Isolation and characterization of an endo- β -galactosidase from *Bacteroides fragilis*

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Six strains of *Bacteroides fragilis* were examined and all found to produce endo- β -galactosidase, an enzyme that hydrolyses internal β -galactosidic linkages of oligosaccharides belonging to the poly-*N*-acetyl-lactosamine series, with the common structure $\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}/\text{Glc}$. The enzyme was produced without the addition of an inducer such as keratan sulphate. It was purified 7000-fold from the culture supernatant and obtained with a yield 4–10-fold greater than from sources described previously. The specificity of the enzyme towards bovine corneal keratan sulphate, milk oligosaccharides and the glycolipids lacto-*N*-neotetraosylceramide and lacto-*N*-tetraosylceramide closely resembled that of the endo- β -galactosidase isolated from *Escherichia freundii*. A novel observation was that both enzymes hydrolysed the type 2 sequence, $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}$, at about twice the rate of the type 1 isomer, $\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}$. Because of the ease of purification of the enzyme and high yield in the absence of contaminating glycosidases and proteinases, *Bacteroides fragilis* is a valuable source of endo- β -galactosidase for the structural analysis of carbohydrate chains.

Oligosaccharides of the poly-*N*-acetyl-lactosamine series, having the structure $(\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3)_n$, are of considerable current interest because the I and i antigens they express behave as tumour-associated and differentiation antigens (Feizi, 1981, 1982; Hakomori, 1981). Endo- β -galactosidase, which specifically cleaves the internal β -galactosidic linkages of these structures, has been invaluable for the structural characterization of these antigens and the analysis of cell-surface glycoproteins during differentiation and maturation (Fukuda & Fukuda, 1981).

The main source of this enzyme at present is *Escherichia freundii*, but this organism produces endo- β -galactosidase only in the presence of an inducer, such as whale cartilage keratan sulphate (Kitamikado & Ueno, 1970) or Smith-degraded pig gastric mucin (Fukuda, 1981).

The observations by Inglis *et al.* (1975) and Doinel *et al.* (1980) have suggested that *Bacteroides fragilis* also produces endo- β -galactosidase, since the culture filtrate of this organism diminishes the reactivity of human erythrocytes with anti-I and anti-i antibodies at the same time as rendering them agglutinable by the *N*-acetylglucosamine-specific lectin of *Bandeiraea simplicifolia* (BSII lectin).

In the present paper we report the isolation and properties of endo- β -galactosidase from *Bacteroides fragilis*, which enzyme, like that from *Flavobacterium keratolyticus* (Kitamikado *et al.*, 1981), is produced without induction, and we compare its substrate specificity with that of the endo- β -galactosidase from *Escherichia freundii*.

Experimental procedures

Materials

Keratan sulphate (KSI fraction), isolated from bovine corneas by the method of Rodén *et al.* (1972) (yield 89 mg from 38 g of corneas), had the following composition: galactose, 19.4%; *N*-acetylglucosamine, 23.8%; mannose, 2.2%; sialic acid, 1.7%; fucose, 0.5% sulphate, 15.6%. Partially desulphated keratan sulphate was prepared by the solvolytic desulphation of the pyridinium salt of keratan sulphate (KSI fraction) (Nagasawa *et al.*, 1977) and had a sulphate content of 3.9%. Lacto-*N*-neotetraosylceramide ($\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{Cer}$) was prepared by treating sialosyl-lacto-*N*-neotetraosyl-ceramide ($\text{NeuAc}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{Cer}$) isolated from

bovine erythrocytes (Uemura *et al.*, 1978) with *Vibrio cholerae* neuraminidase (Iwamori & Nagai, 1981).

The following oligosaccharides were generously given by Dr. Winifred Watkins of the Clinical Research Centre: lacto-*N*-neotetraose (Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc), lacto-*N*-tetraose (Gal β 1 $\rightarrow$ 3GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc), lacto-*N*-fucopentaose I (Fuc α 1 $\rightarrow$ 2Gal β 1 $\rightarrow$ 3GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc), lacto-*N*-fucopentaose II (Gal β 1 $\rightarrow$ 3(Fuc α 1 $\rightarrow$ 4)GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc), lacto-*N*-difucohexose I (Fuc α 1 $\rightarrow$ 2Gal β 1 $\rightarrow$ 3(Fuc α 1 $\rightarrow$ 4)GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc) and the blood-group-A- and -B-active tetrasaccharides [14 C]GalNAc α 1 $\rightarrow$ 3(Fuc α 1 $\rightarrow$ 2)Gal β 1 $\rightarrow$ 4Glc (310 mCi/mmol) and [14 C]Gal α 1 $\rightarrow$ 3(Fuc α 1 $\rightarrow$ 2)Gal β 1 $\rightarrow$ 4Glc (54 mCi/mmol). The trisaccharides [14 C]GalNAc α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc and [14 C]Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc were purified by paper chromatography in solvent 1 after mild acid hydrolysis (0.1 M-HCl for 60 min at 100°C) of the parent 14 C-labelled blood-group-A- and -B-active tetrasaccharides. The chemically synthesized Gal β 1 $\rightarrow$ 4GlcNAc (*N*-acetyl-lactosamine) and Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal were gifts from Dr. S. David, Université Paris-Sud, Paris, France. Lacto-*N*-tetraosylceramide (Gal β 1 $\rightarrow$ 3GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1Cer) was a gift from Dr. K.-A. Karlsson, University of Göteborg, Göteborg, Sweden. [3 H]Lacto-*N*-tetraosylceramide (2.5×10^4 c.p.m./nmol) and [3 H]lacto-*N*-neotetraosylceramide (3×10^5 c.p.m./nmol) were prepared by the galactose oxidase/ NaB^3H_4 method described by Suzuki & Suzuki (1972). Glucose, galactose, *N*-acetylglucosamine and stachyose (Gal α 1 $\rightarrow$ 6Gal α 1 $\rightarrow$ 6Glc α 1 $\rightarrow$ 2Fru) were from Sigma Chemical Co.

