transesterification equilibrium constant and the thioesterase specificity apparently contribute more to the product specificity than the condensation rates. The turnover number for the thioesterase is not known. However, since the turnover numbers of the overall reaction and the other enzymes can be estimated from this and previous (Cox & Hammes, 1983; Cognet & Hammes, 1985) work, a rough estimate for this turnover number of 10–100 s^{-1} can be made. Apparently, all of the individual turnover numbers are similar in magnitude so that a single rate-determining step does not exist for the overall reaction.

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Isolation and Characterization of a New Endo-β-galactosidase from *Diplococcus pneumoniae*†

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ABSTRACT: An endo-β-galactosidase, which hydrolyzes the internal β-galactosidic linkages of R→GlcNAc(or GalNAc)β1→3Galβ1→4GlcNAc(or Glc), was isolated from the culture supernatant of *Diplococcus pneumoniae*. The enzyme, named endo-β-galactosidase D_{II}, hydrolyzed linear *N*-acetyllactosamine repeating structures in glycolipids and glycopeptides to release oligosaccharides. The specificity of endo-β-galactosidase D_{II} is the same as that of *Escherichia freundii* endo-β-galactosidase as far as described above, but the following differences between these two enzymes were found: (1) Branched lactosaminyl glycolipids and H-antigenic glycolipids were resistant to endo-β-galactosidase D_{II}, even when linear structure was present at the inner part. (2) Throughout the enzymic hydrolysis, endo-β-galactosidase D_{II} released mostly small oligosaccharides (tetra-, tri-, and disaccharides) from substrates, suggesting that the enzyme split off the oligosaccharides stepwise from the nonreducing terminal. (3) Lactosaminoglycans were partially hydrolyzed by endo-β-galactosidase D_{II} to produce small oligosaccharides as the major product and residual glycopeptides. The residual glycopeptides were readily hydrolyzed by *E. freundii* endo-β-galactosidase to produce various sizes of oligosaccharides. (4) Keratan sulfate was not degraded by endo-β-galactosidase D_{II}. These properties of endo-β-galactosidase D_{II} characterize it as a new endo-β-galactosidase with a unique specificity.

Lactosaminoglycan glycoproteins and lacto-series glycolipids are characterized by repeating the *N*-acetyllactosamine sequence $(GlcNAc\beta1\rightarrow3Gal\beta1\rightarrow4)_n$ and by their susceptibility to endo-β-galactosidase (Fukuda et al., 1978b, 1979b). These glycoconjugates are responsible for cell surface antigens, such as Ii and ABO blood group systems. The previous studies have shown that expression of these antigens is regulated under

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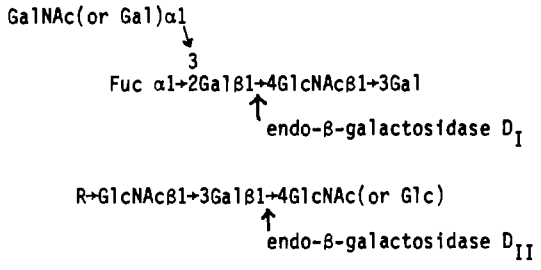
normal ontogenic development and cellular differentiation (Marsh, 1961; Szulman, 1980). Endo-β-galactosidase from *Escherichia freundii* (Fukuda & Matsumura, 1976) has most widely been used for the structural and immunochemical analyses of lactosaminoglycans and glycolipids (Fukuda et al., 1979a,b, 1981, 1984a,b; Muramatsu et al., 1979; Spooncer et al., 1984). Keratanase from *Pseudomonas* sp. (Nakazawa & Suzuki, 1975) was also used as endo-β-galactosidase in some cases (Shur, 1982; Fenderson et al., 1984). Endo-β-galactosidases were also isolated from *Flavobacterium keratolyticus* (Kutamikado et al., 1981) and from *Bacterioides fragilis* (Scudder et al., 1983). Both of the latter enzymes were shown to be similar to the *E. freundii* enzyme.

Diplococcus pneumoniae is a rich source for exo- and endoglycosidases (Hughes & Jeanloz, 1964; Koide & Muramatsu, 1974; Takasaki & Kobata, 1976; Glasgow et al., 1977). Takasaki & Kobata (1976) reported an endo-β-galactosidase activity that hydrolyzed the oligosaccharides with blood group A or B determinant. Later, another endo-β-galactosidase was found, and these two endo-β-galactosidases were, therefore, called D_I and D_{II} (Fukuda, 1982):



Endo-β-galactosidase D_{II} was thought to be similar to the *E. freundii* enzyme. However, further study of endo-β-galactosidase D_{II} revealed a unique property of this enzyme, which distinguished it from other known endo-β-galactosidases. This paper describes the characteristics of endo-β-galactosidase D_{II} with comparison to the *E. freundii* endo-β-galactosidase.

Materials and Methods

Bacterial Strains. The three strains of *D. pneumoniae* type I were kindly provided by Dr. P. Atkinson, Albert Einstein College School of Medicine, Dr. R. Hill, Duke University, and Dr. Y. Shibata, Seikagaku Kogyo Research Institute. Within these strains, there was no difference in the productivity of endo-β-galactosidases.

Enzymes. Endo-β-galactosidase from *E. freundii* was purified as described previously (Fukuda, 1981). Keratanase from *Pseudomonas* sp. (Nakazawa & Suzuki, 1974) is a product of Seikagaku Kogyo Ltd., Japan, and was kindly provided by Dr. Y. Shibata.

Glycolipids. The glycolipids were prepared in our laboratory from human blood cells according to the method described (Hakomori & Watanabe, 1976): Briefly, lipids were extracted from the membranes of whole blood cells with hot ethanol. After Folch's phase partition, the lower layer glycolipids were freed from cholesterol and phospholipids by acetylation procedures. Upper layer glycolipids were subjected to QAE-Sephadex A-25 column chromatography, and neutral and acidic glycolipids were separated in the solvent systems described by Ando & Yu (1977). Purification of each glycolipid was performed by high-performance liquid chromatography with a Varian HPLC[1] apparatus (Model 5000, Varian Associates), on columns (1 × 50 cm or 0.5 × 100 cm) of Iatrobeads (IRS 8010, 10-μm diameter, Iatron, Tokyo), and eluted with a solvent system of 2-propanol/hexane/water (Watanabe & Arao, 1981). Amino-CTH, paragloboside, and N_1 glycolipid were prepared from human granulocytes as described (Fukuda et al., 1985). Lactoisooctaosylceramide and lactoisodecaosylceramide were prepared from H_3 and H_4 glycolipids, respectively, by treatment with 0.1 N trichloroacetic acid at 100 °C for 1 h followed by purification with HPLC. Lacto-*N*-tetraosylceramide and lacto-*N*-fucopentaosyl-II-ceramide, isolated from human meconium (Karlsson & Larson, 1981), were kindly provide by Dr. K.-A. Karlsson, University of Goteborg, Sweden. Lactonorhexaosylceramide was prepared from sialosyllactonorhexaosylceramide by treatment with 0.05 N H_2SO_4 at 80 °C for 1 h.

^{3}H-Labeled Glycolipids. Galactose or *N*-acetylgalactosamine termini of A^a, H_2, and X_2 glycolipids and lactonorhexaosylceramide were labeled by the galactose oxidase/NaB^3H_4 method as described previously (Fukuda et al., 1979a). The double bond of sphingosine of the H_2 glycolipid and C_7-sialosylhexaosylceramide (see below) was reduced with NaB^3H_4 under the presence of $Pd/BaSO_4$ (Sigma) as catalyst, as follows. The glycolipid (100 μg) was dissolved in 500 μL of methanol and 3 mg of $Pd/BaSO_4$, and 2.5 mCi of NaB^3H_4 dissolved in 50 μL of 0.01 N NaOH was added. Reduction was carried out at room temperature for 4 h and then stopped by adding a few drops of glacial acetic acid. ^{3}H-Labeled glycolipid was extracted with methanol, and $Pd/BaSO_4$ was removed by centrifugation. Supernatant was applied on a LH-20 column (1 × 30 cm), equilibrated with chloroform–methanol (2/1 v/v), and eluted with the same solvent.

