The synthesis of biologically significant oligosaccharides constitutes a major challenge in synthetic organic chemistry. In this context, the regioselective protection of sugar hydroxyl groups plays a major role. The known selectivity of enzymatic reactions has led to extensive investigations into the enzyme-catalysed



(1)

Scheme (1).

regioselective hydrolysis of acylated sugars and glycosides [1–8] and the regioselective esterification of sugars and glycosides [9-16]. However, given the possibility of carrying out regioselective ester transformations, a major difficulty remains in the analysis of the product mixtures arising from such reactions. The glucose pentaacetate (scheme (1)) used as a vehicle for the present study, for example, could give rise, in principle, to five tetraacetates, ten triacetates, ten diacetates, five monoacetates, and glucose, a total of thirty two possible products (including the substrate, glucose pentaacetate). Analysis of such a mixture, as individual compounds, would be a formidable problem. However, an approach to a simpler analytical method was suggested by the method of reacetylation of partial hydrolysis products with [^{2}H]-acetic anhydride ("perdeuterioacetylation") [17] as a means of determining net loss of acetyl groups from specific positions during hydrolysis of peracetylated sugars. For this procedure to succeed, it is necessary to be able to distinguish the individual acetyl groups, a problem best solved by NMR.

Suppose, for example, that all of the acetyl methyl signals of β-D-glucose pentaacetate (1) could be resolved and assigned using ^{1}H NMR. The net loss of acetyl groups from individual positions following partial hydrolysis could be determined by perdeuterioacetylation and comparison of the intensities of the NMR signals attributable to individual acetyl groups with those of the non-deuterated, peracetylated material. The significant difference between this approach and others, such as the procedure used by Shaw and Klibanov [3] for the analysis of the products from the hydrolysis of β-D-glucose pentaacetate by the lipase from *Aspergillus niger*, the system used in the present study, is that final analysis is carried out always on a single compound (β-D-glucose pentaacetate) rather than on a mixture that changes in nature and complexity throughout the process being analysed.

The aim of the present study was to hydrolyse β-D-glucose pentaacetate, using the lipase from *Aspergillus niger*, and to follow the evolution of individual, partly acetylated species. As noted above, direct analysis of all possible hydrolysis products would not be feasible, particularly since identification would be required in each case, and isolation of each product, at least on an analytical scale, would be required. However, separation of partly acetylated products as individual *classes* (tetraacetates, triacetates etc.) would be much simpler. The relative amounts of each class in the total hydrolysis product could then be determined by perdeuterioacetylation and mass spectrometric determination, based on the relative intensities of the M, M + 3, M + 6 etc. peaks. If this information were to be combined with the results of perdeuterioacetylation and NMR analysis of individual classes of partly acetylated products, it would be possible, in principle, to determine the amounts of individual isomers in a given class.

Thus, the tetraacetates can be described by the following array, in which the numbers indicate acetylated positions and products are described as $^{x}p_{y}$, where x

indicates the class (4 = tetraacetate) and y indicates the individual isomer.

1 2 3 4 – $^4p_1$

1 2 3 – 6 $^4p_2$

1 2 – 4 6 $^4p_3$

1 – 3 4 6 $^4p_4$

– 2 3 4 6 $^4p_5$

Suppose the percentage decrease in the signals attributable to each acetyl group in the perdeuterioacetylated triacetates are $^4d_1$, $^4d_2$, $^4d_3$, $^4d_4$, and $^4d_5$, then by inspection:

$^4p_5 = d_1$

$^4p_4 = d_2$

etc.

The corresponding array for the triacetates is of dimension 5×10:

1 2 3 – – $^3p_1$

1 2 – 4 – $^3p_2$

1 2 – – 6 $^3p_3$

1 – 3 4 – $^3p_4$

1 – 3 – 6 $^3p_5$

1 – – 4 6 $^3p_6$

– 2 3 4 – $^3p_7$

– 2 3 – 6 $^3p_8$

– 2 – 4 6 $^3p_9$

– – 3 4 6 $^3p_{10}$

The losses in intensity of the NMR signals of the perdeuterioacetylated mixture are given by:

$^3d_1 = {}^3p_7 + {}^3p_8 + {}^3p_9 + {}^3p_{10}$

$^3d_2 = {}^3p_4 + {}^3p_5 + {}^3p_6 + {}^3p_{10}$

etc.

