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Single mid-chain GlcNAcβ1-6Galβ1-4R sequences of linear oligosaccharides are resistant to endo-β-galactosidase of *Bacteroides fragilis*

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Endo-β-galactosidase (EC 3.2.1.103) of *Bacteroides fragilis*, at 250 mU ml^{-1}, did not cleave the internal galactosidic linkage of the linear radiolabelled trisaccharide GlcNAcβ1-6Galβ1-4GlcNAc, or those of the tetrasaccharides Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc and Galβ1-4GlcNAcβ1-6Galβ1-4Glc. The isomeric glycans which contained the GlcNAcβ1-3Galβ1-4GlcNAc/Glc sequence were readily cleaved.

Keywords: endo-β-galactosidase, *Bacteroides fragilis*, oligo-*N*-acetyllactosaminoglycans, GlcNAcβ1-6Galβ1-4R sequences

Abbreviations: GlcNAc, 2-acetamido-2-deoxy-D-glucose; Lact, lactose; MT, maltotriose; MTet, maltotetraose, R_{MTet}, chromatographic migration rate in relation to that of maltotetraose.

Endo-β-galactosidase of *Bacteroides fragilis* cleaves linear GlcNAcβ1-3Galβ1-4GlcNAc/Glc sequences of poly- and oligo-*N*-acetyllactosaminoglycans at the internal galactosidic linkages [1, 2], but it fails to hydrolyse branched structures of the type GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc [2–4]. The substrate properties of linear GlcNAcβ1-6Galβ1-4GlcNAc sequences have been studied recently by Hanisch *et al.* [5], who reported that the internal galactosidic linkages of Galβ1-3/4GlcNAcβ1-6Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAcβ1-6(Galβ1-3)GalNAcOL, and of related saccharides, are cleavable.

The present experiments, too, represent an attempt to define the substrate properties of linear GlcNAcβ1-6Galβ1-4GlcNAc/Glc sequences. Unexpectedly, our data show that the radiolabelled trisaccharide GlcNAcβ1-6Galβ1-4GlcNAc (**2**) and two radiolabelled tetrasaccharides, Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc (**6**) and Galβ1-4GlcNAcβ1-6Galβ1-4Glc (**8**), were not cleaved by the enzyme.

Materials and methods

Substrate trisaccharides

Isolation of radiolabelled GlcNAcβ1-3Galβ1-4GlcNAc (**1**) and GlcNAcβ1-6Galβ1-4GlcNAc (**2**) from teratocarcinoma cells has been described [6]; their specific radioactivities are unknown. Other samples of radiolabelled glycan **1** were synthesized enzymatically as described [7], scaling up the reaction to obtain enough material for NMR spectroscopy. Purified **1** gave a ^{1}H-NMR spectrum containing "structural reporter peaks" [8] that were identical with those reported by Koenderman *et al.* [9] for GlcNAcβ1-3Galβ1-4GlcNAc. A synthetic sample of GlcNAcβ1-6[1-^{14}C]Galβ1-4GlcNAc (**2**) was also prepared [10]. It was obtained from the branched tetrasaccharide GlcNAcβ1-3(GlcNAcβ1-6)[1-^{14}C]-Galβ1-4GlcNAc, synthesized enzymatically *in vitro* as described [7]. The branched tetrasaccharide gave a 500 MHz ^{1}H-NMR spectrum that revealed "structural reporter peaks" identical with those reported by Koenderman *et al.* [9] for GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (a peak that was not listed by Koenderman *et al.* was present in the spectrum of our sample at 2.071 ppm). The tetrasaccharide was subjected to partial β-*N*-acetylhexosaminidase cleavage which gave a mixture containing the original substrate, GlcNAcβ1-3[1-^{14}C]Galβ1-4GlcNAc (**1**), GlcNAcβ1-6[1-^{14}C]-Galβ1-4GlcNAc(**2**), and [1-^{14}C]Galβ1-4GlcNAc. This mixture was fractionated by paper chromatography to yield the pure saccharides, including **2** [10]. The trisaccharide GlcNAcβ1-3Galβ1-4[1-^{14}C]Glc (**3**) was prepared from Galβ1-4[1-^{14}C]Glc (Amersham, UK) and UDP-GlcNAc (Sigma, St Louis, MO, USA) essentially as described by Yates and Watkins [11]. The product was homogeneous in paper chromatography (R_{Lact} = 0.74 in solvent A); it was cleaved completely by treatment [6] with β-*N*-acetylhexosaminidase from jack bean, regenerating labelled lactose.