Analytical methods

Total hexose was monitored by using the phenol/ H_2SO_4 method (Dubois *et al.*, 1956). Individual sugars were analysed by g.l.c. of the trimethylsilyl ethers of their methyl glycosides (Bhatti *et al.*, 1970). Glucose, released from milk oligosaccharides by the action of endo- β -galactosidase, was measured by using an enzymic method described by Finch *et al.* (1969). Mono- and oligo-saccharides were resolved by descending paper chromatography on Whatman no. 40 paper developed with either solvent 1 (ethyl acetate/pyridine/water, 10:4:3, by vol.) or solvent 2 (butan-1-ol/acetic acid/water, 10:3:7, by vol.) and by t.l.c. on Whatman HP-KF silica-gel plates developed with solvent 3 (butan-1-ol/acetone/water, 6:5:4, by vol.). Sugars on paper chromatograms were located with alkaline AgNO_3 (Trevelyan *et al.*, 1950) and on t.l.c. chromatograms with 0.2% orcinol in 2 M- H_2SO_4 . Radioactivity on paper chromatograms was detected as described previously (Scudder & Chantler, 1981).

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Purified endo- β -galactosidase was labelled with ^{125}I (Greenwood *et al.*, 1963) and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970) in 12.5%-(w/v)-acrylamide gels in the presence of 0.1% sodium dodecyl sulphate. After electrophoresis the gel was sliced and the radioactivity measured with a Nuclear Enterprises model 1600 γ -radiation counter. The following proteins were used as M_r markers: phosphorylase *a* (M_r 94 000); bovine serum albumin (M_r 68 000); ovalbumin (M_r 43 000); trypsin (M_r 23 300); cytochrome *c* (M_r 13 000).

Enzyme assays

The activity of endo- β -galactosidase was assayed with bovine corneal keratan sulphate (KSI fraction) as substrate. The incubation mixture contained 100 μg of keratan sulphate and 10 μg of bovine serum albumin in 50 μl of 50 mM-acetate buffer, pH 5.8 (75 mM-sodium acetate adjusted to pH 5.8 with 1 M-acetic acid and diluted to 50 mM), and 10 μl of enzyme solution. Incubation was for 20 min at 37°C, when the increase in reducing power was measured by the method of Park & Johnson (1949). Under these conditions the reaction rate was proportional to the enzyme concentration and linear with respect to time. One unit of enzyme activity is defined as the amount of enzyme catalysing the release of 1 μmol of reducing power as galactose per min; this is equivalent to 0.25–0.30 unit obtained with the assay conditions described previously (Fukuda & Matsumura, 1976) with whale cartilage keratan sulphate as substrate.

The pH optimum of endo- β -galactosidase was determined by assaying the enzyme at pH values between 4.0 and 8.0 in the following 100 mM buffers: pH 4.5–5.7, acetic acid/acetate; pH 5.7–7.4, cacodylate/HCl; pH 7.4–8.0, Tris/HCl (prepared by adjusting the pH of a 150 mM solution of sodium acetate, sodium cacodylate or Tris with a 1 M solution of the appropriate acid and diluting to 100 mM).

The pH-stability was determined by assaying the enzyme after incubation at 20°C for 16 h at pH values between 3.5 and 10.5 in the following 25 mM buffers: pH 3.5, citric acid/citrate (25 mM-trisodium citrate adjusted to pH 3.5 with 25 mM-citric acid); pH 4.0–5.5, acetic acid/acetate; pH 6.0–7.0, cacodylate/HCl; pH 7.5–8.5, Tris/HCl (sodium acetate, sodium cacodylate and Tris buffers prepared by adjusting the pH of a 50 mM solution of the salt with a 1 M solution of the appropriate acid and diluting to 25 mM); pH 9.0–10.5, glycine/NaOH (25 mM-glycine adjusted with 25 mM-NaOH).

To determine the temperature optimum, endo- β -galactosidase was assayed after incubation for 30 min at the appropriate temperature (4–65°C).