This is an array of five equations with ten unknowns, and cannot be solved for each individual product $^3p_1$, $^3p_2$ etc. However, the assumption underlying the

application of enzymatic hydrolysis is that the hydrolysis will be selective. Thus if (as is known [3] and will be confirmed below), the acetate group at C-6 is resistant to hydrolysis and the acetate group at C-1 is rapidly and completely hydrolysed before groups at other positions, species ${}^3p_1$, ${}^3p_2$, ${}^3p_3$, ${}^3p_4$, ${}^3p_5$, ${}^3p_6$, and ${}^3p_7$ will not be produced. We are then left with four equations and three unknowns:

$${}^3d_1 = {}^3p_8 + {}^3p_9 + {}^3p_{10}$$

$${}^3d_2 = {}^3p_{10}$$

$${}^3d_3 = {}^3p_9$$

$${}^3d_4 = {}^3p_8$$

Since the sum total of triacetates can be determined by perdeuterioacetylation of the total hydrolysis mixture, the percentage of each individual triacetate is readily computed.

Alternatively, the calculations may be based on contributions of a given species to each acetate signal. Thus if ${}^3I_1$, ${}^3I_2$, etc represent the integrations of peaks attributable to the C-1, C-2 acetate groups etc respectively, in the NMR spectrum of the perdeuterioacetylated mixture, then:

$${}^3I_2 = {}^3p_8 + {}^3p_9$$

$${}^3I_3 = {}^3p_8 + {}^3p_{10}$$

$${}^3I_4 = {}^3p_9 + {}^3p_{10}$$

The corresponding array for the diacetates (omitting species lacking an acetyl group at C-6) is as follows:

1	–	–	–	6	${}^2p_1$
–	2	–	–	6	${}^2p_2$
–	–	3	–	6	${}^2p_3$
–	–	–	4	–	${}^2p_4$

The corresponding set of simultaneous equations is:

$${}^2d_1 = {}^2p_2 + {}^2p_3 + {}^2p_4$$

$${}^2d_2 = {}^2p_1 + {}^2p_3 + {}^2p_4$$

$${}^2d_3 = {}^2p_1 + {}^2p_2 + {}^2p_4$$

$${}^2d_4 = {}^2p_1 + {}^2p_2 + {}^2p_3$$

Here we have four equations and four unknowns. However, since hydrolysis of the C-1 acetate group is rapid and essentially complete before hydrolysis occurs

at any other position, species $^{2}p_1$ can be ignored. The relative proportions of the remaining diacetates are then obtained by inspection.

The only monoacetate produced in significant amount is the C-6 acetate.

2. Experimental

2.1. GENERAL PROCEDURES

^{1}H NMR spectra were obtained using a Bruker WH 400 spectrometer. Long range ^{13}C-^{1}H shift correlation was carried out by a heteronuclear COSY-type experiment, with the use of a composite 180° carbon pulse, and refocussing delays of 3.7 and 1.85ms [18]. Mass spectra were obtained with a Kratos MS-80 spectrometer. β-D-Glucose pentaacetate and [$^{2}H_6$]acetic anhydride were obtained from the Aldrich Chemical Co. Ltd. The lipase from *Aspergillus niger* was obtained from the Amano Pharmaceutical Company.

2.2. ENZYMATIC HYDROLYSES

A suspension of β-D-glucose pentaacetate (250 mg) in phosphate buffer (Na_2HPO_4/KH_2PO_4, pH 7.9,6.4 ml, 0.5 M) was stirred at 30 °C with Lipase A (Amano). The reaction was stopped at intervals between 40 and 150 min. and the product mixture was analysed.

2.3. SEPARATION OF PARTLY ACETYLATED PRODUCTS

The mixture was lyophilised and the residue was dissolved in ethyl acetate:ethanol (1 : 1, 25 ml). Silica (1 g, silica gel 60, particle size 0.040–0.063 mm) was added. The solvent was evaporated under reduced pressure and the silica gel was packed on top of a column of silica gel 60, (25 mm diam., 60 g). The column was eluted with the following solvents: light petroleum (b.p. 40–60 °C):ethyl acetate (1 : 1, 250 ml; 4 : 6, 125 ml; 3 : 7, 250 ml; 2 : 8, 125 ml; 1 : 9, 125 ml); ethyl acetate (250 ml); ethyl acetate:ethanol (95 : 5, 125 ml; 9 : 1, 125 ml; 8 : 2, 125 ml). Fractions (20 ml) were collected and analysed by TLC (silica gel 60), solvent system light petroleum (b.p. 40–60 °C):ethyl acetate (1 : 9). The spots were visualised by spraying with sulphuric acid (10% in ethanol) followed by heating. If cross-contamination by another class of partial hydrolysis product was observed, the fraction was further spearated by preparative TLC. Solvent was evaporated under reduced pressure and the residue was perdeuterioacetylated using [$^{2}H_6$]acetic anhydride (0.2 ml) in pyridine (0.5 ml) over 12 h. The solution was evaporated under reduced pressure and the residue was examined by ^{1}H NMR and MS. A sample of the total hydrolysis product, before separation, was also perdeuteroacetylated and examined in the same way.