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The unlabelled trisaccharide GlcNAcβ1-6Galβ1-4Glc (**4**) was purchased from Sigma. According to the manufacturer, the structure of this synthetic trisaccharide has been confirmed by ^{13}C-NMR spectroscopy.

Galactosylation of trisaccharides with β(1-4)-galactosyltransferase [12]

The labelled trisaccharides **1–3** were incubated overnight at 37°C under toluene with 50 nmol UDP-Gal (Sigma) and 50 mU bovine milk *N*-acetyllactosamine synthase (EC 2.4.1.90) (Sigma) in a mixture that contained in 100 µl, 5 µmol Tris-HCl, pH 7.5, and 1 µmol $MnCl_2$. The unlabelled trisaccharide **4** (85 nmol) was galactosylated in a similar mixture containing 2 nmol (= 0.5 µCi) UDP-[U-^{14}C]Gal (New England Nuclear, Dreieich, Germany).

Endo-β-galactosidase treatment of oligosaccharides

The labelled oligosaccharides in 40 ml of 50 mM sodium acetate buffer, pH 5.8, containing 0.2 mg ml^{-1} bovine serum albumin and 0.5 mg ml^{-1} NaN_3, were incubated for 24 h at 37°C with 10 mU of endo-β-galactosidase (EC 3.2.1.103) from *Bacteroides fragilis* (Boehringer, Mannheim, Germany (see [1, 2]). The reactions were terminated by boiling for 3 min. Pure GlcNAcβ1-3[1-^{14}C]Galβ1-4GlcNAc was cleaved in control experiments consistently to the extent of 98–99%.

Treatment with Diplococcus pneumoniae β-galactosidase [13]

This enzyme (EC 3.2.1.23) was used as described [14].

Paper chromatography

Prior to paper chromatography, the oligosaccharides were desalted by filtration through Dowex AG-1X8 (AcO$^-$) and Dowex AG-50-WX8 (H$^+$). The paper chromatograms were run as described [14] by using the upper phase of *n*-butanol:acetic acid:water, 4:1:5 by vol (solvent A), or n-butanol:ethanol:water, 10:1:2 by vol (solvent B). Radiolabelled saccharides were localized as described [14]; unlabelled glycans were stained with aniline phthalate.

Marker saccharides

Unlabelled *N*-acetylglucosamine, glucose, galactose, lactose and GlcNAcβ1-6Gal were purchased from Sigma; the malto-oligosaccharides were from Boehringer. Radiolabelled GlcNAcβ1-3Gal and GlcNAcβ1-6Gal have been described previously [6]. [U-^{14}C]Galβ1-4GlcNAcβ1-6Gal was obtained as described [14]. [^{3}H]Galβ1-4GlcNAcβ1-3Gal from labelled paragloboside [15] was obtained from Dr Michiko Fukuda. Radiolabelled teratocarcinoma tetrasaccharides Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc and Galβ1-4GlcNAc-β1-6Galβ1-4GlcNAc have been described [14].

^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis, the oligosaccharide samples (60–130 nmol) were repeatedly treated with ^2H_2O at room temperature. After each exchange treatment the samples were lyophilized. Finally, the samples were dissolved in ^2H_2O, and ^{1}H-NMR spectroscopy was performed at 500 MHz in a Bruker AM 500 spectrometer (BioCarb Chemicals, Lund, Sweden) operating in a Fourier transform mode. The probe temperature was kept at 300 K. Chemical shifts, expressed downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate, were actually measured by reference to internal acetone ($\delta = 2.225$ ppm) with an accuracy of 0.002 ppm.