Linear and Branched Lactosaminoglycans (Band 3 Glycopeptides). Band 3 was purified from both adult and cord (newborn) erythrocytes as described (Fukuda et al., 1978). Each band 3 was digested with Pronase, and glycopeptides were prepared by gel filtration of Sephadex G-50. Large molecular weight glycopeptides were pooled as lactosaminoglycans and were labeled by galactose oxidase/NaB^3H_4 as described previously (Fukuda et al., 1979b).

Other Materials. Keratan sulfate was prepared from bovine cornea according to Meyer et al. (1953). Galactono(1→5)-lactone was purchased from Sigma. *p*-Aminophenyl 1-thio-β-D-galactoside–Sepharose 4B was prepared by adding *p*-aminophenyl 1-thio-β-D-galactoside (Sigma) to cyanogen-activated Sepharose 4B, according to Glasgow et al. (1977). *p*-Aminophenyl 1-thio-β-D-*N*-acetylglucosaminide–Sepharose 4B was prepared by using *p*-aminophenyl 1-thio-β-D-*N*-acetylglucosamine (Bachem, Marina Del Ray, CA) in the same manner.

Hydrolysis of Keratan Sulfate by Endo-β-galactosidase D_{II}. The susceptibility of keratan sulfate to endo-β-galactosidase D_{II} was examined by three different methods. (1) *Keratan* sulfate (150 μg) was dissolved in 50 μL of 0.2 M sodium acetate buffer, pH 5.0, and was incubated with 10 milliunits of endo-β-galactosidase D_{II} at 37 °C for 20 h. The reducing

[1] Abbreviations: HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; TLC, thin-layer chromatography; CMH, glucosylceramide, Glcβ1→1Cer; CDH, lactosylceramide, Galβ1→4Glcβ1→1Cer; CTH, globotriaosylceramide, Galα1→3Galβ1→4Glcβ1→1Cer; aCTH, amino-CTH or lacto-*N*-triaosylceramide; PG, paragloboside or lacto-*N*-neotetraosylceramide; A^a glycolipid, GalNAcα1→3(Fucα1→2)Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; lacto-*N*-tetraosylceramide, Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; lacto-*N*-fucopentaosyl-II-ceramide, Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; Galol, galactitol.

power of the reaction mixture was measured by the method of Park & Johnson (1949). (2) The digest of keratan sulfate by endo-β-galactosidase D_{II} was examined by gel filtration. Keratan sulfate (1 mg) was dissolved in 400 μL of citrate–phosphate buffer, pH 5.0, containing 24 milliunits of enzyme and incubated at 37 °C for 20 h. The digest was applied on a Sephadex G-50 column (1 × 96 cm), equilibrated with 0.2 M NaCl, and eluted with the same solution. Fractions of 0.75 mL were collected, and hexose was measured by anthrone reaction. (3) The digest of keratan sulfate was analyzed by TLC. Keratan sulfate (100 μg) was dissolved in 30 μL of 0.2 M sodium acetate buffer, pH 5.0, and was incubated with 10 milliunits of endo-β-galactosidase D_{II} at 37 °C for 20 h. The digest was spotted on HPTLC (Si-HPF plate, J. T. Baker) and was developed in the solvent system 1-butanol/acetic acid/water (3:2:1 v/v). TLC was sprayed with an orcinol reagent (0.2% orcinol in 4 N H_2SO_4) and was heated at 115 °C in an oven to visualize sugars. In the above analyses, enzymes from *E. freundii* and from *Pseudomonas* sp. were used for comparison.

Enzyme Assay. The assay of endo-β-galactosidase D_I was carried out with ^{3}H-labeled A^a glycolipid as substrate as follows: ^{3}H-labeled A^a glycolipid (see footnote 1 for structure) (3000 cpm, 2 nmol) was dissolved in 10 μL of 0.2 M sodium acetate buffer, pH 5.8, containing 20 μg of sodium deoxytaurocholate and was mixed with 10 μL of enzyme solution. After incubation at 37 °C for 30 min, 80 μL of water and 600 μL of chloroform/methanol (2/1 v/v) were added. After the two phases were separated from each other, 100 μL of the upper water phase was taken to measure the radioactivity. The endo-β-galactosidase D_I releases ^{3}H-labeled trisaccharide, [^{3}H]GalNAcα1→3(Fucα1→2)Gal, from the A^a glycolipid, and this trisaccharide is partitioned into a water phase, while the ^{3}H-labeled A^a glycolipid stays in the organic phase. The increase of radioactivity in water phase represents the activity of endo-β-galactosidase D_I.

For the assay of endo-β-galactosidase D_{II}, sialosylnorhexaosylceramide (C_7-sialosylhexaosylceramide) was prepared in order to minimize the hydrolysis of this substrate by neuraminidase (Suttajit & Winzler, 1971). Sialosylhexaosylceramide (1 μmol) was dissolved in 200 μL of water, and 4 μmol of $NaIO_4$, which was dissolved in 100 μL of 0.05 M sodium acetate buffer, pH 4.0, was added. Oxidation was performed at 4 °C for 2 h, and the reaction was stopped by adding a few drops of ethylene glycol. The pH of the reaction mixture was adjusted to 8.0 by adding 0.2 M sodium borate; then, 5 mg of $NaBH_4$ was added to reduce the C_7 aldehyde of *N*-acetylneuraminic acid. C_7-Sialosylhexaosylceramide was then desalted by a LH-20 column and the double bond of ceramide was reduced with NaB^3H_4 as described above.

The assay of endo-β-galactosidase D_{II} was carried out as follows: C_7-sialosylhexaosyl[^{3}H]ceramide (8000 cpm, 2 nmol) was dissolved in 10 μL of 0.2 M sodium citrate–phosphate buffer, pH 5.0, containing 20 μg of sodium deoxytaurocholate and was added to 10 μL of enzyme solution. After incubation at 37 °C for 10 min, 80 μL of water and 600 μL of chloroform/methanol (2/1, v/v) was added. The upper water phase (100 μL) was taken to measure the radioactivity. The C_7-sialosylhexaosyl[^{3}H]ceramide is soluble in the upper phase, while GlcNAcβ1→3Galβ1→4Glc[^{3}H]Cer and Glc[^{3}H]Cer, which are released by endo-β-galactosidase D_{II}, are partitioned into the lower organic phase. The decrease of radioactivity from the upper phase represents the activity of endo-β-galactosidase D_{II}. This method allowed detection of 1×10^{-5} unit of endo-β-galactosidase D_{II} activity.

Exoglycosidases were measured by using *p*-nitrophenyl glycosides. One enzyme unit is defined as 1 μmol of substrate hydrolyzed per minute under the assay conditions described above.

Purification of Endo-β-galactosidase D_{II}. D. pneumoniae was cultured according to Hughes & Jeanloz (1964) under the presence of 5% bovine serum. To the culture filtrate of *D. pneumoniae* (1 L, step 1), ammonium sulfate was added to the 75% saturation level. The resulting precipitate was collected and was dialyzed against 0.02 M Tris-HCl buffer, pH 7.5 (step 2). Enzyme solution was applied to the column (3 × 50 cm) of DEAE-Sephadex A-25 equilibrated with 0.02 M Tris-HCl buffer, pH 7.5. Fractions of 10 mL were collected. Endo-β-galactosidases D_I and D_{II} and exo-β-galactosidase were not bound to DEAE-Sephadex column (step 3). These enzymes were precipitated with a 75% saturation of ammonium sulfate, dialyzed against 0.03 M sodium phosphate buffer, pH 6.0, containing 0.2 M NaCl, and then separated by a gel filtration column (2 × 120 cm) of Sephacryl S-200 equilibrated with 0.02 M sodium phosphate buffer, pH 6.0, containing 0.2 M NaCl. Fractions of 6.5 mL were collected. Endo-β-galactosidase D_{II} eluted slightly after the exo-β-galactosidase (step 4) and was dialyzed against 0.2 M sodium cacodylate buffer. In order to remove the exo-β-galactosidase and exo-β-*N*-acetylglucosaminidase activity contaminating in this fraction, the enzyme solution was successively passed through to each column (1 × 3 cm) of *p*-aminophenyl 1-thio-β-D-galactoside–Sepharose 4B and *p*-aminophenyl 1-thio-β-D-*N*-acetylglucosaminide–Sepharose 4B. The endo-β-galactosidase D_{II} thus obtained was dialyzed against 0.02 M sodium phosphate buffer, pH 6.0, and was stored at −20 °C. The culture filtrate (1 L) contained 9.7 units of endo-β-galactosidase D_{II} with a specific activity of 0.00054 unit/mg of protein. The final preparation (step 5) yields 1.9 units of endo-β-galactosidase D_{II} with a specific activity of 0.070 unit/mg of protein.