3. Results and discussion

The procedure described in the Introduction was applied to the analysis of the product mixture from hydrolysis of β-D-glucose pentaacetate using the lipase from *Aspergillus niger*. Before proceeding to a full analysis, a further complication needed resolution. As noted above, hydrolysis of the C-1 acetate group was extremely rapid. Mutarotation was therefore possible and perdeuterioacetylation gave, at all stages, a mixture of α- and β-glucose pentaacetates (partly deuterioacetylated). Accordingly, for the NMR analysis, it was necessary to define conditions that would permit resolution and identification of the ten signals attributable to the acetyl methyl signals of the anomeric pentaacetates. After

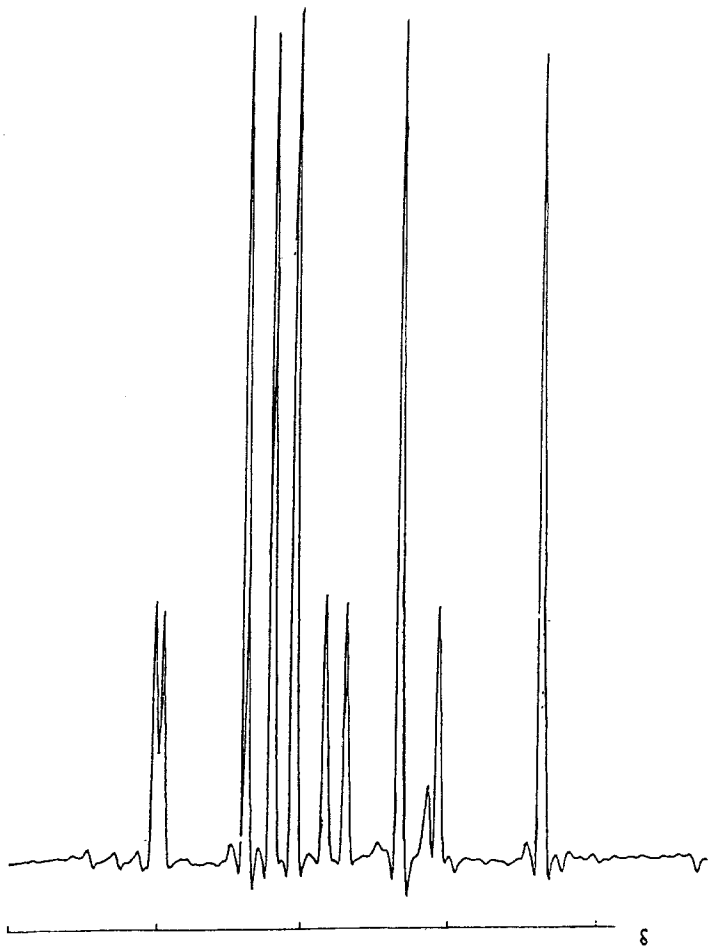


Fig. 1a. Resolution of acetate methyl signals by ^1H NMR in d_5-pyridine:d_6-benzene (0.35:0.3). The assignments of the peaks, reading from left to right, (α, β, refer to the anomer, α-D-glucopyranose pentaacetate, or β-D-glucose pentaacetate respectively, numerals refer to the position of attachment of the acetyl group) are: α-4, α-3, β-3, β-2, β-4, α-6, α-1, β-6, α-2, β-1.