Keratosulphatase activity was assayed under the same conditions as endo- β -galactosidase except that incubation was for 8 h. After incubation, free sulphate was measured by using the method described by Terho & Hartiala (1971).

Neuraminidase activity was measured spectrofluorometrically with 4-methylumbelliferyl-*N*-acetyl- α -D-neuraminic acid as substrate. Enzyme (100 μ l) was incubated with 0.04 μ mol of substrate and 50 μ g of bovine serum albumin in 250 μ l of 50 mM-acetate buffer, pH 5.8, for 16 h at 37°C, and then 3 ml of 0.5 M-glycine/NaOH buffer, pH 10.4 (0.5 M-glycine adjusted to pH 10.4 with 0.5 M-NaOH) was added. Released β -methylumbelliferone was measured with a Perkin-Elmer model 204 fluorescence spectrophotometer at excitation and emission wavelengths of 365 nm and 460 nm respectively.

Various exoglycosidases were assayed with the appropriate *p*-nitrophenyl glycoside as substrate (Li & Li, 1972). Proteinase activity was monitored with the use of azo-albumin (Tomarelli *et al.*, 1949).

Purification of endo- β -galactosidase from E. freundii

Endo- β -galactosidase was isolated from a culture filtrate of *E. freundii* as described previously (Fukuda & Matsumura, 1976; Fukuda, 1981).

Purification of endo- β -galactosidase from B. fragilis

Selection of B. fragilis strain and preparative-scale culture. Each of six strains of *B. fragilis* (obtained from the Microbiology Laboratory, Northwick Park Hospital, Harrow, Middx., U.K., as clinical isolates from six individual patients) produced 30–230 munits endo- β -galactosidase/ml when cultured anaerobically at 37°C. The strain (M 66741/80) producing the highest concentration of enzyme was grown anaerobically on blood/agar plates for 24 h, and the growth from one plate was inoculated into 4 $\times$ 0.5-litre volumes of 3% Difco Bacto-Tryptone broth and cultured anaerobically at 37°C for 40 h, at which time production of endo- β -galactosidase had reached a maximum. The culture fluid was removed from the cells (which had negligible endo- β -galactosidase activity) by centrifugation at 8000 *g* for 60 min at 4°C. All remaining steps were performed at 4°C.

Ammonium sulphate precipitation. The culture fluid was made 80% saturated with (NH₄)₂SO₄ and left to stand overnight. The precipitate was recovered by centrifugation at 2500 *g* for 30 min, dissolved in 75 ml of distilled water, dialysed against distilled water (with Spectrapor no. 1 dialysis tubing, *M_r* cut-off 6000–8000) and freeze-dried.

Sephadex G-100 chromatography. The freeze-dried preparation was dissolved in, and dialysed

against, 10 mM-acetate buffer, pH 5.8, containing 0.2 M-NaCl, and two 10 ml portions were chromatographed separately on a column (3.2 cm $\times$ 100 cm) of Sephadex G-100 equilibrated against the same buffer; the flow rate was 40 ml/h and the fraction size 7.2 ml. Fractions having endo- β -galactosidase activity were pooled as indicated in Fig. 1(a), dialysed against distilled water and freeze-dried.

Sephadex CM-50 chromatography. The freeze-dried preparation from the previous step was dissolved in 10 ml of 75 mM-acetate buffer, pH 5.0 (125 mM-sodium acetate adjusted to pH 5.0 with 1 M-acetic acid and diluted to 75 mM), and dialysed against the same buffer overnight. The dialysed sample was applied to a column (2.4 cm $\times$ 12 cm) of Sephadex CM-50 equilibrated against the same buffer. The column was washed with 180 ml of buffer at a flow rate of 30 ml/h, and then eluted with a linear gradient composed of 125 ml of starting buffer and 125 ml of 500 mM-acetate buffer, pH 5.0 (750 mM-sodium acetate adjusted to pH 5.0 with 5 M-acetic acid and diluted to 500 mM). Fractions having enzyme activity were pooled as indicated in Fig. 1(b), concentrated by using an Amicon ultra-filtration cell with a UM-2 membrane, and stored at –20°C.

Results

Isolation and properties of B. fragilis endo- β -galactosidase

Production of endo- β -galactosidase by B. fragilis. *B. fragilis* produced endo- β -galactosidase in the absence of an inducer such as keratan sulphate; when 0.2% keratan sulphate was included in the culture medium endo- β -galactosidase activity was not detectable.

Purity. Endo- β -galactosidase from *B. fragilis* was purified 7000-fold with 47% recovery of activity (Table 1). The preparation was free from proteinase, keratosulphatase and the following exoglycosidases, which had been present at 12 munits/ml or less in the original culture supernatant: α - and β -galactosidase, α - and β -*N*-acetylglucosaminidase, α -*N*-acetylglactosaminidase, α -mannosidase, α -L-fucosidase and neuraminidase. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a ¹²⁵I-labelled enzyme preparation revealed a major radiolabelled band having an apparent *M_r* of 32 000 and a minor band of *M_r* 23 000 (Fig. 2).

