Carbohydrate Research, 186 (1989) 217–223
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SYNTHESIS OF 2-ACETAMIDO-2-DEOXY-3-*O*-β-D-GALACTOPYRANOSYL-D-GALACTOSE BY THE SEQUENTIAL USE OF β-D-GALACTOSIDASES FROM BOVINE TESTES AND *Escherichia coli*

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(Received June 11th, 1988; accepted for publication, September 26th, 1988)

ABSTRACT

β-D-Gal*p*-(1→3)-D-GalNAc (**1**) was synthesised from lactose and GalNAc on a mmolar scale by transgalactosylation using β-D-galactosidase from bovine testes. The large proportions of unwanted oligosaccharides in the product mixture were removed by treatment with β-D-galactosidase from *E. coli*, which left **1**, monosaccharides, and a small proportion of trisaccharides. Carbon–Celite chromatography then gave **1** in a yield of 21% based on the GalNAc added.

INTRODUCTION

As the understanding of the biological role of glycoconjugates has increased, so has the need for cheap, synthetically produced oligosaccharides. We have been concerned with the synthesis of glycosidic linkages, using glycosidases which are usually readily available and comparatively cheap. Although some elegant examples have been presented[1,2], we believe that glycosyltransferases are not suitable at present for preparative work since they have limited stability, are difficult to purify, and involve nucleotide–sugar regeneration.

Glycosidases may be used for the synthesis of glycosides either by reversal of the hydrolytic reaction[3] or by the transglycosylation reaction[4–6]. They are usually specific with respect to the terminal non-reducing sugar and the linkage type, but the orientation of the aglycon is often of less importance. This limited specificity is a drawback when using glycosidases for synthesis. However, by using a suitable combination of enzymes with partly overlapping specificities, the desired overall specificity may be achieved.

We now report the synthesis of the T-antigenic determinant β-D-Gal*p*-(1→3)-

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0008-6215/89/$ 03.50

D-GalNAc (**1**), which is the immunodominant structure of the Thomsen–Friedenreich receptor[7,8] and an auto-immune marker present on the external surface membrane of human carcinomas[9].

EXPERIMENTAL

Materials. — *E. coli* β-D-galactosidase (EC 3.2.1.23) grade 6 (specific activity, 80–150 U/mg of protein) was obtained from Sigma, and bovine testes β-D-galactosidase (EC 3.2.1.23) was from Boehringer Mannheim. Celite Hyflo Super-Cel was from KEBO Lab AB (Stockholm, Sweden).

Enzyme assays. — β-D-Galactosidase from bovine testes was incubated with 2mM *o*-nitrophenyl β-D-galactopyranoside (ONPG) in 50mM sodium phosphate–citrate buffer (pH 4.3) containing 0.02% of sodium azide. Samples were withdrawn at intervals and diluted 8 times with 0.2M Na_2CO_3, prior to spectrophotometry at 420 nm.

β-D-Galactosidase from *E. coli* was assayed by direct spectrophotometry at 420 nm. The spectrophotometric cell contained 2mM ONPG in 50mM sodium phosphate buffer (pH 7.0) with mM $MgCl_2$ and 0.02% of sodium azide.

All assays were run at 37°. One unit (U) is defined as the hydrolysis of 1μmol of ONPG/min under the above conditions.

Purification of β-D-galactosidase from bovine testes. — The enzyme was a crude preparation obtained by a modification of the method of Distler and Jourdian[10]. All manipulations were performed at 0–4° unless otherwise indicated.

Bovine testes were obtained from a local slaughterhouse and stored at −20° until used. One testis (~250 g) was partially thawed and homogenised for 4 min with a blender, during which ice was added to maintain the correct temperature. 0.1M Acetic acid (300 mL) was added, and the pH was adjusted to 4.0 by dropwise addition of 2M HCl. The homogenate was stirred for 30 min, filtered through a fine nylon net, and centrifuged for 20 min at 10,000*g*, and the supernatant solution was poured through a glass wool pad to remove lipid.