Results

Preparation and characterization of the tetrasaccharide substrates

The structures of the radiolabelled oligosaccharides **1–8** of the present experiments are given in Table 1. The trisaccharides **1–4** were β(1-4)-galactosylated enzymatically to yield radiolabelled tetrasaccharides **5–8**, respectively. These were isolated by paper chromatography using solvent A. The tetrasaccharides **5** and **6** migrated on paper like the radiolabelled marker saccharides from teratocarcinoma cells, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc and Galβ1-4GlcNAc-β1-6Galβ1-4GlcNAc, respectively (data not shown). The glucose-containing tetrasaccharide **7** was slower (R_{MTet} = 0.91) than its *N*-acetylglucosamine analogue **5** (R_{MTet} = 1.27). In the same way, the glucose-containing tetrasaccharide **8** was slower (R_{MTet} = 0.80) than its *N*-acetylglucosamine analogue **6** (R_{Mtet} = 1.07). The tetrasaccharides **6** and **8**, containing the mid-chain GlcNAcβ1-6Gal sequence, were slower than their GlcNAcβ1-3Gal-containing isomers **5** and **7**, respectively.

Degalactosylation of the tetrasaccharides **5–7** with β-galactosidase from *D. pneumoniae* regenerated the original trisaccharides **1–3**, respectively. This was shown by paper chromatography in solvents A and B (data not presented). The tetrasaccharide **8** was also degraded completely by this enzyme, liberating [^{14}C]galactose (data not shown). These

Table 1. Oligosaccharides studied in the present experiments.

Number	*Saccharide*	*Digestibility with endo-β-galactosidase*
1	GlcNAcβ1-3Galβ1-4GlcNAc	+
2	GlcNAcβ1-6Galβ1-4GlcNAc	–
3	GlcNAcβ1-3Galβ1-4Glc	ND[a]
4	GlcNAcβ1-6Galβ1-4Glc	ND[a]
5	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc	+
6	Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc	–
7	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	+
8	Galβ1-4GlcNAcβ1-6Galβ1-4Glc	–

[a] ND = Not determined in the present experiments.