M_r, temperature and pH optima. Purified endo- β -galactosidase had apparent *M_r* 30 000 as judged by its elution (*K_{av}*) on a column of Sephadex G-100 (Fig. 1a). With keratan sulphate (KSI fraction) as substrate, the enzyme had a pH optimum of 5.7 and a temperature optimum of 55°C.

Fig. 1. Purification of endo- β -galactosidase from *B. fragilis*

(a) Sephadex G-100 chromatography. The arrows indicate the void volume (V_0) and positions of elution of the following protein standards: bovine serum albumin (M_r 68 000); ovalbumin (M_r 43 000); carbonic anhydrase (M_r 30 000); myoglobin (M_r 18 500). (b) Sephadex CM-50 chromatography. Fractions were monitored for protein by A_{280} (●) and for endo- β -galactosidase activity by A_{690} (○). Conditions were as described in the Experimental procedures section.

Table 1. Purification of endo- β -galactosidase from *B. fragilis*

Purification procedures, assay conditions and units are as described in the Experimental procedures section.

Procedure	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Cell-free culture fluid	460	20 600	0.022	100	1
80%-satn.-(NH ₄) ₂ SO ₄ precipitate	445	144	3.1	97	141
Sephadex G-100 chromatography	327	20	16.35	71	743
Sephadex CM-50 chromatography	218	1.4	156	47	7090

Fig. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of endo- β -galactosidase preparation labeled with ^{125}I

For details see the Experimental procedures section. The arrows indicate the positions of standards: phosphorylase *a* (M_r 94 000); bovine serum albumin (M_r 68 000); ovalbumin (M_r 43 000); trypsin (M_r 23 300); cytochrome *c* (M_r 13 000). BPB indicates the position of Bromophenol Blue.

Temperature and pH-stability. Storage for at least 12 months at -20°C , or 2–3 months at 4°C , at a protein concentration of $44\text{ }\mu\text{g/ml}$ did not affect the activity of endo- β -galactosidase. However, under standard assay conditions at 37°C , when the protein concentration was lowered to about $3\text{ }\mu\text{g/ml}$, there was a 40% loss of activity in 30 min (20% loss in 3 h at 4°C), unless bovine serum albumin (0.2 mg/ml) was present. Unlike the *F. keratolyticus* endo- β -galactosidase, the enzyme was not stabilized by 2 mM-Ca^{2+} (Kitamikado *et al.*, 1981). In the presence of 0.2 mg of albumin/ml the enzyme (125 munits/ml) retained its activity over a wide range of pH and was unaffected by preincubation at 25°C for 16 h at pH values between 3.5 and 9.0.

Effect of metal cations and thiol-reactive compounds. The effect on endo- β -galactosidase in the presence of 0.2 mg of albumin/ml of various bivalent metal cations was investigated after each had been pre-incubated with 125 munits of enzyme/ml for 60 min at 37°C . At a concentration of 1 mM , Cu^{2+} , Zn^{2+} and Fe^{2+} caused partial inhibition of enzyme activity; Ca^{2+} , Mn^{2+} , Mg^{2+} and EDTA had no demonstrable effect. Endo- β -galactosidase was totally inhibited by $1\text{ mM-}p$ -chloromercuribenzoic acid, but at the same concentration *N*-ethylmale-

imide (an alternative thiol-reactive compound) had no effect.

Inhibition of endo- β -galactosidase by oligosaccharides. Fig. 3 shows the inhibitory effect of the oligosaccharides Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal and GlcNAc β 1 $\rightarrow$ 3Gal on the activity of *B. fragilis* endo- β -galactosidase when assayed with [^3H]lacto-*N*-neotetraosylceramide as substrate. The trisaccharide gave 60% inhibition at a concentration of 20 mM , and GlcNAc β 1 $\rightarrow$ 3Gal caused a 95% loss of enzyme activity at the concentration tested (30 mM). At a concentration of 20 mM , galactose, *N*-acetyl-

Fig. 3. Effect of various mono- and oligo-saccharides on the activity of endo- β -galactosidase from *B. fragilis* [^3H]Lacto-*N*-neotetraosylceramide ($4 \times 10^4\text{ c.p.m.}$) was dissolved in $10\text{ }\mu\text{l}$ of 50 mM -acetate buffer, pH 5.8, containing 19.2 nmol of sodium taurodeoxycholate, $2\text{ }\mu\text{g}$ of bovine serum albumin, 2.5 munits of endo- β -galactosidase and $0\text{--}300\text{ nmol}$ of the appropriate saccharide. Incubation was for 20 min at 37°C , and then $40\text{ }\mu\text{l}$ of distilled water was added and the mixture partitioned with $300\text{ }\mu\text{l}$ of chloroform/methanol (2:1, v/v). Percentage hydrolysis was assessed by measuring the ^3H -labelled oligo-saccharide in the upper phase by liquid-scintillation counting. Values were corrected by subtracting the radioactivity of the upper phase of a control incubation mixture that contained heat-inactivated enzyme. Results are expressed as percentages of the hydrolysis in the absence of inhibitor. Saccharides used were galactose (O), *N*-acetylglucosamine (●), Gal β 1 $\rightarrow$ 4GlcNAc (▲), Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal (Δ) and GlcNAc β 1 $\rightarrow$ 3Gal (■).

glucosamine and *N*-acetyl-lactosamine (Gal β 1 $\rightarrow$ 4GlcNAc) had no inhibitory effect.