Ammonium sulfate was added to 40% saturation and, after stirring for 1 h, the extract was centrifuged for 10 min at 10,000*g*, the precipitate was collected and dissolved in assay buffer (50 mL), and the solution was incubated at 50° for 15 min before centrifugation for 10 min at 20,000*g*. The supernatant solution was dialysed against assay buffer and lyophilised.

The purification resulted in a 10-fold increase in the specific activity, typically 0.14 U/mg, with a total activity of 16 U. The recovery of activity was 50% of that in the crude extract.

Protein determination. — Protein concentrations were determined by the method of Bradford[11], with a reagent supplied by BioRad. Chicken-egg albumin was used as the reference.

H.p.l.c. — Transgalactosylations and hydrolyses were monitored by h.p.l.c. analysis on a LiChrosorb NH_2-column as previously described[12]. During the purifi-

cation of **1**, h.p.l.c. was performed on a Nucleosil C_{18}-column, with water as eluant.

Transgalactosylation. — Reactions were performed in 50mM sodium phosphate–citrate buffer (pH 4.3) containing 0.02% of sodium azide, at 37°. Normally, 20% of lactose and 5% of GalNAc were used. With 0.7 U of bovine testes enzyme/g of lactose, optimal yields were obtained after 20–30 h. The reaction was terminated by heating to 90° for 10 min.

Hydrolysis of by-products. — The mixture of products from the transgalactosylation was diluted ten times with 50mM sodium phosphate buffer (pH 7.0) containing 0.02% of sodium azide and mM $MgCl_2$. The pH was checked and, if necessary, adjusted to 7.0. *E. coli* enzyme was added (380 U/g of added lactose) and the reaction was allowed to proceed at 37° until the contaminating disaccharides could no longer be detected by h.p.l.c. (typically 48 h). The reaction was terminated by heating to 90° for 10 min after adjusting the pH to 4.3.

Isolation of β-D-Galp-(1→3)-D-GalNAc (**1**). — Equal parts by weight (250 g) of dry charcoal and Celite were slurried in water and packed into a glass column (5 × 68 cm) with a thin layer of Celite at the bottom. The column was washed slowly with conc. hydrochloric acid for deactivation and to wash out traces of iron and alkali, then with water until the effluent was neutral.

A solution of the mixture of products from GalNAc (1 g) and lactose (4 g) in water (100 mL) was applied to the column and eluted successively with H_2O (5.4 L) and H_2O containing 1% (2.5 L), 2.5% (3.6 L), 5% (2.9 L), 10% (2.4 L), and 25% of ethanol (2.3 L). The effluent was monitored by refractometry. Fractions were assayed with the anthrone–sulfuric acid reagent and positive fractions analysed by h.p.l.c. Elution with aqueous 1% ethanol removed all of the galactose and glucose, GalNAc was eluted at 2.5% of ethanol, and **1** was eluted as a sharp peak at 10% of ethanol. The fractions containing **1** were combined, concentrated to dryness, and lyophilised.

Analytical procedures. — G.l.c. of reduced and methylated[13] oligosaccharides was carried out on a Perkin–Elmer Sigma 1 gas chromatograph equipped with a split–splitless injector and a flame-ionisation detector. Separations were performed on a DB-1 fused-silica capillary column (30 m × 0.25 mm) at 160–330°. G.l.c.–m.s. was performed on a VG Masslab 1250 quadropole instrument linked to a Hewlett–Packard 5790 gas chromatograph equipped with a split–splitless injector and the appropriate column. The spectra were recorded at 70 eV with an ion-source temperature of 200° and processed by a PDP 11/23 on-line computer system.

Methylation analysis was performed as previously described[14].

^{1}H-N.m.r. spectra were obtained for solutions in D_2O (internal acetone, 2.225 p.p.m.) at 27° with a Bruker 500-MHz instrument.

RESULTS AND DISCUSSION

The aim of the present study was to show that β-D-galactosidase preparations obtained from two sources with different specificities could be used in sequence to

produce a desired disaccharide (**1**), and that this could be done with a crude enzyme preparation.

It is known that β-D-galactosidase from bovine testes readily hydrolyses (1→3) linkages[15], indicating also an ability to synthesise this linkage under suitable conditions. However, in transgalactosylations with GalNAc and lactose, **1** was formed together with its isomer β-D-Gal*p*-(1→6)-D-GalNAc (**2**).