Isoelectric Focusing Column Chromatography. As part of step 4, enzyme solution was dialyzed extensively against distilled water followed by 0.025 M ethanolamine–acetate buffer, pH 9.25, and applied to a column (1 × 23 cm) of PBE 94 gel (Pharmacia). After the column was washed with 10 mL of the same buffer, pH gradient elution was performed by eluting 130 mL of diluted (1:10) polybuffer 96–acetate, pH 5.5. Fractions of 2 mL were collected. Each glycosidase activity and the pH of the eluate were measured as described in the section on enzyme assay. Endo-β-galactosidases D_I and D_{II} and exo-β-galactosidase were eluted at pH 7.5, 6.8 and 6.6, respectively.

RESULTS

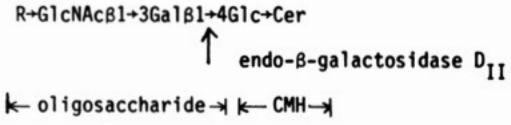
Purification of Endo-β-galactosidase D_{II}. Endo-β-galactosidase D_{II} was purified from culture filtrate of *D. pneumoniae* through DEAE-Sephadex column chromatography, gel filtration on Sephacryl S-200, and affinity chromatography. On DEAE-Sephadex column chromatography, endo-β-galactosidase D_{II} and exo-β-galactosidase were eluted together followed by endo-β-galactosidase D_I [data not shown; see Takasaki & Kobata (1976) and Glasgow et al. (1977)]. In order to remove exo-β-galactosidase and exo-β-*N*-acetylglucosaminidase, affinity columns of *p*-aminophenyl 1-thio-β-galactoside–Sepharose and *p*-aminophenyl 1-thio-β-*N*-acetylglucosaminide–Sepharose were used. Enzyme preparation, which was achieved to a 130-fold increase of specific activity with 20% recovery, was used for the study described below.

Properties of Endo-β-galactosidase D_{II}. The above partially

purified preparation of endo-β-galactosidase D_{II} was free from β-*N*-acetylglucosaminidase, α-mannosidase, α-L-fucosidase, and sialidase but contained exo-β-galactosidase (about 5% based on enzyme units). In order to minimize the effect of exo-β-galactosidase, galactono(1→5)lactone was added in the reaction mixture. As shown previously (Fukuda et al., 1984b), the addition of galactonolactone does not effect the activity of endo-β-galactosidase from *E. freundii*. The isoelectric point of endo-β-galactosidase D_{II} was found to be pH 6.8 from the elution profile of the chromatofocusing column. The optimum activity of endo-β-galactosidase D_{II} was found between pH 4.8 and pH 5.2 in sodium citrate–phosphate buffer.

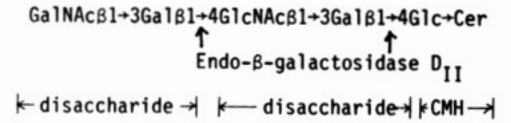
The effect of the following reagents were examined after preincubation of the enzyme for 30 min at room temperature. Ethylenediaminetetraacetate (1 mM), β-mercaptoethanol (at 1 and 10 mM), and the ions Ca^{2+}, Mg^{2+}, and Zn^{2+} (at 5 mM) did not exert any effect on enzyme activity. One millimolar of the ions Hg^{2+}, Ag^{+}, and *p*-(chloromercuri)benzoate completely inactivated the enzyme activity.

Hydrolysis of Glycolipids by Endo-β-galactosidase D_{II}. Endo-β-galactosidase D_{II} hydrolyzed glycolipids endoglycosidically. Figure 1 shows the hydrolysis of glycolipids examined by TLC. Amino-CTH (lacto-*N*-triaosylceramide) and its analogues were hydrolyzed to oligosaccharides (R→-GlcNAcβ1→3Gal) and glucosylceramide (Figure 1A). Thus, endo-β-galactosidase D_{II} hydrolyzes these substrates as follows:

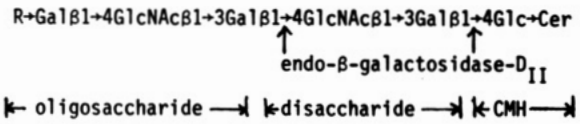


Glycolipids with a type 1 carbohydrate chain were found to be susceptible to endo-β-galactosidase D_{II}, as follows. These glycolipids, of which R in the above structure is Galβ1→3 in lacto-*N*-tetraosylceramide or Galβ1→3(Fucα1→4) in lacto-*N*-fucopentaosyl-II-ceramide, were labeled by the galactose oxidase/NaB^3H_4 method and were subjected to enzymic hydrolysis in the same manner as described in the enzyme assay for endo-β-galactosidase D_I. The released ^3H-labeled oligosaccharide was analyzed by HPLC (for the method, see the legend for Figure 2). By estimation from the radioactivity recovered as oligosaccharides after 30 min of reaction, hydrolysis of lacto-*N*-tetraosylceramide and lacto-*N*-fucopentaosyl-II-ceramide was 70% and 48%, respectively. Under the same condition, paragloboside, Galβ1→-4GlcNAcβ1→3Galβ1→4Glc→Cer, was hydrolyzed completely.

Glycolipids, which are made of two *N*-acetyllactosaminyl units, were hydrolyzed to the terminal oligosaccharides, disaccharide and glucosylceramide (Figure 1B):



The X_2 glycolipid was also hydrolyzed to disaccharides and glucosylceramide. Therefore, endo-β-galactosidase D_{II} hydrolyzed the β-galactosidic linkage of the GalNAcβ1→-3Galβ1→4 sequence as follows:



As shown in Figure 1B, the H_2 glycolipid was not hydrolyzed by endo-β-galactosidase D_{II}. Since relatively slow hydrolyz-