Substrate-specificity studies

Hydrolysis of keratan sulphate. Digestion of bovine corneal keratan sulphate (KSI fraction) by *B. fragilis* endo- β -galactosidase and chromatography of the hydrolysis products on a column of Bio-Gel P4 gave three major oligosaccharide peaks (Fig. 4a). Two of these, A1 and A2, were identified as a disaccharide and tetrasaccharide respectively by the following criteria: (a) oligosaccharides A1 and A2 were eluted at positions corresponding to 3 and 6 hexose units respectively on the Bio-Gel P4 column calibrated with standard glucose oligomers of

Fig. 4. Bio-Gel P4 chromatography of the hydrolysis products of endo- β -galactosidase-treated bovine corneal keratan sulphate

Keratan sulphate (2.5 mg) or partially desulphated keratan sulphate (2.0 mg) was dissolved in 150 μ l of 50 mM-acetate buffer, pH 5.8, containing 30 μ g of bovine serum albumin and 37.5 munits of endo- β -galactosidase, and incubated for 24 h at 37°C. The enzyme was inactivated by heating at 100°C for 3 min, and the mixture was chromatographed on a column (2.6 cm $\times$ 180 cm) of Bio-Gel P4 (–400 mesh) equilibrated against 0.1 M-ammonium acetate at a flow rate of 18 ml/h. Hexose-positive fractions (●) were pooled as indicated. (a) Oligosaccharide hydrolysis products of enzyme-treated keratan sulphate KSI fraction. (b) Oligosaccharide hydrolysis products of enzyme-treated partially desulphated keratan sulphate KSI fraction. The vertical arrows indicate the point of elution of glucose oligomers, from di- to octa-saccharide, obtained by acid hydrolysing Dextran T2000 (Turvey & Whelan, 1957).

partially hydrolysed dextran. In this chromatographic system hexosamine has a K_{av} corresponding to 2–3 hexose units; (b) g.l.c. analysis revealed that oligosaccharides A1 and A2 were composed of galactose and *N*-acetylglucosamine in the ratio of 1:1; (c) oligosaccharide A1, which was free from sulphate, had R_{Gal} 0.66 in solvent 2 and R_F 0.50 in solvent 3, corresponding to those of the disaccharide GlcNAc β 1 $\rightarrow$ 3Gal (Fukuda & Matsumura, 1976; Hounsell *et al.*, 1979); (d) oligosaccharide A2 had R_{Gal} 0.2 in solvent 2 and R_F 0.64 in solvent 3, and was composed of sulphate, galactose and *N*-acetylglucosamine in the proportions of 1.4:1:1, which, from a knowledge of the structure of bovine corneal keratan sulphate (KSI fraction) (Stein *et al.*, 1982), corresponds to:

The same products were obtained when keratan sulphate (KSI fraction) was digested with the *E. freundii* endo- β -galactosidase [results not shown; see also Fukuda & Matsumura (1976)].

Fig. 4(b) shows the effect of partial desulphation on the hydrolysis of keratan sulphate (KSI fraction) by *B. fragilis* endo- β -galactosidase: the amounts of products such as oligosaccharide A2 and the larger uncharacterized oligosaccharide A3, which were hitherto resistant to enzymic hydrolysis, were markedly decreased, and the major product, oligosaccharide B1, corresponded to GlcNAc β 1 $\rightarrow$ 3Gal.

Hydrolysis of oligosaccharides. Table 2 shows that preparations of the *B. fragilis* and *E. freundii* endo- β -galactosidases, which had been standardized (see the Experimental procedures section) with bovine corneal keratan sulphate (KSI fraction) as substrate, were similar in their ability to hydrolyse the internal Gal β 1 $\rightarrow$ 4Glc linkage of lacto-*N*-neotetraose, lacto-*N*-tetraose and the fucosylated derivatives of the latter. The extent of hydrolysis depended on the position of linkage between terminal galactose and sub-terminal *N*-acetylglucosamine and on the degree and position of fucosylation. The type 2 oligosaccharide, lacto-*N*-neotetraose, was hydrolysed to a greater extent than was the type 1 isomer, lacto-*N*-tetraose, and the fucosylated derivatives of lacto-*N*-tetraose were hydrolysed less readily than was the parent tetrasaccharide. Lacto-*N*-fucopentaose II was the most susceptible, and the difucosyl derivative, lacto-*N*-difucohexaose I, was scarcely hydrolysed after prolonged incubation at an elevated enzyme concentration (24 h at 250 munits/ml).