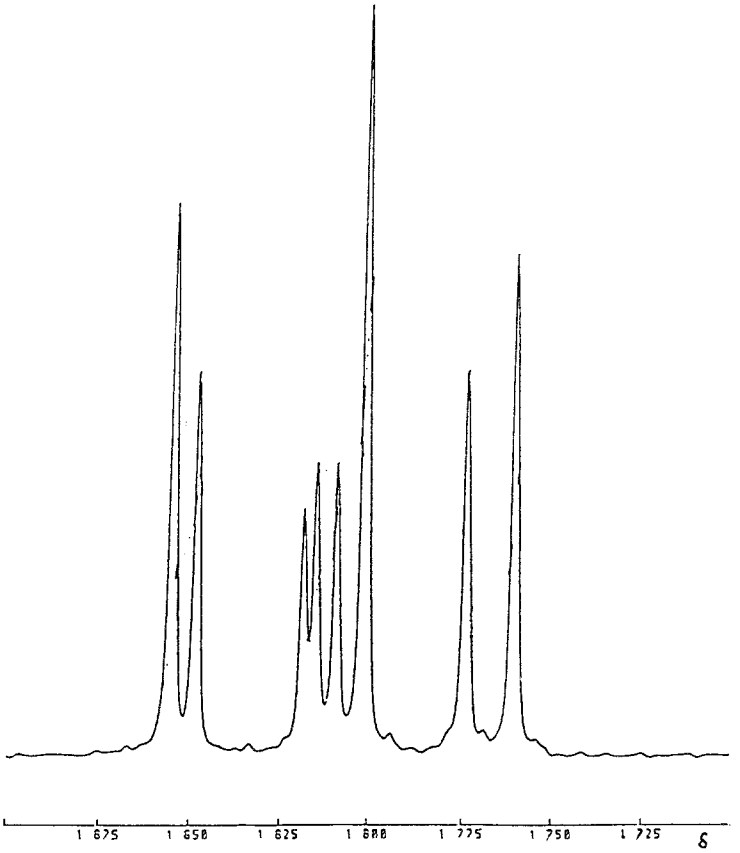


Fig. 1b. ^{1}H NMR spectrum of perdeuterioacetylated product from the partial enzymatic hydrolysis product from β-D-glucose pentaacetate.

trials, it was found that in a mixture of d_5-pyridine: d_6-benzene (0.35 : 0.3), all ten signals were resolved at 400 MHz (fig. 1a). Although the formation of α- and β-D-glucose pentaacetates gave rise to this complication, at the same time, because integration of both sets of peaks was possible, two independent sets of data could be obtained from each NMR experiment. From these two sets of data, it was possible also to make an estimate of the experimental errors. These were found to be $12 \pm 8\%$.

A long-range ^{13}C-^{1}H 2D shift correlation experiment simultaneously revealed ^{3}J H (ring) to CO and ^{2}J CO to CH_3 coupling. Each ring proton resonance was thus unambiguously correlated with one methyl proton resonance. The ring protons were assigned by standard methods. A similar indirect shift correlation using the Coloc method has been reported by Morris et al. [19].

A typical spectrum of a perdeuterioacetylated product mixture is shown in fig. 1b. As noted above, hydrolysis of the C-1 acetate group was extremely rapid. Accordingly, in this spectrum, only eight signals are seen, the C-1 hydroxyl group having been completely perdeuterioacetylated in both anomers.

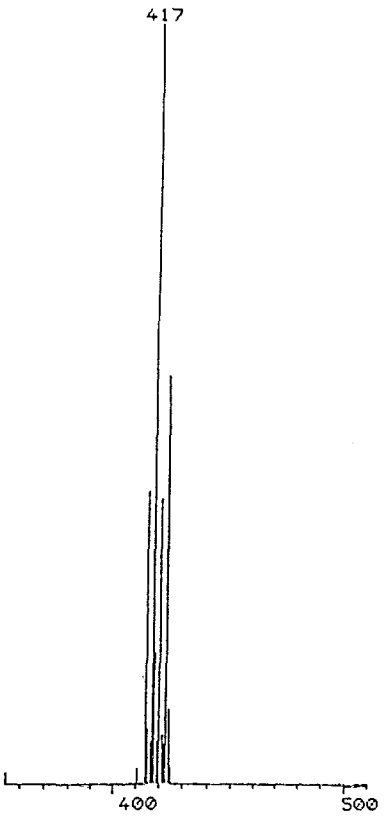


Fig. 2. Ion cluster in the mass spectrum of perdeuterioacetylated partial enzymatic hydrolysis product of β-D-glucose pentaacetate.