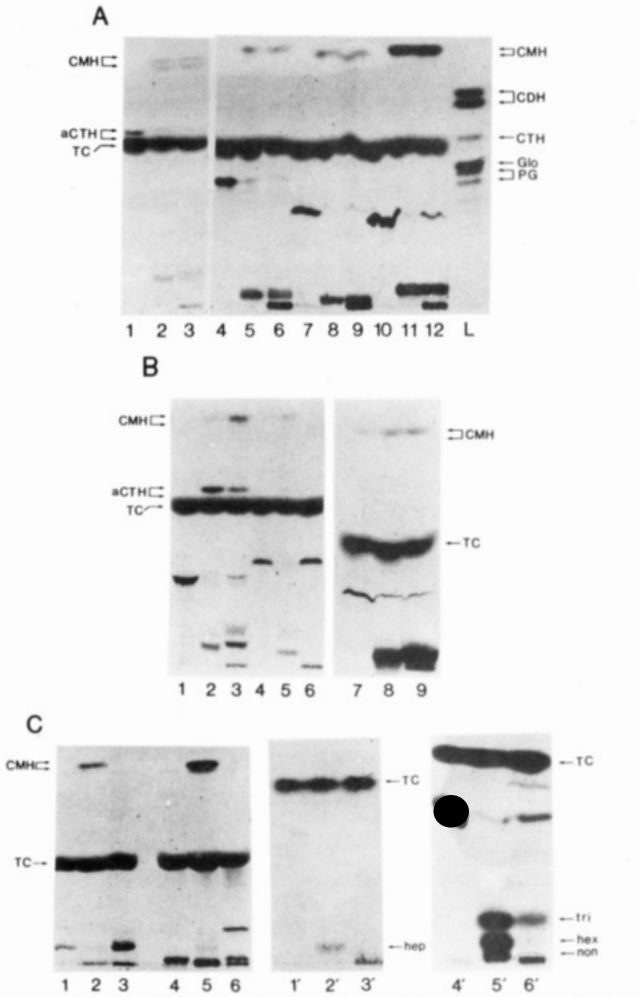


FIGURE 1: HPTLC of the digests of glycolipids by endo-β-galactosidases. Glycolipids (5–15 μg) were dissolved in 5 μL of 0.1 M sodium citrate–phosphate buffer, pH 5.0, containing 10 μg of sodium deoxytaurocholate and 60 mM galactono(1→5)lactone, and 5 μL of 1.2 milliunits (A and B) or 12 milliunits (C) of endo-β-galactosidase D_{II} was added. The hydrolysis of glycolipids by *E. freundii* endo-β-galactosidase was carried out in the same manner except for the following: 1.25 milliunits of enzyme and 0.1 M sodium acetate buffer, pH 5.8, was used. After incubation at 37 °C for 20 h, the digest was spotted on a HPTLC plate (Si-HPF plate, J. T. Baker) and chromatography was developed in a solvent of chloroform/methanol/water (56:38:10 v/v). (A) Hydrolysis of amino-CTH and its analogous glycolipids: (lanes 1–3) amino-CTH; (lanes 4–6) paragloboside; (lanes 7–9) N_1 glycolipid; (lanes 10–12) sialosylparagloboside. (L) Folch's lower phase glycolipid mixture prepared from whole blood cells. Glycolipids were incubatedd without enzyme (lanes 1, 4, 7, and 10), with *E. freundii* endo-β-galactosidase (lanes 2, 5, 8, and 11), and with endo-β-galactosidase D_{II} (lanes 3, 6, 9, and 12). TC, taurodeoxycholate. (B) Hydrolysis of analogous glycolipids of lactonorhexaosylceramide: (lanes 1–3) sialosyllactonorhexaosylceramide; (lanes 4–6) H_2 glycolipid; (lanes 7–9) X_2 glycolipid. Glycolipids were incubated without enzyme (lanes 1, 4, and 7), with *E. freundii* endo-β-galactosidase (lanes 2, 5, and 8), and with endo-β-galactosidase D_{II} (lanes 3, 6, and 9). (C) Hydrolysis of branched lactosaminyl glycolipids: (lanes 1–3 and 1′–3′) lactoisooctaosylceramide (defucosylated H_3 glycolipid); (lanes 4–6 and 4′–6′) lactoisodecaosylceramide (defucosylated H_4 glycolipid). Glycolipids were incubated without enzyme (lanes 1, 4, 1′, and 4′), with *E. freundii* endo-β-galactosidase (lanes 2, 5, 2′, and 5′), and with endo-β-galactosidase D_{II} (3, 6, 3′, and 6′). The digests were analyzed after partition of chloroform/methanol/water (4:2:1 v/v). The upper water phase was used for analysis of released oligosaccharides in the solvent system 1-butanol/acetic acid/water (3:2:1 v/v) as shown in lane 1′–6′, and the lower organic phase was used for analysis of shortened glycolipid products in the solvent system chloroform/methanol/water (60:35:8 v/v) as shown in lanes 1–6. Glycolipids and oligosaccharides were detected by an orcinol–H_2SO_4 reagent. The arrows for tri, hex, hep, and non indicate the trisaccharide, hexasaccharide, and heptasaccharide, and nonasaccharide, respectively.

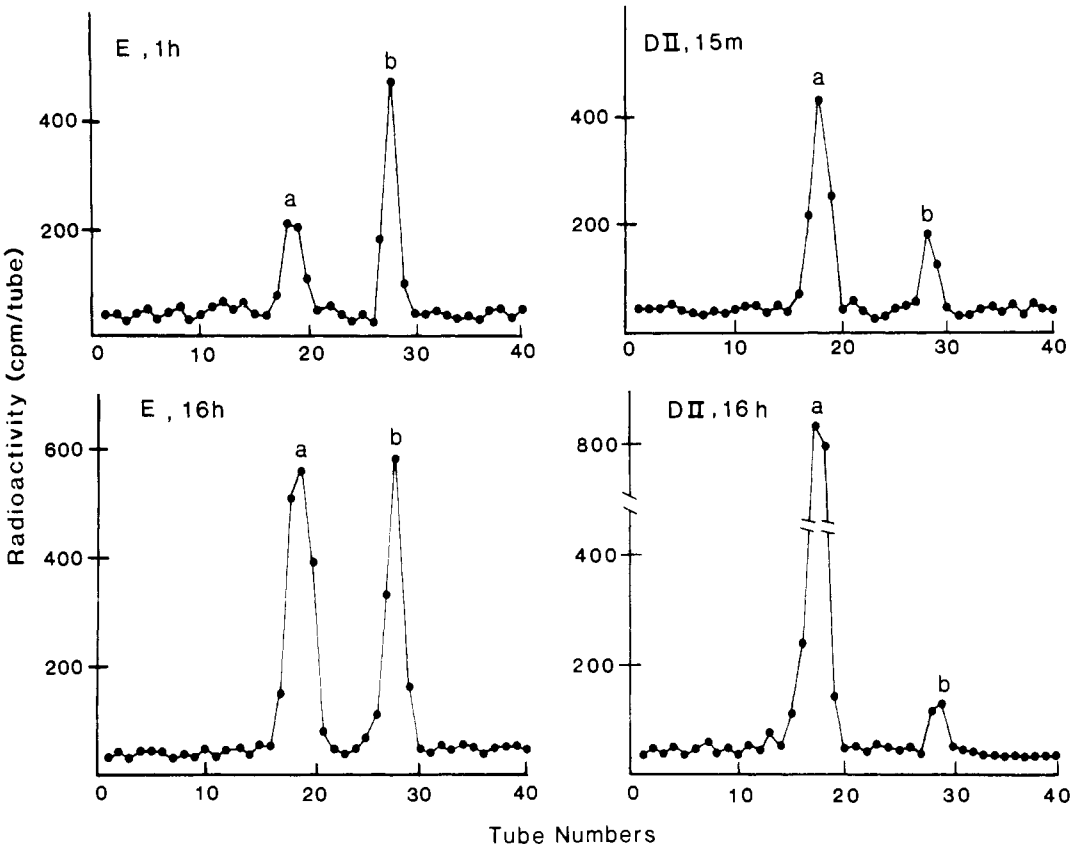
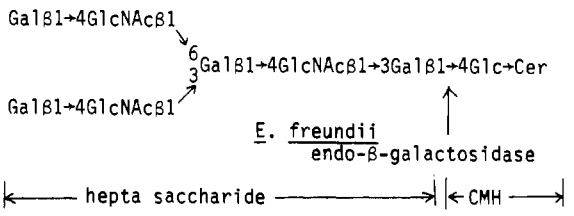


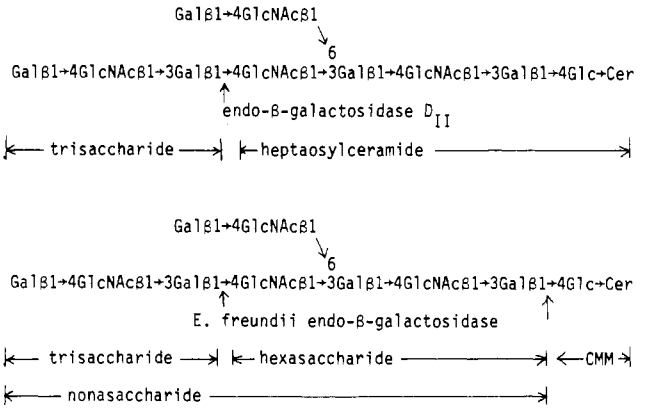
FIGURE 2: Analysis of released oligosaccharides by HPLC. Galactose oxidase/NaB^3H_4 labeled X_2 glycolipids were hydrolyzed by endo-β-galactosidase D_{II} or endo-β-galactosidase from *E. freundii*. Reaction mixtures were partitioned by chlloroform/methanol/water (4:2:1 v/v), and the upper water phase was applied on the HPLC column (4 × 250 mm packed with Bio-Sil Amino-5S, Bio-Rad) equilibrated with acetonitrile/water (60:40). The solvent gradient was programmed to acetonitrile/water (30:70) for over 30 min. Flow rate was constant at 0.5 mL/min, and fractions were collected every 0.5 min. (a) GalNAcβ1→3Gal; (b) GalNAcβ1→3Galβ1→4GlcNAcβ1→3Gal. Galactose eluted at tube 7 in this chromatography. E, *E. freundii* endo-β-galactosidase; D_{II}, endo-β-galactosidase D_{II}; 15m, 1h, and 16h are enzymic reaction times for 15 min, 1 h, and 16 h, respectively.