Fig. 5 shows a t.l.c. chromatogram of the hydrolysis products obtained after incubation of the five milk oligosaccharides with 250 munits of *B. fragilis* endo- β -galactosidase/ml for 24 h. The type 1

Table 2. Hydrolysis of oligosaccharides by endo- β -galactosidases from *B. fragilis* and *E. freundii*

Unlabelled oligosaccharides (0.8 mM) and ^{14}C -labelled oligosaccharides (10000 c.p.m.) were incubated for 8 h in 50 mM-acetate buffer, pH 5.8, containing 0.2 mg of bovine serum albumin/ml and 100 munits of endo- β -galactosidase/ml; in addition, the substrates were incubated for 24 h at an elevated concentration (250 munits/ml) of the *B. fragilis* enzyme. Hydrolysis of unlabelled oligosaccharides was assessed by the amount of free glucose released, and of ^{14}C -labelled oligosaccharides after paper chromatography of the reaction products with solvent 1. The vertical dotted line indicates the site of enzymic hydrolysis as deduced from t.l.c. of the reaction products as shown in Fig. 5.

Substrate	Hydrolysis (%)		
	<i>E. freundii</i> enzyme (100 munits/ml)	<i>B. fragilis</i> enzyme	
		(100 munits/ml)	(250 munits/ml)
Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc	23	28	95
Gal β 1 $\rightarrow$ 3GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc	6.0	10	48
Gal β 1 $\rightarrow$ 3GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc ↑ α 1,4 Fuc	2.4	4.2	22
Gal β 1 $\rightarrow$ 3GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc ↑ α 1,2 Fuc	2.0	2.4	4.0
Gal β 1 $\rightarrow$ 3GlcNAc β 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc ↑ α 1,2 ↑ α 1,4 Fuc Fuc	0	0	2.0
[^{14}C]GalNAc α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc ↑ α 1,2 Fuc	0	0	0
[^{14}C]Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc ↑ α 1,2 Fuc	0	0	0
[^{14}C]GalNAc α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc	0	0	0
[^{14}C]Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc	0	0	0

and type 2 oligosaccharides lacto-*N*-tetraose and lacto-*N*-neotetraose were hydrolysed to give products that migrated with a mobility corresponding to a trisaccharide (Hounsell *et al.*, 1979) and glucose. However, lacto-*N*-neotetraose was completely hydrolysed, whereas there was only partial hydrolysis of lacto-*N*-tetraose. Small amounts of glucose and a tetrasaccharide were detectable as products of partial hydrolysis of lacto-*N*-fucopentaose II, but no degradation products were detected with lacto-*N*-fucopentaose I and lacto-*N*-difucohexaose I.

The ^{14}C -labelled blood-group-A and -B tetrasaccharides, shown in Table 2, were not hydrolysed by either endo- β -galactosidase; this effect was not due to the presence of fucose, since their afucosyl trisaccharide derivatives were also resistant to hydrolysis.

Hydrolysis of lacto-*N*-neotetraosylceramide and lacto-*N*-tetraosylceramide. As with the oligosaccharides, the higher rate of hydrolysis of type 2 than type 1 chains was also observed with the tritium-labelled glycolipids lacto-*N*-neotetraosylceramide (paragloboside) and lacto-*N*-tetraosylceramide. Fig. 6 shows that *B. fragilis* endo- β -galactosidase hydrolysed the type 2 glycolipid at about twice

the rate of the type 1 isomer. The same difference in the rate of the hydrolysis of the type 1 and 2 glycolipids was noted with the *E. freundii* enzyme, and similar rate curves were obtained (results not shown).

T.l.c. of the hydrolysis products (results not shown) revealed only one oligosaccharide product, which had R_F 0.4, corresponding to the trisaccharide Gal β 1 $\rightarrow$ 4(3)GlcNAc β 1 $\rightarrow$ 3Gal (Hounsell *et al.*, 1979). Thus the site of enzymic cleavage was restricted to the Gal β 1 $\rightarrow$ 4Glc linkage.

Discussion

Our studies have shown that the *B. fragilis* endo- β -galactosidase can extensively degrade keratan sulphate but does not hydrolyse the blood-group-A- and -B-active tetrasaccharides. It is therefore of the keratanase type, and unrelated to the *Diplococcus pneumoniae* endo- β -galactosidase [designated DI by Fukuda (1982) in order to differentiate from another endo- β -galactosidase DII], which only cleaves blood-group-A- and -B-active structures on type 2 oligosaccharide chains, with the release of the corresponding trisaccharide (Takasaki & Kobata, 1976).

Fig. 5. T.l.c. chromatogram of milk oligosaccharides treated with endo- β -galactosidase from *B. fragilis*. Oligosaccharides (0.8 mM) were incubated for 24 h with 250 munits of endo- β -galactosidase/ml, as described in the legend to Table 2, and the hydrolysis products were analysed by t.l.c. with solvent 3. Lane 1, lacto-*N*-tetraose; lane 2, lacto-*N*-tetraose + enzyme; lane 3, lacto-*N*-neotetraose; lane 4, lacto-*N*-neotetraose + enzyme; lane 5, lacto-*N*-fucopentaose I; lane 6, lacto-*N*-fucopentaose I + enzyme; lane 7, lacto-*N*-fucopentaose II; lane 8, lacto-*N*-fucopentaose II + enzyme; lane 9, lacto-*N*-difucohexaose I; lane 10, lacto-*N*-difucohexaose I + enzyme. St and Glc are standards of stachyose and glucose.

