Note

Stereochemistry of products of hydrolysis of 2-acetamido-2-deoxy-β-D-glucosides by boar epididymis 2-acetamido-2-deoxy-β-D-glucosidase

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The evidence of the steric course of hydrolysis catalyzed by glycosidases is an important part in the study of these enzymes, in particular for classification of carbohydrases[1,2]. Several approaches based on examination of the anomeric configuration of the sugars released during enzymic hydrolysis have been described for solving this problem[2–7]. It was shown that the application of n.m.r. spectroscopy[2] is more advantageous than polarimetry[3,4] and g.l.c. methods[5–7].

As a part of the study of 2-acetamido-2-deoxy-β-D-glucosidase from boar epididymis[8] we used n.m.r. spectroscopy for examination of the initial products released by the action of this enzyme on benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside. In the n.m.r. spectrum of the substrate (Fig. 1A), the H-1-α and *N*-acetyl methyl signals appear at 3.58 and 0.72 p.p.m., respectively. During the 10 min following the enzyme addition (Fig. 1B), the signal at 0.82 p.p.m. of the *N*-acetyl methyl group of the hydrolyzed substrate (2-acetamido-2-deoxy-D-glucose) appears also. The ratio (calculated from the signal intensity) of the formed 2-acetamido-2-deoxy-D-glucose to the unhydrolyzed substrate is approximately 1:2, which is in agreement with the kinetic measurements (Fig. 2, curve A). The absence of the H-1-β signal in the n.m.r. spectrum (Fig. 1B) points out that there is no inversion of the configuration in this process. The substrate is completely hydrolyzed within 2 h, as shown from the disappearance of the signal at 0.72 p.p.m. (Fig. 1C). The H-1 signal of 2-acetamido-2-deoxy-α-D-glucopyranose (3.98 p.p.m., $J_{1,2}$ ~3 Hz) results from the mutarotation of 2-acetamido-2-deoxy-β-D-glucopyranose produced by the enzymic hydrolysis.

The attribution of the signals in the spectra is confirmed by the spectrum of the equilibrium mixture of 2-acetamido-2-deoxy-α- and β-D-glucopyranose (Fig. 1D), where the H-1-β (3.97 p.p.m., $J_{1,2}$ 3.3 Hz), the H-1-α (3.48 p.p.m., $J_{1,2}$ 7.7 Hz), and the *N*-acetyl methyl (0.83 p.p.m.) signals are observed together. It is important to note that the methyl signals of 2-acetamido-2-deoxy-α- and β-D-glucopyranose have the same chemical shift.

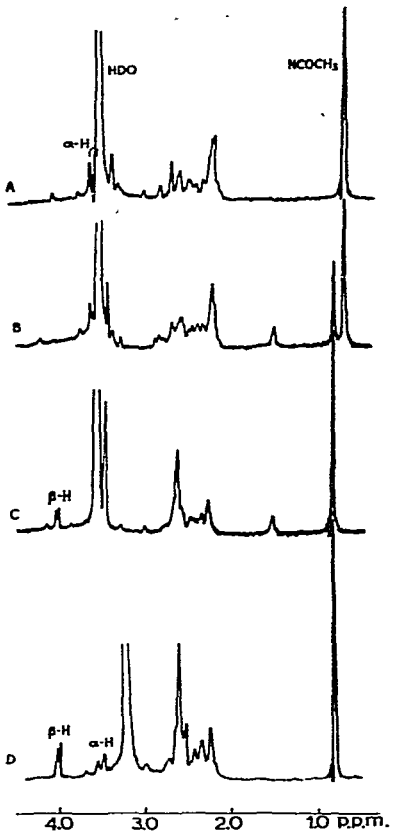

Fig. 1. N.m.r. spectra of (A) benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside, (B) the enzymic digest 10 min after the enzyme addition, (C) 2 h after the enzyme addition, and (D) 2-acetamido-2-deoxy-α,β-D-glucose.

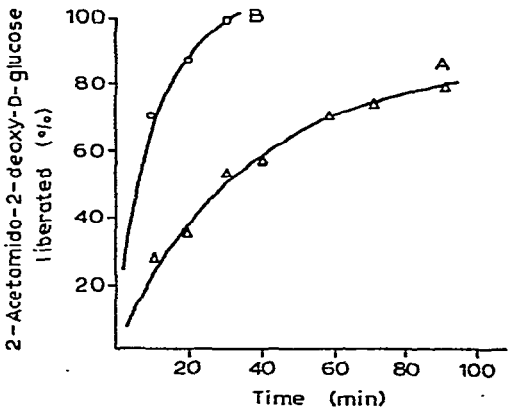

Fig. 2. Kinetics of the enzymic hydrolysis: (A) at 25° and concentration of the substrate of 35 mg/ml and of the enzyme of 8 mg/ml; (B) at 37° and concentrations of 3.5 and 1.4 mg/ml, respectively.