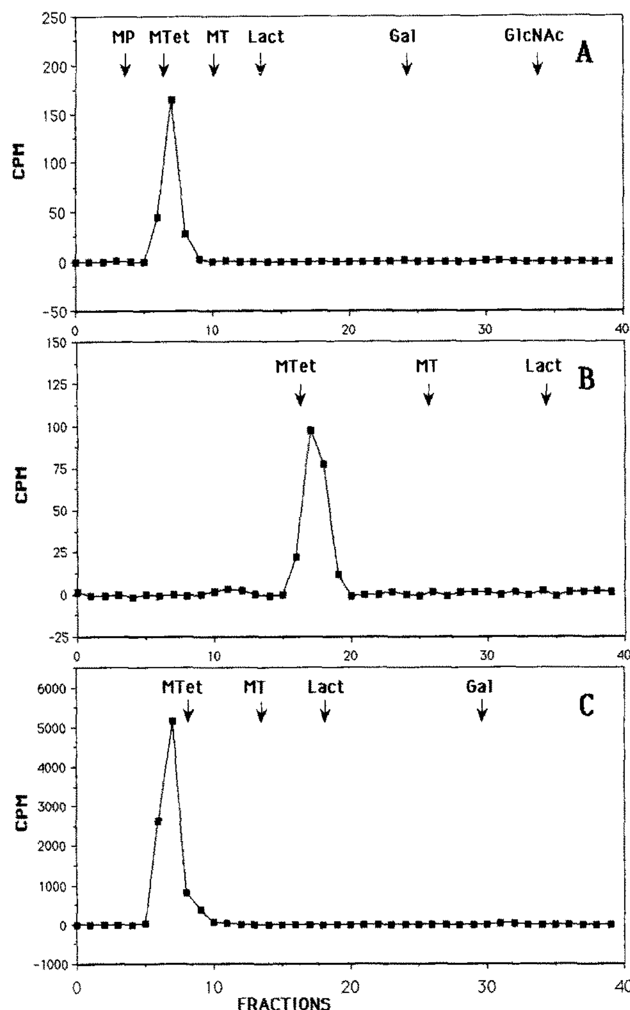

Figure 1. Endo-β-galactosidase digests of radiolabelled tetrasaccharides **6** and **8**, analysed by paper chromatography in solvent A. A. A 40 h chromatogram of a digest from Galβ1-4[^{3}H]GlcNAc-β1-6Galβ1-4[^{3}H]GlcNAc ([^{3}H] **6**). If Galβ1-4[^{3}H]GlcNAcβ1-6Gal had been present, its peak would have been at fraction 7.8, not clearly separated from the intact substrate. B. A 110 h chromatogram of a digest from Galβ1-4GlcNAcβ1-6[^{14}C]Galβ1-4GlcNAc [^{14}C] **6**). If Galβ1-4GlcNAcβ1-6[^{14}C]Gal had been present, its peak would have been at fraction 20.0 [14], clearly discernible. C. A 63 h chromatogram of a digest from [^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc (**8**). If [^{14}C]Galβ1-4GlcNAcβ1-6Gal had been present, its peak had been at fraction 10.3 [14], clearly discernible. Arrows show positions of unlabelled markers.

experiments proved that all four tetrasaccharides **5**–**8** contained Galβ1-4GlcNAc sequences at their nonreducing termini [16].

Endo-β-galactosidase treatment of the tetrasaccharides (**6**) *and* (**8**)

The tetrasaccharide **6**, Galβ1-4[^{3}H]GlcNAcβ1-6Galβ1-4[^{3}H]-GlcNAc, resisted the action of endo-β-galactosidase from *B. fragilis* (Fig. 1a). Neither labelled *N*-acetylglucosamine nor other cleavage products were detected in the digest; the unchanged substrate was the only labelled compound present.

Another radiolabelled form of the tetrasaccharide **6**, Galβ1-4GlcNAcβ1-6[^{14}C]Galβ1-4GlcNAc, also resisted the enzyme (Fig. 1b). In this experiment, a rather long paper chromatogram was run to separate Galβ1-4GlcNAcβ1-6[^{14}C]Gal, a possible cleavage product, from the substrate. However, no radiolabelled Galβ1-4GlcNAcβ1-6Gal was detected; instead, the original tetrasaccharide **6** was recovered in good yield. The experiment of Fig. 1b was repeated with the same result also using a large sample (3764 counts min^{-1}, 7 pmol) of [^{14}C]**6** that had been prepared by *in vitro* enzymatic synthesis (data not shown). On the basis of this experiment, a cleavage exceeding 0.5% can be excluded.

The tetrasaccharide **8**, [^{14}C]Galβ1-4GlcNAcβ1-6Galβ1-4Glc (11 574 counts min^{-1}, 18 pmol) also resisted the enzyme (Fig. 1c).

Endo-β-galactosidase treatment of the tetrasaccharides **5** *and* **7**

The tetrasaccharide **5**, Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc, was cleaved in the expected manner, releasing Galβ1-4GlcNAcβ1-3[U-^{14}C]Gal (Fig. 2a). The glucose-containing tetrasaccharide **7**, Galβ1-4GlcNAcβ1-3Galβ1-4[^{14}C]Glc, was also cleaved at the internal galactosidic linkage; it released labelled glucose in a yield of 90% (Fig. 2(b)).

Endo-β-galactosidase treatment of trisaccharides **1** *and* **2**

Endo-β-galactosidase treatment of the teratocarcinoma trisaccharide GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc (**1**) cleaved 87% of the material into GlcNAcβ1-3[U-^{14}C]Gal, which was identified chromatographically in solvent B (data not shown). The radiolabelled samples of **1** that were synthesized enzymatically were cleaved even more completely.

The teratocarcinoma trisaccharide **2**, [^{3}H]GlcNAcβ1-6Galβ1-4[^{3}H]GlcNAc, also resisted the enzyme (data not shown). Neither labelled *N*-acetylglucosamine, nor labelled GlcNAcβ1-6Gal was detected in the digest in a chromatographic run with solvent B; the original trisaccharide **2** was the only labelled compound observed.

Even another form of the teratocarcinoma trisaccharide **2**, GlcNAcβ1-6[U-^{14}C]Galβ1-4GlcNAc (189 counts min^{-1}), resisted the enzyme; prolonged paper chromatography of the digest with solvent B revealed the presence of the original substrate, but GlcNAcβ1-6[U-^{14}C]Gal was not found (data not shown). This experiment was repeated with the same result by using a large sample (3349 counts min^{-1}, 6 pmol) of the trisaccharide **2** that had been prepared by *in vitro* synthesis.