ability of H-active structures by endo-β-galactosidases is known (Nakagawa et al., 1980; Fukuda, 1981; Scudder et al., 1983), the susceptibility of H_2 glycolipid to endo-β-galactosidase D_{II} was further examined by using the ^3H-labeled H_2 glycolipid. The H_2 glycolipid, of which the terminal galactose was labeled by the galactose oxidase/NaB^3H_4 method, was incubated with endo-β-galactosidase D_{II}. The released oligosaccharide was examined by HPLC but no ^3H-labeled oligosaccharide was detected after 20 h of incubation. The H_2 glycolipid, of which ceramide was labeled by tritium, was also subjected to hydrolysis. Digests were separated by TLC, but neither lacto-*N*-triaosyl[^3H]ceramide nor glucosyl[^3H]ceramide was detected. These results indicate the complete resistance of the H_2 glycolipid toward endo-β-galactosidase D_{II}. Consistently, the H-antigenic side chains of glycopeptides were not susceptible to endo-β-galactosidase D_{II} (see below).

Glycolipids that have branched lactosaminyl structures were examined. As shown in Figure 1C, lactoisooctaosylceramide was not hydrolyzed by endo-β-galactosidase D_{II}, whereas the *E. freundii* enzyme hydrolyzed it to heptasaccharide and glucosylceramide:



Lactoisodecaosylceramide was hydrolyzed to endo-β-galactosidase D_{II} to trisaccharide and a glycolipid, possibly heptaosylceramide, whereas it was converted to three oligosaccharides and glucosylceramide by *E. freundii* endo-β-galactosidase (Figure 1C). These results suggest that endo-β-galactosidase D_{II} hydrolyzes the β-galactosidic linkage located at the peripheral areas but cannot attack the linkages at the branched points or innermost β-galactosidic bond:



Hydrolysis of glycolipids by endo-β-galactosidase D_{II} is summarized by Table I. We also examined *Pseudomonas* sp. keratanase to determine whether it hydrolyzed any of these glycolipids and found that it did not (data not shown).

Release of Oligosaccharides from Glycolipids by Endo-β-galactosidase D_{II}. The nonreducing terminal galactose and *N*-acetylgalactosamine of lacto-*N*-hexaosylceramide and X_2 glycolipid were labeled by the galactose oxidase/NaB^3H_4

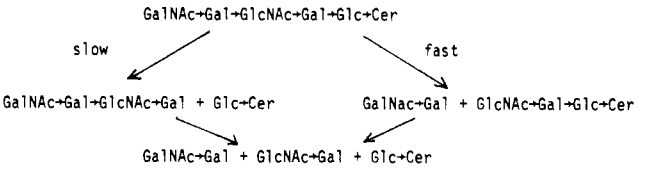
Table I: Hydrolysis of Glycolipids by Endo-β-galactosidase D_{II}

trivial names	glycolipid structure[a]	β-galactosidic bond cleaved[b]		
		VIGalβ 1→	IVGalβ→	IIGalβ→
amino-CTH	GlcNAcβ1→3Galβ1→4Glcβ1→Cer			+
paragloboside	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer			+
lacto-*N*-tetroasylceramide	Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→Cer			+
lacto-*N*-fucopentaosyl-II-ceramide	Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glcβ1→Cer			+
N_1 glycolipid	Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4Glcβ1→Cer			+
sialosylparagloboside	NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer		+	+
X_2 glycolipid	GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer		+	+
lactonorhexaoxylceramide	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer		+	+
H_2 glycolipid	Fucα1→2Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer		-*[c]	-*
sialosyllactonorhexaosylceramide	NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer		+	+
lactoisooctaosylceramide (defucosylated H_3 glycolipid)	Galβ1→4GlcNAcβ1 6 Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer		-	-*
lactoisodecaosylceramide (defucosylated H_4 glycolipid)	Galβ1→4GlcNAcβ1 6 Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer	+	-	-*

[a] The structures of the glycolipids were described in the following: Fukuda et al. (1984c), Fukuda & Hakomori (1982), Kannagi et al. (1982), and Watanabe et al. (1975). [b] IIGal, IVGal, and VIGal are second, fourth, and sixth galactose residues counted from ceramide, respectively. [c] Positions that are hydrolyzed by *E. freundii* enzyme are marked by an asterisk. All listed positions that can be hydrolyzed by endo-β-galactosidase D_{II} are also hydrolyzed by *E. freundii* enzyme.

method. After endo-β-galactosidase digestion, released ^{3}H-labeled oligosaccharides from this glycolipid were analyzed by HPLC (Figure 2). *E. freundii* endo-β-galactosidase released more [^{3}H]GalNAc→Gal→GlcNAc→Gal (peak b) than [^{3}H]GalNAc→Gal (peak a) in the early reaction period. This indicates that the *E. freundii* enzyme attacks the IIGalβ1→ linkage more easily than the IVGalβ1→ linkage. The increase of disaccharides in the prolonged reaction period suggests the formation of disaccharides GalNAc→Gal and GlcNAc→Gal as secondary fragments of tetrasaccharide GalNAc→Gal→GlcNAc→Gal. On the other hand, endo-β-galactosidase D_{II} released primarily the disaccharides, and relatively small amount of the tetrasaccharides was found throughout the reaction time.

As shown in Figure 1B, endo-β-galactosidase D_{II} hydrolyzed the X_2 glycolipid to produce glucosylceramide. Thus, this enzyme can hydrolyze the innermost β-galactosidic bond of the X_2 glycolipid as well. These combined results suggest that the D_{II} enzyme preferentially attacked the peripheral β-galactosidic linkage and then hydrolyzed the penultimate β-galactosidic bond. The hydrolysis of the X_2 glycolipid by endo-β-galactosidase D_{II} can be illustrated as follows:



The released oligosaccharide profiles of lactonorhexaosylceramide examined in the same manner also showed the preferential release of terminal trisaccharides by endo-β-galactosidase D_{II}, while *E. freundii* endo-β-galactosidase releases pentasaccharide, as well as trisaccharide (data not shown).

Hydrolysis of Lactosaminoglycan Peptides by Endo-β-galactosidase D_{II}. It was shown previously that *E. freundii* endo-β-galactosidase hydrolyzed linear lactosaminoglycans to give small oligosaccharides, while it hydrolyzed branched ones to give various-sized oligosaccharides (Fukuda et al., 1979b, 1984a,b). Upon exhaustive digestion of endo-β-galactosidase D_{II}, branched lactosaminoglycans were converted mainly to small oligosaccharides and the residual large molecular weight glycopeptides (Figure 3B). When the digestion products of shorter hydrolysis period were examined, a greater ratio of glycopeptides to small oligosaccharides was obtained, but no appreciable amount of intermediate-size products appeared. The residual glycopeptides were reisolated after gel filtration and were subjected to digest with 3 times the amount of endo-β-galactosidase D_{II}, but the second digest did not show any release of oligosaccharides. Thus, it was concluded that residual glycopeptides are fairly resistant to endo-β-galactosidase D_{II}. The residual glycopeptides were, however, readily hydrolyzed by *E. freundii* endo-β-galactosidase to give various sizes of products (Figure 3C).