B. fragilis appears to be the best bacterial source thus far described for the isolation of endo- β -galactosidase. Firstly, like *F. keratolyticus*, it does not have an absolute requirement for an inducer, such as keratan sulphate, for production of the enzyme. Secondly, it produces endo- β -galactosidase at a concentration [230 munits/ml of culture supernatant, assayed with bovine corneal keratan sulphate (KSI fraction)] equivalent to 58–69 munits/ml when assayed with whale cartilage keratan sulphate as substrate (see the Experimental procedures section). This is substantially higher than the yields reported from other sources, such as *F. keratolyticus*, 9 munits/ml (Kitamikado *et al.*, 1981), and *E. freundii*, 5–24 munits/ml (Fukuda & Matsumura, 1976; Nakagawa *et al.*, 1980; Fukuda, 1981). Thirdly, the enzyme from *B. fragilis* is obtained free from detectable exoglycosidase and proteinase activity, in a form suitable for the structural analysis of glycoconjugates.

When tested with bovine corneal keratan sulphate (KSI fraction) and various oligosaccharides, it was apparent that the *B. fragilis* endo- β -galactosidase had a substrate-specificity almost identical

Fig. 6. Hydrolysis of ^3H -labelled lacto-*N*-neotetraosylceramide and lacto-*N*-tetraosylceramide by endo- β -galactosidase from *B. fragilis*.

A portion (3.5×10^4 c.p.m.) of ^3H -labelled lacto-*N*-neotetraosylceramide (●) or lacto-*N*-tetraosylceramide (○) was dissolved in 35 μl of 50 mM-acetate buffer, pH 5.8, containing 7 μg of bovine serum albumin, 67 nmol of sodium taurodeoxycholate and 8.75 munits of endo- β -galactosidase and incubated at 37°C. Samples (5 μl) were removed at 5, 10, 15, 20 and 30 min, diluted with 45 μl of distilled water, and partitioned with 300 μl of chloroform/methanol (2:1, v/v). Percentage hydrolysis was assessed by measuring the ^3H -labelled oligosaccharide in the upper phase by liquid-scintillation counting. Values were corrected by subtracting the radioactivity of the upper phase of a control incubation mixture that contained heat-inactivated enzyme. The first-order rate constant (k) is shown for each plot.

with that of the enzyme isolated from *E. freundii*. Both enzymes hydrolyse keratan sulphate (KSI fraction) endoglycosidically to give a major trisulphated tetrasaccharide product, which was further hydrolysed after removal of sulphate, giving a disaccharide corresponding to GlcNAc β 1 $\rightarrow$ 3Gal. This confirms previous findings (Fukuda & Matsumura, 1976) that endo- β -galactosidase will not hydrolyse internal β -galactosidic linkages having sulphated galactose residues.

Substrate-specificity studies with several oligosaccharides isolated from human milk demonstrated that lacto-*N*-tetraose was more readily hydrolysed than were its fucosylated derivatives lacto-*N*-fucopentaose I and lacto-*N*-fucopentaose II.

Therefore, as with the enzymes from *E. freundii* (Nakagawa *et al.*, 1980; Fukuda, 1981) and *F. keratolyticus* (Kitamikado *et al.*, 1981), the action of *B. fragilis* endo- β -galactosidase is inhibited by fucosylation of residues in close proximity to the internal β -galactosidic linkage. Thus the sequence

of lacto-*N*-fucopentaose II is less readily hydrolysed than is the non-fucosylated parent tetrasaccharide and, in agreement with earlier studies (Fukuda, 1981), the lacto-*N*-fucopentaose I sequence

is less well hydrolysed than is lacto-*N*-fucopentaose II. In addition, we have shown that the difucosylated sequence

is the poorest of the type 1 fucosylated substrates.

Precise quantitative information is not yet available on the relative susceptibilities of fucosylated oligosaccharides of the type 2 series. However, from studies with glycolipids as substrates (Fukuda *et al.*, 1978; Fukuda, 1981) the sequences

and

are known to be hydrolysed by *E. freundii* endo- β -galactosidase. In addition, it has been shown (Kannagi *et al.*, 1982) that this enzyme cannot hydrolyse the difucosylated sequence

Additional information on the specificity of endo- β -galactosidase has been obtained from the present study, namely that the enzymes from *B. fragilis* and *E. freundii* are more effective at hydrolysing type 2 (lacto-*N*-neotetraosyl) than type 1 (lacto-*N*-tetraosyl) structures. This aspect of the specificity of the *E. freundii* enzyme has not been studied in detail previously, although it has been reported that a preparation of the endo- β -galactosidase from a *Pseudomonas* sp. did not hydrolyse

lacto-*N*-tetraose (Nakazawa & Suzuki, 1975). The possibility of obtaining enzymes capable of discriminating between these two sequences is attractive, especially since it has been shown that type 1 and type 2 chains are antigenically distinct (Kabat *et al.*, 1982; Gooi *et al.*, 1983) and behave as differentiation antigens in man (Feizi, 1981; Gooi *et al.*, 1983) and mouse (Kapadia *et al.*, 1981).