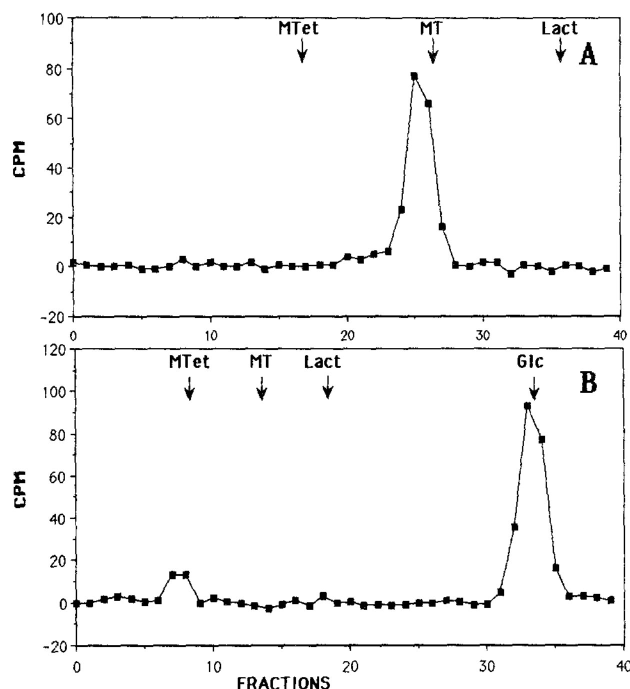

Figure 2. Endo-β-galactosidase digests of radiolabelled tetrasaccharides **5** and **7**, analysed by paper chromatography in solvent A. A. A 110 h chromatogram of a digest from Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc (**5**); the calculated positions of radiolabelled markers Galβ1-4GlcNAcβ1-3Gal and Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc are 24.8 and 20.7, respectively. B. A 63 h chromatogram of a digest from Galβ1-4GlcNAcβ1-3Galβ1-4[^{14}C]Glc (**7**); the calculated position of undigested substrate is 7.5. Arrows show positions of unlabelled markers.

Discussion

The present data show that radiolabelled samples of the trisaccharide **2**, and the tetrasaccharides **6** and **8** (see Table 1 for the structures) resisted the action of endo-β-galactosidase from *B. fragilis* at 250 mU ml^{-1}, under conditions generally used for digestion with this enzyme. All three resistant saccharides contained a linear mid-chain GlcNAcβ1-6Galβ1-4R sequence. In a marked contrast, the saccharides **1**, **5** and **7** of Table 1, all of which contained a linear mid-chain GlcNAcβ1-3Galβ1-4R sequence, were readily cleaved by the enzyme under the same conditions.

These findings should prove useful for the detection, isolation and characterization of unbranched GlcNAcβ1-6Gal backbone sequences in natural oligo- and polysaccharides. Such sequences have been reported rarely so far in natural saccharides [5, 17], but their *in vitro* biosynthesis has been observed in several systems [18–21].

We have previously found that endo-β-galactosidase from *Escherichia freundii* cleaves unbranched GlcNAcβ1-6Galβ1-4GlcNAc sequences poorly [14]. The substrate specificities of the endo-β-galactosidases of *E. freundii* and *B. fragilis* are known to resemble each other in many other ways as well [1, 2]; only in their ability to cleave branch point galactosidic linkages located at the non-reducing end of extended linear sequences containing at least two *N*-acetyllactosamine units, may the two enzymes differ [3, 4, 22].

Our present findings appear to be at variance with the cleavage of linear GlcNAcβ1-6Galβ1-4Gal sequences reported recently by Hanisch *et al.* [5]. This difference may be partly semantic: Even Hanisch *et al.* found incomplete reactions. If a real difference exists, it may arise from the different substrate concentrations used; Hanisch *et al.* used typically 70 nmol of unlabelled substrates, whereas we used radiolabelled samples as small as 3–20 pmol in comparable experiments. A much more interesting possibility is that the GlcNAcβ1-6Galβ1-4R sequences of our samples, and those present in the oligosaccharides of Hanisch *et al.*, may have different properties. The saccharide chains studied by Hanisch *et al.* contained several adjoining GlcNAcβ1-6Gal units, whereas ours contained only one. Examples are known where a given saccharide sequence appears to possess variable properties in different molecules [23].

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