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RAPID PROCEDURES FOR DETERMINATION OF ENDO-*N*-ACETYL-α-D-GALACTOSAMINIDASE IN *Clostridium perfringens*, AND OF THE SUBSTRATE SPECIFICITY OF EXO-β-D-GALACTOSIDASES*†

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ABSTRACT

Culture fluid of *Clostridium perfringens* hydrolyzed the synthetic, chromogenic substrates β-Gal-(1→3)-α-GalNAc-1→OPh and β-Gal-(1→3)-α-GalNAc-1→$OC_6H_4NO_2$-*o* or -*p* to β-Gal-(1→3)-GalNAc and the aglycon. Such assays facilitated the characterization and purification of this endo-*N*-acetyl-α-D-galactosaminidase activity. This activity was purified 1200-fold by fractionation with ammonium sulfate and chromatography on columns of Sephadex-G200, DEAE-Sephadex, and hydroxylapatite. The final preparation showed activity over a broad range of pH, with an optimum at 9.0, but less-pure material had two pH optima, 4.0 and 9.0. Another assay method, which employed the synthetic, chromogenic substrates β-Gal-(1→3)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*, β-Gal-(1→4)-βGlcNAc-1→$OC_6H_4NO_2$-*p*, and β-Gal-(1→6)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*, was developed for the rapid identification of the linkage specificity of exo-β-D-galactosidases from any source *via* a coupled reaction with *N*-acetyl-β-D-hexosaminidase.

INTRODUCTION

Glycosidases are important in the physiological catabolism of glycoproteins, and as tools for structural and functional analysis of glycoproteins[1,2]. Both exo- and endo-glycosidases have been described, and these enzymes can show specificity for the type and D or L configuration of the sugar hydrolyzed off, as well as for the anomeric form and positional linkage of the glycosidic bond. Ideally, specific assays that are quantitative and rapid are desirable for the characterization and purification of glycosidases. We now describe the use of synthetic, chromogenic substrates in such assays for the determination of endo-*N*-acetyl-α-D-galactosaminidase activity from *Clostridium perfringens*, and for the characterization of the specificity of positional linkage of exo-β-D-galactosidase in crude preparations from various sources.

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EXPERIMENTAL

Materials and methods. — The following glycosides were synthesized in our laboratory as described: β-Gal-(1→3)-α-GalNAc-1→$OC_6H_4NO_2$-*p* (ref. 3), β-Gal-(1→3)-β-GalNAc-1→$OC_6H_4NO_2$-*p* (ref. 3), β-Gal-(1→3)-α-GalNAc-1→$OC_6H_4NO_2$-*o* (ref. 4), β-Gal-(1→3)-α-GalNAc-1→OPh (ref. 4), β-Gal-(1→3)-β-GlcNAc-1→$OC_6H_4NO_2$-*p* (ref. 5), β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p* (ref. 4), β-Gal-(1→6)-β-GlcNAc-1→$OC_6H_4NO_2$-*p* (ref. 5), α-GalNAc-1→$OC_6H_4NO_2$-*o* (ref. 6), and β-Gal-(1→3)-GalNAc (ref. 7). All other glycosides, and almond emulsin, *Escherichia coli* exo-β-D-galactosidase, and *Aspergillus niger* exo-*N*-acetyl-β-D-hexosaminidase, were obtained from Sigma Chemical Co. Asialofetuin and asialoagalactofetuin were prepared according to Spiro[8]. Crude preparations of exo-β-D-galactosidase from *Aspergillus niger* and *Diplococcus pneumoniae*, which also contained exo-*N*-acetyl-β-D-hexosaminidase, were prepared by fractionation with ammonium sulfate[9,10].

Routine assay-mixtures for exo-β-D-galactosidase, exo-*N*-acetyl-β-D-glucosaminidase, and exo-*N*-acetyl-α-D-galactosaminidase contained 0.05M cacodylate–HCl buffer, pH 6.0, and 8mM *p*-nitrophenyl β-D-galactoside, 3mM *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside, or 3mM *o*-nitrophenyl 2-acetamido-2-deoxy-α-D-galactoside, respectively. Assay mixtures for endo-*N*-acetyl-α-D-galactosaminidase contained either 0.05M acetate buffer, pH 4.0, or 0.05M Tris acetate buffer, pH 9.0, and 2mM β-Gal-(1→3)-α-GalNAc-1→OPh, 2mM β-Gal-(1→3)-α-GalNAc-1→$OC_6H_4NO_2$-*o*, or 2mM β-Gal-(1→3)-α-GalNAc-1→$OC_6H_4NO_2$-*p*. Mixtures containing enzyme, along with controls lacking enzyme or substrate, were incubated in a total volume of 50 μL for various times at 37°.