In a similar transgalactosylation with the β-D-galactosidase from *E. coli*, only **2** was formed[12]. Furthermore, the *E. coli* enzyme has low activity against (1→3) linkages compared to (1→6) linkages[16,17]. Thus, **2** should be a considerably better substrate than **1** under hydrolytic conditions, thereby allowing the selective removal of **2** from a mixture of **1** and **2**. This proved to be the case as shown below.

Transgalactosylation. — The rates of formation of **1** and **2** in the transgalactosylation reaction catalysed by the bovine testes enzyme differed markedly (Fig. 1). The maximum yield of **1** was 37% based on added GalNAc and occurred at 18% consumption of lactose. The yield of **1** at equilibrium (not shown) was considerably lower, since transgalactosylation is a kinetically controlled reaction and the competing hydrolysis reaction is the thermodynamically more favourable reaction. The formation of **2** was much slower and the maximum yield occurred at 90–95% consumption of lactose (not shown).

Several products other than **1** and **2** were formed (Fig. 2A) for which the acceptor was not GalNAc but Gal, Glc, or lactose.

Hydrolysis. — The hydrolysis reaction was performed with a high concentration of enzyme compared to that used for the transgalactosylation since product inhibition occurred and several of the contaminant oligosaccharides were poor substrates to the *E. coli* β-D-galactosidase[18]. Under these conditions, **2** was hydrolysed

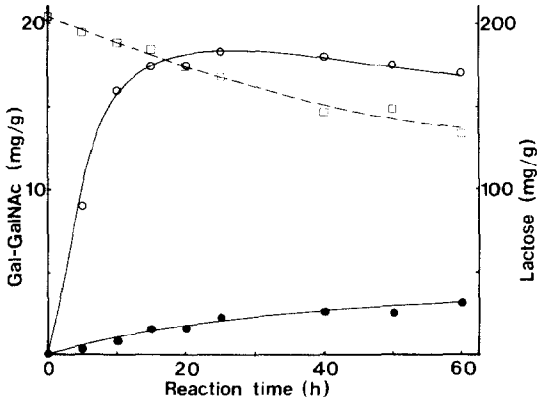

Fig. 1. Formation of β-D-Gal*p*-(1→3)-D-GalNAc (**1**) and β-D-Gal*p*-(1→6)-D-GalNAc (**2**) during a transgalactosylation reaction based on 5% of GalNAc and 20% of lactose (see Experimental). Concentrations expressed as mg/g of reaction solution: —○—, β-Gal-(1→3)-GalNAc; —●—, β-Gal-(1→6)-GalNAc; – –□– –, lactose.

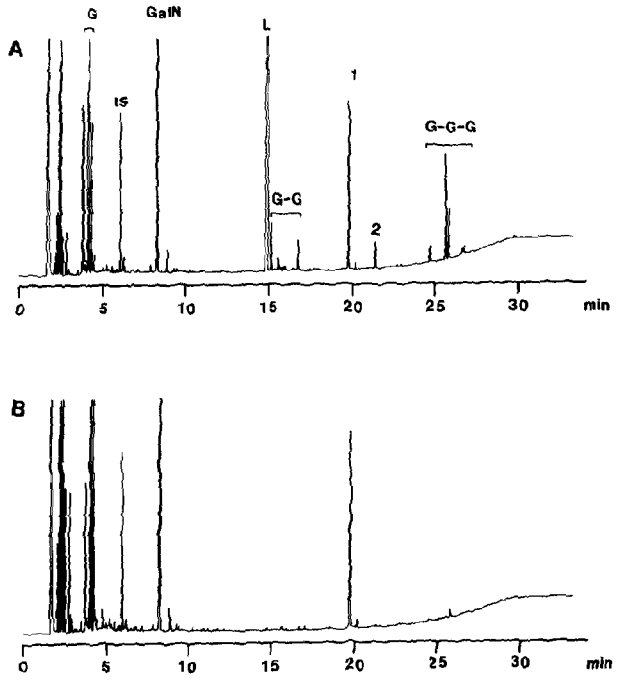

Fig. 2. Gas–liquid chromatogram of the reduced and methylated products after A, transgalactosylation for 20 h; and B, subsequent hydrolysis for 48 h: G, Gal and Glc; IS, perseitol (internal standard); GalN, GalNAc; L, lactose; G-G, Gal- and Glc-containing disaccharides; 1, β-Gal-(1→3)-GalNAc; 2 β-Gal-(1→6)-GalNAc, G-G-G, Gal- and Glc-containing trisaccharides.

completely after 4 h, whereas 48 h were necessary to remove the rest of the contaminating disaccharides and most of the trisaccharides.