To prepare for the more detailed analysis, hydrolysis of β-D-glucose pentaacetate was carried out and the total hydrolysis mixture was analysed at intervals by perdeuterioacetylation followed by mass spectrometric analysis in the chemical ionisation mode with ammonia as the ionising gas. Analysis of the mixture was based on the quasimolecular ion $C_{16}H_{22}O_{11}.NH_4^+$ ($m/Z = 408$). Penta-, tetra-, tri-, di- and monoacetates and glucose in the product mixture gave peaks of $m/Z = 408$, 411, 414, 417, 420 and 423 respectively. A typical ion cluster is shown in fig. 2. This has peaks of m/Z (% abundance relative to base peak) 408(0.7), 411(17.7), 414(41.7), 417(20.0), 420(5.2) and 423(3.4) corresponding to a mixture of pentaacetate (0.8%), tetraacetates (19.9%), triacetates (47.0%), diacetates (22.5%), monoacetates (5.9%) and glucose (3.8%). Typical results from two hydrolyses are shown in fig. 3a,b. In the first experiment, the hydrolysis products had evolved further than in the second experiment, but the product profile is similar in both. At 50 min, tetraacetates predominated and at 100 min triacetates were the most abundant species. Thereafter, di- and monoacetates and glucose evolved at the expense of triacetates. These product profiles are similar to those obtained by Shaw and Klibanov and which were based on gas chromato-

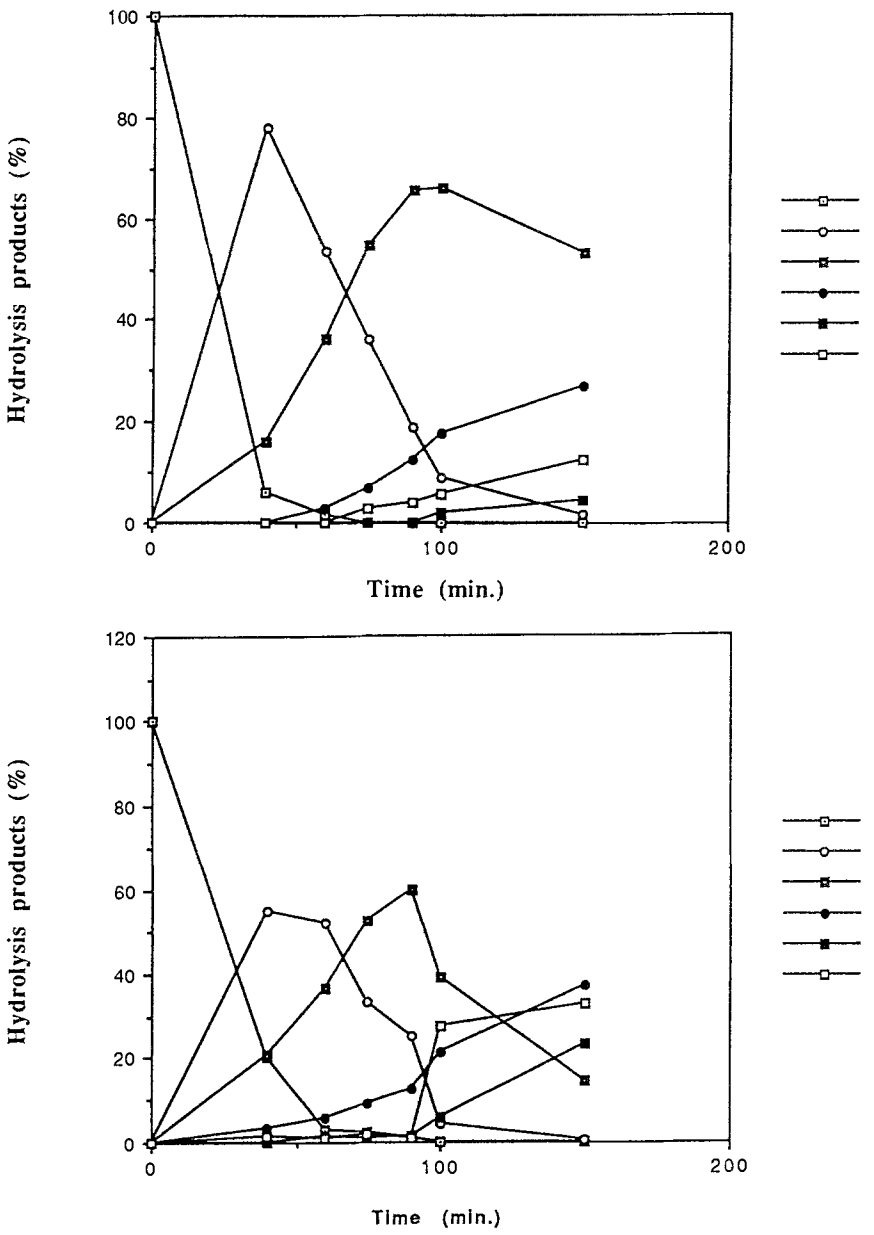


Fig. 3. a, b. Evolution of partial hydrolysis products by class in the enzymatic hydrolysis of β-D-glucose pentaacetate.