For nitrophenyl substrates, reactions were terminated by addition of 1 mL of 0.2M Na_2CO_3, and either A_{420} or A_{400} was measured for *o*- or *p*-nitrophenyl substrates, respectively. For phenyl substrate, reactions were terminated by addition of 0.1 mL of M Folin reagent, followed by 1 mL of 0.4M Na_2CO_3. After 30 min at room temperature, A_{625} was determined. All reactions were linear with respect to time and to protein concentration. An enzyme unit is μmol of nitrophenol released per h. Reactions of assay samples analyzed by paper chromatography were terminated by addition of 100 μL of ice-cold, absolute ethanol, and centrifuged to give a pellet of precipitated protein. Supernatant liquors that contained the products of low molecular weight and appropriate reference compounds were chromatographed on Whatman No. 1 paper using 3:2:1 butyl acetate–glacial acetic acid–water for 16 h. Compounds were detected with silver nitrate reagent[11].

Purification of endo-*N*-acetyl-α-D-galactosaminidase is summarized in Table I. *Clostridium perfringens* NTCC3626 was cultured, and 3 L of culture fluid (step 1) was concentrated to 45 mL by fractionation with ammonium sulfate[12]. The ammonium sulfate fraction (step 2), in 0.01M Tris HCl, pH 8.0, containing 0.1M NaCl and 0.1mM 1,4-dithiothreitol, was applied in an ascending manner to a column (5 × 85 cm) of Sephadex G-200 and eluted with the same buffer. Endo-*N*-acetyl-α-D-

TABLE I

PURIFICATION OF ENDO-*N*-ACETYL-α-D-GALACTOSAMINIDASE ACTIVITIES FROM *C. perfringens*

Step	*Total protein (mg)*	*Endo-N-acetyl-α-D-galactosaminidase*			
		Total units		*Specific activity (units/mg of protein)*	
		pH 4.0	*pH 9.0*	*pH 4.0*	*pH 9.0*
1 Culture fluid	600	300	240	0.5	0.4
2 $(NH_4)_2SO_4$, 0–80% saturation	487	294	197	0.6	0.4
3 Sephadex-G200	58	218	198	3.8	3.4
4 DEAE-Sephadex A-50	4	80	88	20.0	22.0
5 Hydroxylapatite	0.04	13	19	325.0	475.0

galactosaminidase, assayed at pH 4.0 and 9.0, and the major activities of exo-β-D-galactosidase, exo-*N*-acetyl-β-D-glucosaminidase, and exo-*N*-acetyl-α-D-galactosaminidase were all co-eluted in the void volume. Fractions (380 mL) containing these activities were concentrated to 64 mL with an Amicon ultrafiltration cell using a PM-10 filter, and were designated step 3.

Step 3 material was applied to a column (1.5 × 15 cm) of DEAE-Sephadex A-50, extensively washed with the loading buffer, and eluted with a linear gradient (300 mL) of 0.1M–0.6M NaCl in 0.01M Tris HCl, pH 8.0, containing 0.1M 1,4-dithiothreitol. Endoglycosidase activity, assayed at pH 4.0 and 9.0, and the major exoglycosidase activities already mentioned co-eluted at 0.38M NaCl. Fractions (90 mL) containing these activities were concentrated by ultrafiltration to 14 mL, and were designated step 4.

Step 4 material was dialyzed against 0.05M phosphate buffer, pH 7.5, containing 0.1M 1,4-dithiothreitol, applied to a column (1.5 × 5 cm) of hydroxylapatite, and extensively washed with the loading buffer. The column was eluted stepwise with 100-mL portions of 0.10, 0.15, 0.20, and 0.25M phosphate buffer, pH 7.5, containing 0.1mM 1,4-dithiothreitol. Endoglycosidase activity, assayed at pH 4.0 and 9.0, and the major activities of the exoglycosidases, co-eluted at 0.15M phosphate. Fractions (34 mL) containing these activities were concentrated to 4 mL by ultrafiltration, and were designated step 5. The protein content of each step was determined by the method of Lowry *et al.*[13]. Specific activities of the step-5 material, assayed at pH 6.0 for exo-β-D-galactosidase, exo-*N*-acetyl-β-D-glucosaminidase, and exo-*N*-acetyl-α-D-galactosaminidase, were 5300, 4050, and 12 units per mg of protein, respectively. When assayed at pH 4.0 or 9.0, step-5 preparations of exo-β-D-galactosidase and exo-*N*-acetyl-β-D-glucosaminidase exhibited 10–20% of the activity assayed at pH 6.0, whereas exo-*N*-acetyl-α-D-galactosaminidase had no activity.