The smallest oligosaccharide released by endo-β-galactosidase D_{II} (P-1 D_{II} in Figure 3B,E) was eluted at the same position of standard Galβ1→4GlcNAcβ1→3Gal in a HPLC analysis. On the other hand, the smallest oligosaccharide that was released from the residual glycopeptide by the *E. freundii* enzyme (P-1 E in Figure 3C,F) eluted at the same position of standard Fucα1→2Galβ1→4GlcNAcβ1→3Gal (data not shown).

Released oligosaccharides were also analyzed after reduction with NaB^{3}H$_4$. Upon digestion of endo-β-galactosidase D_{II}, ^{3}H-labeled oligosaccharide alcohols showed three components

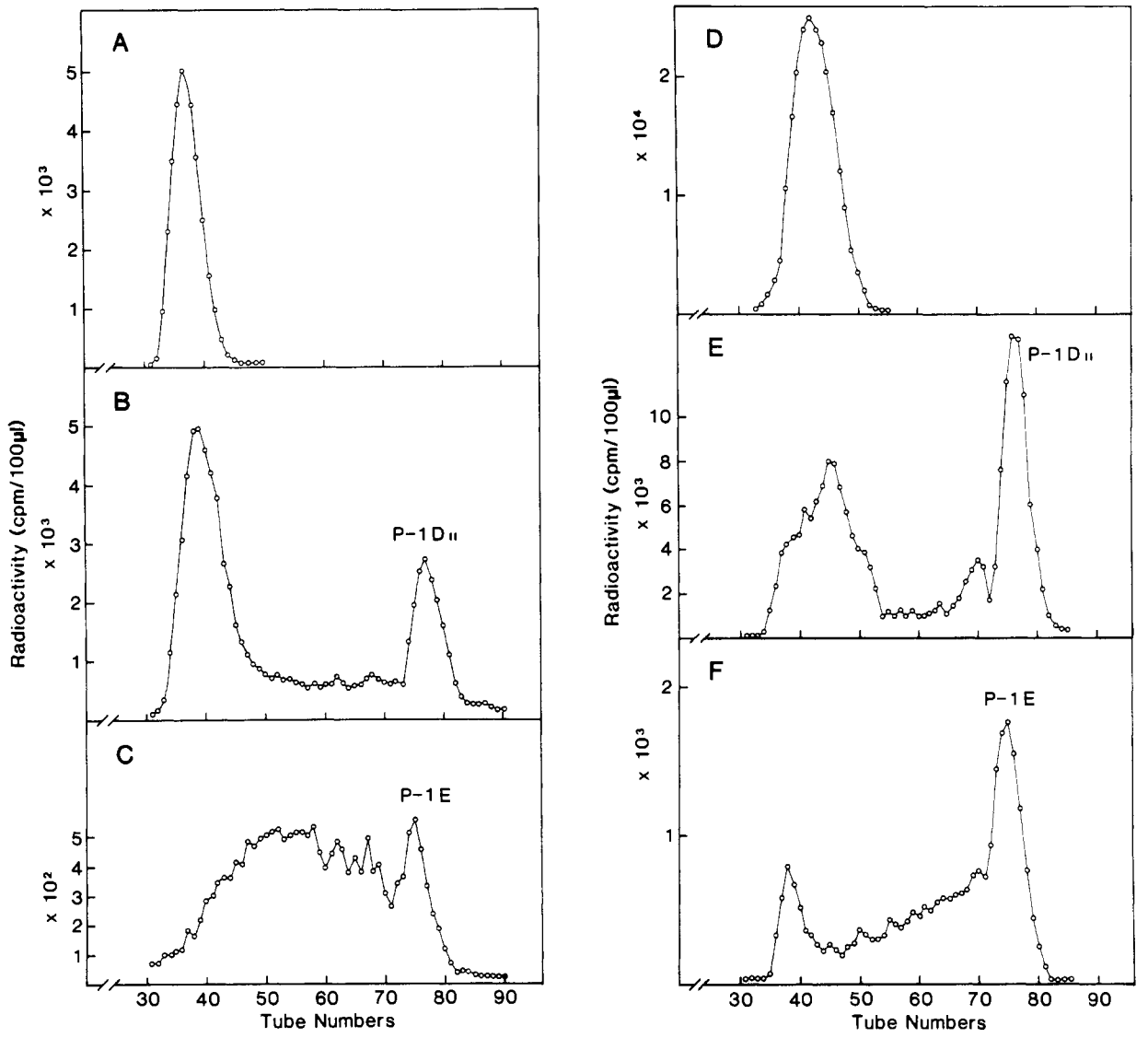


FIGURE 3: Analysis of the digestion products of erythrocyte lactosaminoglycans by gel filtration. Band 3 glycopeptides prepared from cord and adult erythrocytes were labeled by galactose oxidase/NaB^3H_4 and were hydrolyzed by endo-β-galactosidases. Sephadex G-50 (superfine) column (1 × 94 cm) equilibrated with 0.2 M NaCl was used for gel filtration analysis of digests. Samples were eluted with the same solution, and fractions of 0.75 mL were collected. Free galactose eluted at the tube 85 in this column chromatography. (A) [^{3}H]Lactosaminoglycans (4×10^5 cpm, 30 nmol as galactose) prepared from adult erythrocytes were dissolved in 200 μL of 0.1 M sodium citrate–phosphate buffer, pH 5.0, containing 60 mM of galactono(1→5)lactone. After incubation at 37 °C for 18 h without enzyme. (B) [^{3}H]Lactosaminoglycans from adult erythrocytes were treated in the same manner as described above, except 24 milliunits of endo-β-galactosidase D_{II} were added into the reaction mixture. (C) Residual glycopeptides (tube 34–43 in panel B) were dissolved in 200 μL of 0.1 M sodium acetate buffer, pH 5.8, and incubated with 2.5 milliunits of *E. freundii* endo-β-galactosidase at 37 °C for 18 h. (D) [^{3}H]Lactosaminoglycans (2×10^5 cpm, 10 nmol as galactose) prepared from cord erythrocytes were treated in the same manner as described in the legend for panel A. (E) [^{3}H]Lactosaminoglycans from cord erythrocytes were treated in the same manner as described in the legend for panel A, except 24 milliunits of endo-β-galactosidase D_{II} were added into the reaction mixture. (F) Residual glycopeptides (tubes 35–52 in panel E) were treated with *E. freundii* endo-β-galactosidase in the same manner as described in the legend for panel C.

on a paper chromatogram corresponding to GlcNAcβ1→3Galol, Galβ1→4GlcNAcβ1→3Galol, and NeuAcα2→3Galβ1→4GlcNAcβ1→3Galol (Figure 4A). The corresponding oligosaccharide fraction obtained after *E. freundii* enzyme digestion showed that an additional component migrated at the same position as Fucα1→2Galβ1→4GlcNAcβ1→3Galol (b in Figure 4B). These results indicate that endo-β-galactosidase D_{II} hydrolyzed the side chains with the ±NeuAc→Gal→GlcNAc→Gal terminal sequence and released the terminal oligosaccharides and internal disaccharides, but not ones with the Fuc→Gal→GlcNAc→Gal terminal sequence.

Linear lactosaminoglycans from cord erythrocytes were digested with endo-β-galactosidase D_{II} and products were analyzed in the same manner as branched lactosaminoglycans (Figure 3D–F). They also gave small oligosaccharides and residual glycopeptides. The residual glycopeptide was only degraded by *E. freundii* enzyme to give fucosyltetrasaccharide and a lesser amount of branched oligosaccharides.

Taking into consideration the significant amount of residual glycopeptides, the action of endo-β-galactosidase D_{II} on lactosaminoglycans might be restricted to the terminal area. Methylation analysis of the residual glycopeptides did not show a significant difference from that of intact glycopeptides, which also suggests that this enzyme only hydrolyzed the peripheral part of the lactosaminoglycans. In addition to the above results, it was found that *Pseudomonas* sp. keratanase did not hydrolyze these lactosaminoglycans at all.