graphic analysis of silylated derivatives of the products in the total hydrolysis mixture.

Each aliquot was subjected to separation into acylation classes by chromatography over silica gel. For the analytical procedure described above, it was important that the individual classes were isolated free of contamination by other

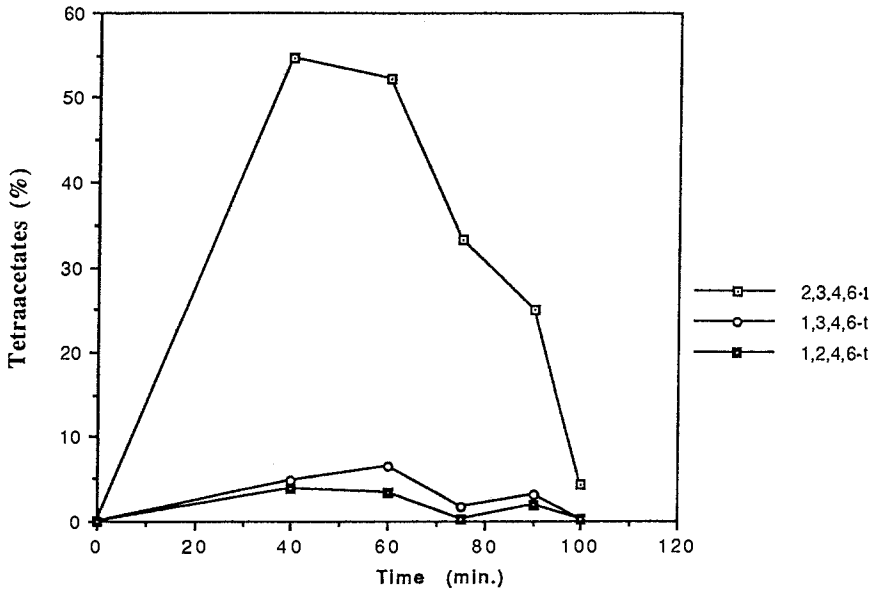


Fig. 4. Evolution of tetraacetates in the enzymatic hydrolysis of β-D-glucose pentaacetate.

classes. That this condition obtained was checked by mass spectrometric analysis of the perdeuterioacetylated fractions, which readily revealed any cross contamination (cf fig. 2). Occasionally, small amounts of cross contamination were observed in the diaacetate fractions, and where appropriate, corrections were made for contaminating monoacetates and glucose. Evolution of tetraacetates is

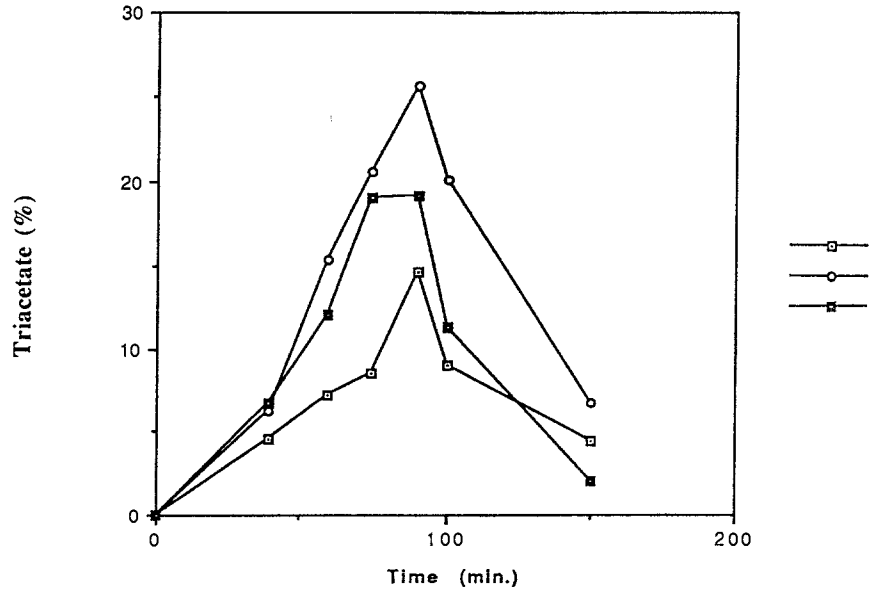


Fig. 5. Evolution of triacetates in the enzymatic hydrolysis of β-D-glucose pentaacetate.