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References

- Bhatti, T., Chambers, R. E. & Clamp, J. R. (1970) *Biochim. Biophys. Acta* **222**, 339–347
- Doinel, C., Andreu, G., Cartron, J. P., Salmon, C. & Fukuda, M. N. (1980) *Vox Sang.* **38**, 94–98
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Feizi, T. (1981) *Trends Biochem. Sci.* **6**, 333–335
- Feizi, T. (1982) *Adv. Exp. Med. Biol.* **152**, 166–167
- Finch, P. R., Yuen, R., Schachter, H. & Moscarello, M. A. (1969) *Anal. Biochem.* **31**, 296–305
- Fukuda, M. & Fukuda, M. N. (1981) *J. Supramol. Struct. Cell. Biochem.* **17**, 313–324
- Fukuda, M. N. (1981) *J. Biol. Chem.* **256**, 3900–3905
- Fukuda, M. N. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 1160
- Fukuda, M. N. & Matsumura, G. (1976) *J. Biol. Chem.* **251**, 6218–6225
- Fukuda, M. N., Watanabe, K. & Hakomori, S.-I. (1978) *J. Biol. Chem.* **253**, 6814–6819
- Gooi, H. C., Williams, L. K., Uemura, K., Hounsell, E. F., McIlhinney, R. A. J. & Feizi, T. (1983) *Mol. Immunol.* in the press
- Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- Hakomori, S.-I. (1981) *Semin. Hematol.* **18**, 39–62
- Hounsell, E. F., Wood, E. & Feizi, T. (1979) *Proc. Int. Symp. Glycoconjugates 5th* 122–123
- Inglis, C., Bird, G. W. G., Mitchell, A. A. B., Milne, G. R. & Wingham, J. (1975) *J. Clin. Pathol.* **28**, 964–968
- Iwamori, M. & Nagai, Y. (1981) *Biochim. Biophys. Acta* **665**, 205–213
- Kabat, E. A., Liao, J., Shyong, J. & Osserman, E. F. (1982) *J. Immunol.* **128**, 540–544
- Kannagi, R., Nudelman, E., Lavery, S. B. & Hakomori, S.-I. (1982) *J. Biol. Chem.* **257**, 14865–14874
- Kapadia, A., Feizi, T. & Evans, M. J. (1981) *Exp. Cell Res.* **131**, 185–195
- Kitamikado, M. & Ueno, R. (1970) *Bull. Jpn. Soc. Sci. Fish.* **36**, 1175–1180
- Kitamikado, M., Ito, M. & Li, Y.-T. (1981) *J. Biol. Chem.* **256**, 3906–3909
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

- Li, Y.-T. & Li, S.-C. (1972) *Methods Enzymol.* **28**, 699–720
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Nagasawa, K., Inoue, Y. & Kamata, T. (1977) *Carbohydr. Res.* **58**, 47–55
- Nakagawa, H., Yamada, T., Chien, J.-L., Gardas, A., Kitamikado, M., Li, S.-C. & Li, Y.-T. (1980) *J. Biol. Chem.* **255**, 5955–5959
- Nakazawa, K. & Suzuki, S. (1975) *J. Biol. Chem.* **250**, 912–917
- Park, J. T. & Johnson, M. J. (1949) *J. Biol. Chem.* **181**, 149–151
- Rodén, L., Baker, J. R., Cifonelli, J. A. & Mathews, M. B. (1972) *Methods Enzymol.* **28**, 110–112
- Scudder, P. R. & Chantler, E. N. (1981) *Biochim. Biophys. Acta* **660**, 128–135
- Stein, T., Keller, R., Stuhlsatz, H. W., Greiling, H., Ohst, E., Müller, E. & Scharf, H.-D. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 825–833
- Suzuki, Y. & Suzuki, K. (1972) *J. Lipid Res.* **13**, 687–690
- Takasaki, S. & Kobata, A. (1976) *J. Biol. Chem.* **251**, 3603–3609
- Terho, T. T. & Hartiala, K. (1971) *Anal. Biochem.* **41**, 471–476
- Tomarelli, R. M., Charney, J. & Harding, M. L. (1949) *J. Lab. Clin. Med.* **34**, 428–433
- Trevelyan, W. E., Proctor, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
- Turvey, J. R. & Whelan, W. J. (1957) *Biochem. J.* **67**, 49–52
- Uemura, K., Yuzawa, M. & Taketomi, T. (1978) *J. Biochem. (Tokyo)* **83**, 463–471