Thus, n.m.r. analysis shows that the enzyme hydrolyzes the glucoside with retention of the anomeric configuration. The same conclusion is suggested by the polarimetric data of the hydrolysis performed under conditions where the rate of hydrolysis (Fig. 2, curve B) is much greater than the rate of mutarotation. A smooth

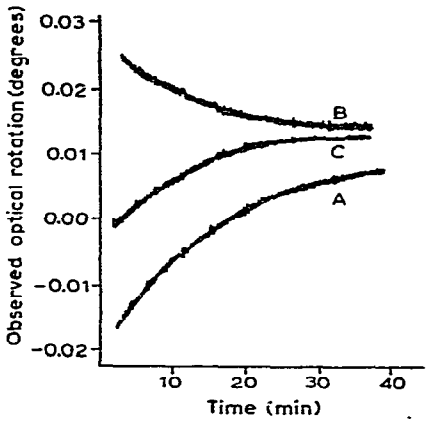

Fig. 3. Optical rotations observed during hydrolysis of the substrate (A) and during mutarotation of 2-acetamido-2-deoxy-α-D-glucopyranose (B) and of 2-acetamido-2-deoxy-β-D-glucopyranose (C) at 36.5–37.0°. The concentration of the sugars was 3.5 mg/ml and of the enzyme 1.4 mg/ml.

increase in the positive direction (Fig. 3, curve A) and the absence of a maximum in this region allow to conclude that the initial product of the enzymic hydrolysis is 2-acetamido-2-deoxy-β-D-glucopyranose.

Reese *et al.*[1] have demonstrated that *exo*-glucanases differ from glucosidases not only in the anomeric configuration of the sugars released on enzymic hydrolysis, but also in such other characteristics as substrate specificity, inhibition with δ-gluconolactone, and transfer of glucosyl groups. On the basis of the results obtained with our system, of the similar rate constants observed for the hydrolysis of β-(1→3)-, β-(1→4)-, and β-(1→6)-linked 2-acetamido-2-deoxy-D-glucose disaccharides and of some other 2-acetamido-2-deoxy-β-D-glucosides[8], of the transfer of glycosyl groups[8], and of the high inhibition observed with 2-acetamido-2-deoxy-D-glucono-1,5-lactone[9], we have classified the 2-acetamido-2-deoxy-β-D-glucosidase from boar epididymis as a glycosidase rather than as an *exo*-glycanase.

MATERIALS AND METHODS

Enzyme. — The 2-acetamido-2-deoxy-D-glucoside 2-acetamido-2-deoxy-β-D-glucohydrolase (E. C. 3.2.1.30) was isolated and purified as described previously[8], and was found to be homogeneous on disc electrophoresis. It released[10] 420 μmoles of *p*-nitrophenol/mg of protein/min from mM *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in the presence of 0.01% bovine serum albumine at 37°.

Substrate. — Reaction of 2-methyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyrano)-2-oxazoline[11] with dry benzyl alcohol in the presence of 2% hydrogen chloride gave benzyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (in 75% yield), m.p. 168–169°, $[\alpha]_D^{25}$ −39° (*c* 1, methanol). Lit.[12]: m.p. 170°, $[\alpha]_D^{26}$ −43° (*c* 1, methanol). De-*O*-acetylation (Zemplén) of the latter compound gave benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside, m.p. 207–208°, $[\alpha]_D^{25}$ −49.7° (*c* 0.28, water). Lit.[11]: m.p. 207–208°, $[\alpha]_D^{26}$ −48° (*c* 1, water).

Enzymic hydrolysis. — In a typical experiment the substrate (17.9 mg) was dissolved in 0.1M sodium potassium phosphate buffer, pH 5.1 (0.51 ml), and the lyophilized enzyme was added to the solution. The release of 2-acetamido-2-deoxy-D-glucose was determined by a modified Morgan–Elson procedure[13].

N.m.r. spectroscopic determination. — Spectra were recorded with a Jeolco JNM-4H-100 spectrometer (100 MHz) in buffered (D_2O) solutions at a concentration of substrate of 35 mg/ml and of enzyme of 8 mg/ml at *ca.* 30°. The chemical shifts were measured relative to the methyl groups signal of the internal reference *tert*-butyl alcohol. The accuracy of the chemical shifts measurements was ± 0.01 p.p.m.

Polarimetric procedure. — A recording spectropolarimeter Cary-60 was used. The hydrolysis was performed at a concentration of substrate of 3.5 mg/ml and of enzyme of 1.4 mg/ml at 36.5–37.0°.

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