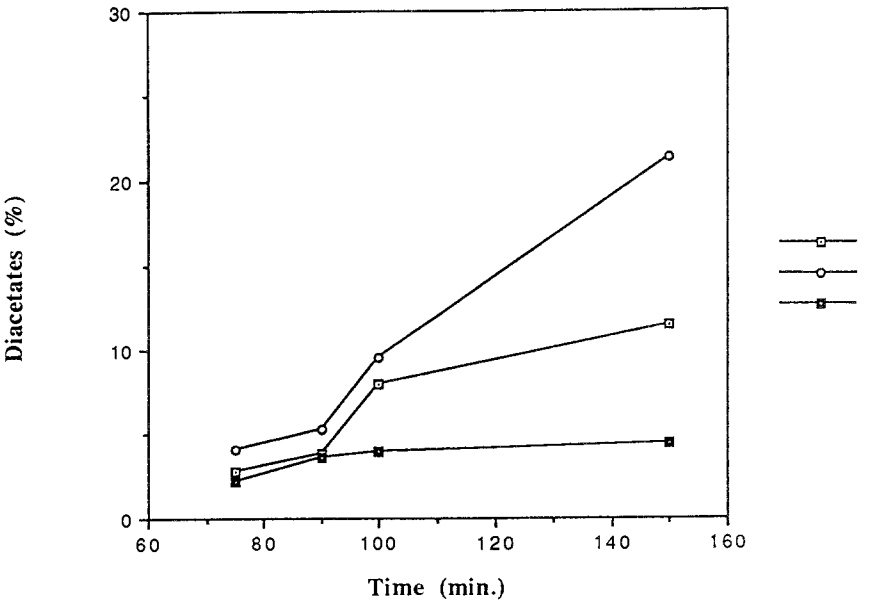


Fig. 6. Evolution of diacetates in the enzymatic hydrolysis of β-D-glucose pentaacetate.

shown in fig. 4. The predominant species at all times was the 2,3,4,6-tetracetate, the minor amounts measured of the 1,3,4,6- and 1,2,4,6-tetraacetates are barely statistically significant. Evolution of the triacetates is shown in fig. 5. The major isomers were the 2,4,6- and the 3,4,6-triacetates, showing that after the 6-acetoxy group, the 4-acetoxy group was the ester function most resistant to hydrolysis. The 2,3,6-triacetate was also formed. With these substrates, however, selectivity appeared to change, since in the diacetate class the 3,6- and 2,6-diacetates were the most abundant species in this class, with lesser amounts of the 4,6-diacetate (fig. 6). The only monoacetate produced was the 6-acetate, the evolution of which is shown in fig. 7, together with that of glucose.

The method described, as this demonstration shows, makes possible the identification and estimation of individual species in a potentially highly complex mixture of products. The method is of general applicability provided that a certain minimum selectivity is observed in the system under investigation. Whether or not selectivity is exhibited is readily determined by perdeuterioacetylation and NMR analysis of total hydrolysis products, without need for separation of any kind. In the present case, for example, the complete and rapid hydrolysis of the C-1 acetoxy group was readily seen in the absence, shortly after incubation had begun, of any signals attributable to the C-1 acetoxy group in the NMR spectrum of the total hydrolysis mixture (cf. fig. 1b). By the same token, the resistance of the C-6 acetoxy group to hydrolysis was revealed in the ratio of the integration of the corresponding NMR signal compared with the combined integrations of sugar ring methine and methylene protons.