Keratan Sulfate Is Not Susceptible to Endo-β-galactosidase D_{II}. Since keratan sulfates have been used as substrates for all the other endo-β-galactosidases, the hydrolyzability of kertan sulfate by endo-β-galactosidases D_{II} was examined as

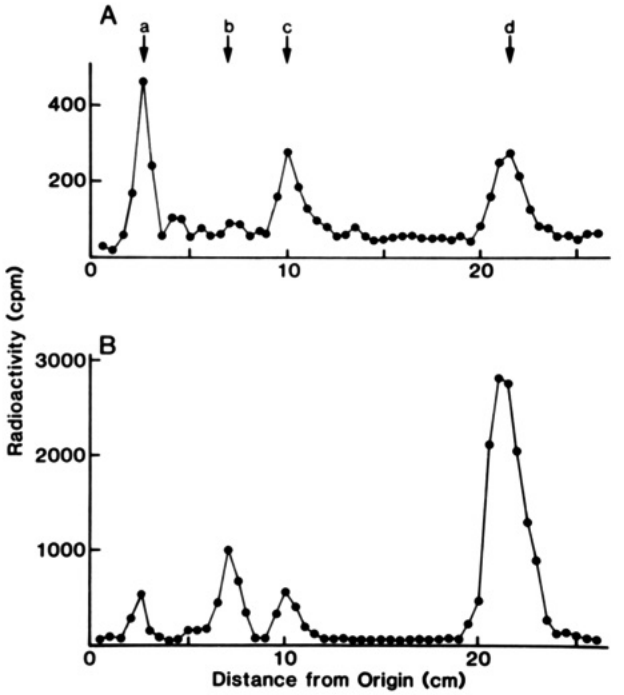


FIGURE 4: Paper chromatogram of oligosaccharide released from erythrocyte lactosaminoglycans by endo-β-galactosidases. Lactosaminoglycans from cord erythrocytes (100 μg) were dissolved in 0.1 M sodium citrate–phosphate buffer, pH 5.0, containing 60 mM of galactono(1→5)lactone and were incubated with 25 milliunits of endo-β-galactosidase D_{II} or 2.5 milliunits of *E. freundii* endo-β-galactosidase at 37 °C for 24 h. Oligosaccharides, which were eluted between tubes 71 and 85 from Sephadex G-50 column as shown in Figure 3, were reduced with NaB^3H_4, desalted with a Sephadex G-10 column and analyzed by paper chromatography in a solvent system of ethyl acetate/pyridine/water (5:4:3). The chromatogram was cut every 0.5 cm, and radioactivity was measured. The arrows show the migration positions of standard oligosaccharides: (a) NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galol; (b) Fucα1→2Galβ1→4GlcNAcβ1→3Galol; (c) Galβ1→4GlcNAcβ1→3Galol; (d) GlcNAcβ1→3Galol. The migration positions of galactose and lactose were 25 and 13 cm, respectively. (A) Oligosaccharide alcohols obtained by endo-β-galactosidase D_{II} digestion; (B) oligosaccharide alcohols obtained by *E. freundii* endo-β-galactosidase digestion. Note the difference in oligosaccharide at position b.

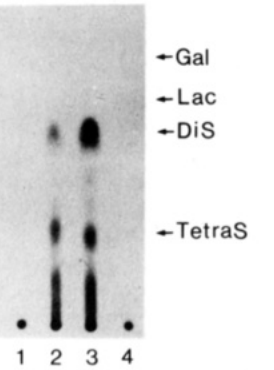


FIGURE 5: TLC pattern of the digests of keratan sulfate: (lane 1) keratan sulfate after incubation without enzyme; (lane 2) digests by *E. freundii* endo-β-galactosidase; (lane 3) digests by *Pseudomonas* keratanase; (lane 4) digests by endo-β-galactosidase D_{II}. Gal, galactose; Lac, lactose; DiS, GlcNAc-6-*O*-sulfate-β1→3Gal; TetraS, GlcNAc-6-*O*-sulfate-β1→3Galβ1→4GlcNAc-6-*O*-sulfate-β1→3Gal. TLC was developed in the solvent of 1-butanol/acetic acid/water (3:3:2 v/v), and sugars were visualized by an orcinol–H_2SO_4 reagent.

follows: (1) Keratan sulfate (KS-I, from bovine cornea) was incubated with endo-β-galactosidases D_{II}, and the digests were analyzed by TLC (Figure 5). No oligosaccharide was detected in the endo-β-galactosidase D_{II} digest. In a parallel experiment, endo-β-galactosidases from *E. freundii* and from *Pseudomonas* sp. hydrolyzed keratan sulfate to oligosaccharides. (2) The maximum increase of reducing power by endo-β-galactosidase D_{II} was 0.2% of that obtained by the *E. freundii* enzyme. (3) The digest of keratan sulfate with endo-β-galactosidase D_{II} was analyzed by gel filtration of the Sephadex G-50 column. Sugars were recovered at the void volume, and no fragment was detected. In a parallel experiment with the *E. freundii* enzyme and *Pseudomonas* keratanase, keratan sulfates were completely depolymerized to oligosaccharides, and sugars were eluted in the included volume.

These results indicate that endo-β-galactosidase D_{II} cannot degrade keratan sulfate. Crude enzyme preparation, steps 1 and 2 of the enzyme preparation, did not hydrolyze keratan sulfate either. Therefore, *D. pneumoniae* apparently lacks enzyme systems that hydrolyze keratan sulfate endoglycosidically.

DISCUSSION

This paper describes the presence of a new endo-β-galactosidase produced by *D. pneumoniae*. The enzyme termed as endo-β-galactosidase D_{II} is specific to the internal β-galactosidic bond of the R→GlcNAc(or GalNAc)β1→3Galβ1→4GlcNAc(or Glc) structure. This specificity is the same as that of *E. freundii* endo-β-galactosidase (Fukuda & Matsumura, 1976; Fukuda et al., 1978b; Fukuda, 1981; Kannagi et al., 1982; Fukuda & Fukuda, 1984). The differences between these two endo-β-galactosidases were seen in the profiles of released oligosaccharides from the polylactosaminyl chain, which has more than two susceptible sites. Endo-β-galactosidase D_{II} hydrolyzed these substrates to release mostly the small oligosaccharides but produced very small amounts of large oligosaccharides (Figures 2 and 3). In contrast, *E. freundii* endo-β-galactosidase released large oligosaccharides as well, particularly in the early reaction period [Figure 2 and 3; also see Fukuda & Matsumura (1976)]. Thus, two distinct action modes of these endo-β-galactosidases are suggested. Endo-β-galactosidase D_{II} preferentially splits off the oligosaccharides from the nonreducing terminal, while *E. freundii* endo-β-galactosidase depolymerizes the carbohydrate chain in a random manner.

The "nonrandom" manner of endo-β-galactosidase D_{II} may result in the limited hydrolysis of the substrates. The branched carbohydrates, such as lactoisooctaosylceramide or lactoisodecaosylceramide, were not hydrolyzed or hydrolyzed only linear parts of the nonreducing terminal area (Figure 1C).

Lactosaminoglycan peptides were also partially hydrolyzed by endo-β-galactosidase D_{II} to small oligosaccharides and residual large molecular weight glycopeptides. The possibility that such a limited hydrolysis of substrates is due to the low concentration of enzymic activity is not likely because that depolymerization of linear lactosaminoglycan by *E. freundii* enzyme can be detected under conditions of diluted enzyme concentration, such as 2.5 microunits/mL, and for various reaction periods between 2 and 20 min. These digests gave the similar profile shown in parts C or F in Figure 3. The pattern of digests that was obtained by endo-β-galactosidase D_{II} is, therefore, characteristic to the action mode and specificity of this enzyme.