The result of a typical hydrolysis reaction is shown in Fig. 2 in which **2**, lactose, and the major part of the oligosaccharides were hydrolysed readily, whereas <25% of **1** was hydrolysed. In a separate experiment, it was shown that the rate of hydrolysis of **1** was $<10^4$ of that of lactose.

The reaction mixture from the transgalactosylation was diluted 10 times prior to hydrolysis in order to promote hydrolysis instead of further synthetic reactions by the *E. coli* β-D-galactosidase[19]. When the hydrolysis was complete, it was necessary to lower the pH before heating because of the instability of **1** in solution at pH 7.0 at 90°.

Crude enzyme preparations. — The commercial bovine testes enzyme is supplied in a pure form, but is too expensive to be used in large-scale syntheses. Therefore, a simplified purification procedure was developed which followed the initial steps described by Distler and Jourdian[10] with the exception of the incubation for 15 min at 50°. This step resulted in precipitation of 60–70% of the remaining protein and an acceptable loss of 20% of the β-D-galactosidase activity. Elaborate

chromatography was avoided in this revised purification scheme. The resulting enzyme preparation was stable at −20° for at least 7 months. When performing the transgalactosylation reaction, we did not notice any loss of enzyme activity under the reaction conditions used.

When working with crude enzyme preparations, there is always a risk of losing enzyme activity due to proteases and there may be undesirable reactions due to other contaminant enzymes. However, in the work described, significant problems of this kind were not observed. A transgalactosylation with a commercial enzyme preparation (2.1 U/mg of protein) gave results equivalent to those for the crude enzyme preparation.

The commercial β-D-galactosidase from *E. coli* was pure and inexpensive, and an alternate purification scheme was therefore not necessary.

Characterisation of products. — Two disaccharide products **1** and **2** (Fig. 2A) were identified by g.l.c.–m.s., after reduction and methylation, as isomeric structures of Hex-HexNAc-ol[20]. After hydrolysis of the product mixture by *E. coli* β-D-galactosidase, g.l.c. revealed pure **1** (Fig. 2B). Carbon–Celite chromatography gave **1** in >95% purity, according to h.p.l.c. (chromatogram not shown). Methylation analysis of **1** showed non-reducing terminal Gal and 3-linked GalNAc. The mass spectrum of reduced and methylated **1** was equivalent to that reported[21] for methylated Gal-(1→3)-GalNAc-ol. ^{1}H-N.m.r. spectroscopy of **1** confirmed the purity and that the Gal unit was β (Gal H-1, δ 4.44 and 4.49, $J_{1,2}$ 7.8 Hz; the difference in chemical shifts reflects the α and β configuration of the GalNAc residue). Compound **2** was identified in the same way as β-Gal-(1→6)-GalNAc.

The yield of isolated **1** was 208.3 mg (21% based on added GalNAc). This compares to a yield of 27% estimated by g.l.c. of the reaction mixture prior to Carbon–Celite column chromatography.

Conclusions. — The limited aglycon specificity ascribed to glycosidases may be circumvented by the two-enzyme strategy used here. The first enzyme produces the desired product, whereas the second enzyme, with its matching specificity, hydrolyses contaminant oligosaccharides formed by the first enzyme. The strategy should be applicable in other systems, provided enzyme pairs with complementary activities are available.

ACKNOWLEDGMENTS

We thank Ms. Ann-Margret Olsson for technical assistance, Mr. Gunnar Grönberg (BioCarb AB, Lund) for performing the n.m.r. spectroscopy, and the National Swedish Board for Technical Development and BioCarb AB for financial support.

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