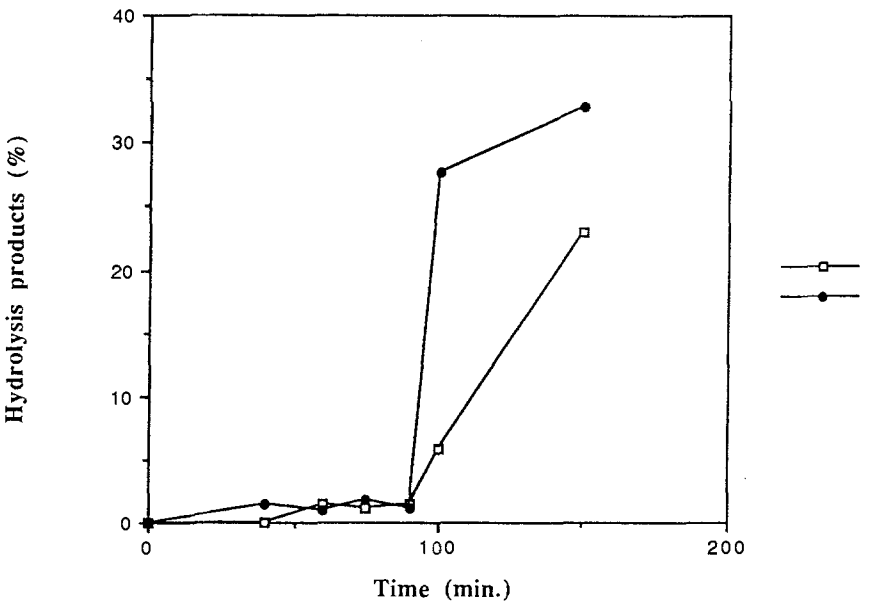


Fig. 7. Evolution of glucose 6-acetate and glucose in the enzymatic hydrolysis of β-D-glucose pentaacetate.

Interpretation of the results presented here in terms of the selectivity of the enzyme must be made with caution, since the possibility of acetyl migration is always present and such rearrangements would not have been detected in these experiments.

Acknowledgement

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References

[1] H.M. Sweers and C-H. Wong, J. Am. Chem. Soc. 108 (1986) 6421.
[2] J. Zemek, S. Kucas and D. Anderle, Coll. Czech. Chem. Commun. 52 (1987) 2347.
[3] J-F. Shaw and A.M. Klibanov, Biotechnol. Bioeng. 29 (1987) 648.
[4] M. Kloosterman, E.W. Mosmüller, H.E. Schoemaker and E.M. Meijer, Tetrahedron. Lett. 28 (1987) 2989.
[5] M. Kloosterman, M.P. de Nijs, J.G.J. Weijnen, H.E. Schoemaker and E.M. Meijer, J. Carbohydr. Chem. 8 (1989) 333.
[6] A. Uemera, K. Nozaki, J. Yamashita and M. Yasumoto, Tetrahedron. Lett. 30 (1989) 3819.
[7] A. Ballesteros, M. Bernabé, C. Cruzado, M. Martin-Lomas and C. Otero, Tetrahedron 45 (1989) 7077.
[8] H. Hönig, P. Seufer-Wasserthal, A.E. Stüts and E. Zenz, Tetrahedron Lett. 30 (1989) 811.

[9] M. Therisod and A.M. Klibanov, J. Am. Chem. Soc. 108 (1986) 5638.
[10] M. Therisod and A.M. Klibanov, J. Am. Chem. Soc. 109 (1987) 3977.
[11] S. Riva, J. Chopineau, A.P.G. Kieboom and A.M. Klibanov, J. Am. Chem. Soc. 110 (1988) 584.
[12] A. Uemera, K. Nozaki, J. Imashita and M. Yasumoto, Tetrahedron Lett. 30 (1989) 3817.
[13] F. Nicotra, S. Riva, F. Secundo and L. Zuchelli, Tetrahedron Lett. 30 (1989) 1703.
[14] G. Carrea, S. Riva, F. Secundo and B. Danieli, J. Chem. Soc., Perkin Trans 1 (1989) 1057.
[15] F. Björkling, S.E. Godtfredsen and S.E. Kirk, J. Chem. Soc., Chem. Commun. (1989) 934.
[16] K. Adelhorst, F. Björkling, S.E. Godtfredsen and O. Kirk, Synthesis (1990) 112.
[17] T. Suami, T. Otake, S. Ogawa, T. Shoji and N. Kato, Bull. Chem. Soc. Jpn 43 (1970) 1219.
[18] A. Bax and G.A. Morris, J. Magn. Res., 42 (1981) 501.
[19] T. Nishida, G.A. Morris and C.R. Enzell, Magn. Reson. Chem. 24 (1986) 179.