As shown under Results, endo-β-galactosidase D_{II} did not hydrolyze the terminal sequence with the type 2 H determinant and subsequent linear structure. The slower hydrolyzability of the H structure has also been noticed in *E. freundii* endo-β-galactosidase (Nakagawa et al., 1980; Fukuda, 1981; Scudder et al., 1984). According to the study by ^{13}C NMR

(Rosevear et al., 1982), the most probable solution conformation of oligosaccharide Fucα1→2Galβ1→4GlcNAc is that C1, O5, C5, and C6 of fucose face C1′ and C2′ of galactose and C4″ and C5″ of *N*-acetylglucosamine. The Fucα1→2 substitution sterically covers the GlcNAc residue, which eventually blocks the recognition by the binding site on the endo-β-galactosidase. On the other hand, the substitution of sialic acid enhances the hydrolysis of susceptible structures, since both endo-β-galactosidases hydrolyze sialosylhexaosylceramide and sialosylparagloboside at about 10 times faster than their desialylated compounds (Fukuda et al., 1978b). This also suggests that the terminal sialylation effects the conformation of the subsequent Gal and GlcNAc residues so that endo-β-galactosidases can interact efficiently with the substrate.

Endo-β-galactosidase D_{II} did not hydrolyze the β-galactosidic bond at the branched point of the R→GlcNAcβ1→6-(R′→GlcNAcβ1→3)Gal structure (Figure 1C). Similarly, *E. freundii* endo-β-galactosidase did not hydrolyze or poorly hydrolyzed the branched point of glycolipids (Fukuda & Hakomori, 1982; Scudder et al., 1984; Fukuda et al., 1978b). However, in the *E. freundii* endo-β-galactosidase digestion products of branched lactosaminoglycans, a significant amount of oligosaccharides having the structure Galβ1→4GlcNAcβ1→6(R→Galβ1→4GlcNAcβ1→3)Gal was detected (Fukuda et al., 1984b). Obviously, a branched point can also be hydrolyzed by *E. freundii* endo-β-galactosidase, if it is present at the nonreducing terminal regions of a long polylactosaminyl chain. Further study will be needed to clarify whether endo-β-galactosidase D_{II} can hydrolyze the β-galactosidic bond at the branched point of lactosaminoglycan. The presence of small amounts of oligosaccharides with larger molecular weights (Figure 3B,E) suggests such a possibility.

Another unique feature of endo-β-galactosidase D_{II} is that this enzyme does not hydrolyze keratan sulfate. It is possible that sulfation of the polylactosaminyl backbone hinders the access of this enzyme. Moreover, the stepwise hydrolysis manner of this enzyme may limit the hydrolysis of keratan sulfate. It is true that all endo-β-galactosidases, except endo-β-galactosidases D_I and D_{II}, are keratan sulfate degrading enzymes, and no clear distinction between keratanase and endo-β-galactosidase has been described as yet. However, as we mentioned in this paper, *Pseudomonas* keratanase did not hydrolyze any glycolipids listed in Table I nor erythrocyte lactosaminoglycans. The oligosaccharide lacto-*N*-teraose was not hydrolyzed by this enzyme (Nakazawa & Suzuki, 1974). *Pseudomonas* keratanase might require sulfated *N*-acetylglucosamine for its specificity. For this reason, it may not be appropriate to use *Pseudomonas* keratanase for the detection of lactosaminoglycans. On the other hand, this enzyme will be useful for analyzing the status of sulfation of keratan sulfates or sulfated lactosaminoglycans.

Summarizing these studies, endo-β-galactosidases can be placed into three groups: Group 1 is enzymes that hydrolyze or depolymerize keratan sulfates, lactosaminoglycans, glycolipids and oligosaccharides. Enzymes isolated from *E. freundii* (Kitamikado & Ueno, 1979; Fukuda, 1981), *F. keratolyticus* (Kitamikado et al., 1981), and *B. fragilis* (Scudder et al., 1983) are included in this group. Group 2 is enzymes that hydrolyze the sulfated poly(*N*-acetyllactosamines) or keratan sulfates but not lactosaminoglycans or glycolipids. This type of enzyme is exemplified by *Pseudomonas* keratanase. Group 3 is enzyme that hydrolyze lactosaminoglycans and glycolipids but not keratan sulfate. Endo-β-galactosidase D_{II} was found to belong to this third group. It will be interesting to differentially determine various types of lactosaminoglycans (sulfation, fucosylation) by using endo-β-galactosidases with different specificities.

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Covalent Modification of Both cAMP Binding Sites in cAMP-Dependent Protein Kinase I by 8-Azidoadenosine 3′,5′-Monophosphate†

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ABSTRACT: Reconstituted porcine cAMP-dependent protein kinase type I was labeled with 8-azidoadenosine 3′,5′-monophosphate (8-N_3cAMP) to study cyclic nucleotide binding and to identify amino acid residues that are either in or in close proximity to the cAMP binding sites. The photoaffinity analogue 8-N_3cAMP behaved as cAMP itself with respect to cyclic nucleotide binding. For both cAMP and 8-N_3cAMP, (1) 2 mol of nucleotide was bound per mole of type I regulatory subunit monomer (R^I), (2) the apparent K_d's observed were approximately 10–17 nM on the basis of either Millipore filtration assays, equilibrium dialysis, or ammonium sulfate precipitation, (3) Scatchard plots showed positive cooperativity, and (4) the Hill coefficients were approximately 1.5–1.6. After photolysis and addition of an excess of cAMP, approximately 1 mol of 8-N_3cAMP/mol of R^I monomer was covalently incorporated. Tryptic digestion of the labeled protein revealed that two unique tryptic peptides were modified. Proline-271 and tyrosine-371 were identified as the two residues that were covalently modified by 8-N_3cAMP in R^I. These results contrast with the type II regulatory subunit (R^{II}) where 8-N_3cAMP modified covalently a single tyrosine residue [Kerlavage, A. R., & Taylor, S. S. (1980) *J. Biol. Chem. 255*, 8483–8488]. R^I contains two adjacent regions of sequence homology in the COOH-terminal fragment that binds two molecules of cAMP. On the basis of the homology between the cAMP binding domain of the *Escherichia coli* catabolite gene activator protein (CAP) and the two cAMP-binding domains of R^I, a model has been proposed that is consistent with the covalent modifications described above. The model suggests that the modification of proline-271 is due to 8-N_3cAMP bound to cAMP binding domain A and modification of tyrosine-371 results from 8-N_3cAMP bound to cAMP binding domain B.

The major receptor protein for cAMP, and as such the primary mediator of cAMP action in eukaryotic cells, is cAMP-dependent protein kinase (EC 2.7.1.37). The native holoenzyme exists as an inactive tetramer consisting of two regulatory (R)[1] and two catalytic (C) subunits. On binding cAMP, the holoenzyme is dissociated into two active catalytic subunits carrying the ATP:protein phosphotransferase activity and a dimeric regulatory subunit that binds cAMP, according to the following scheme:

$$R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C$$

Several lines of evidence support the idea that dissociation involves the formation of a ternary complex involving R, C, and cAMP (Builder et al., 1980; Chau et al., 1980; Armstrong & Kaiser, 1978). Fluorescence data with etheno-cAMP further suggested that dissociation and thus activation do not occur until all four cAMP binding sites were occupied (Smith et al., 1981).

There are two major classes of cAMP-dependent protein kinase that are designated as type I and type II on the basis of elution from DEAE-cellulose (Corbin et al., 1975; Corbin & Keely, 1977). The two isozymes have virtually identical catalytic subunits but dissimilar regulatory subunits (Hofmann et al., 1975; Zoller et al., 1979). In both types of regulatory subunits, however, limited proteolysis has demonstrated a similar type of domain structure (Corbin et al., 1978; Potter & Taylor, 1979; Takio et al., 1982). The NH_2-terminal domain accounts for approximately 25% of the polypeptide chain and appears to be involved primarily in maintaining the dimeric structure of the native protein. The COOH-terminal domain on the other hand retains the cAMP binding sites although it is monomeric. Initial studies indicated that the R subunit bound only one molecule of cAMP per monomer; however,

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[1] Abbreviations: 8-N_3cAMP, 8-azidoadenosine 3′,5′-monophosphate; R subunit, regulatory subunit of the cAMP-dependent protein kinase; C subunit, catalytic subunit of the cAMP-dependent protein kinase; CAP, *Escherichia coli* catabolite gene activator protein; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; K_d, apparent equilibrium dissociation constant; BSA, bovine serum albumin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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