RESULTS

Preliminary experiments on the culture fluid of *Clostridium perfringens*, purified by fractionation with ammonium sulfate, showed that enzyme activity hydrolyzing

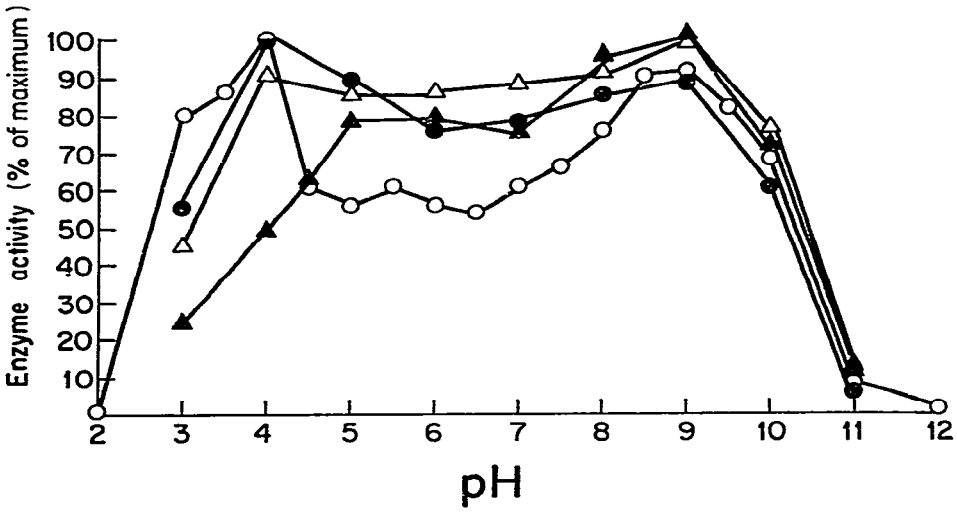

Fig. 1. Endo-*N*-acetyl-α-D-galactosaminidase activity as a function of pH. [Standard assays were conducted by using β-Gal-(1→3)-α-GalNAc-1→$OC_6H_4NO_2$-*p*, various buffers, and step-2 (-●-), step-3 (-○-), step-4 (-△-), or step-5 (-▲-) enzyme preparations. Buffers used were: pH 2–3, phosphate; pH 3.5–5.0, acetate; pH 5.5–6.5, cacodylate–HCl; pH 7–8, phosphate; pH 8.5–10, Tris acetate; and pH 11–12, phosphate–NaOH.]

the chromogenic substrate β-Gal-(1→3)-α-GalNAc-(1→$OC_6H_4NO_2$-*p* had two pH optima, 4.0 and 9.0. These activities were subsequently purified 650- and 1200-fold, respectively (see Table I). Through each purification step, the activities at pH 4.0 and 9.0 were co-purified, but, after step 3, there was a shift from two pH optima to one optimum at pH 9.0, with retention of substantial activity at pH 4.0 (see Fig. 1). At all purification steps, the disaccharide β-Gal-(1→3)-GalNAc was the only product released from chromogenic substrates (see Fig. 2). Also, step-5 preparations assayed at pH 4.0 or 9.0 did not hydrolyze α-GalNAc-1→$OC_6H_4NO_2$-*o*. Therefore, it is considered that identification of this activity as endo-*N*-acetyl-α-D-galactosaminidase is justified. This activity, assayed at pH 4.0, also released the disaccharide β-Gal-(1→3)-GalNAc from asialofetuin, but not from fetuin from which the glycosidically linked oligosaccharide chains that contain this disaccharide had previously been removed by β-elimination (results not given).

The apparent K_m values for phenyl and *o*- and *p*-nitrophenyl substrates of endo-*N*-acetyl-α-D-galactosaminidase, assayed at pH 4.0 with the step-5 preparation, were 1.1, 1.0, and 0.5mM, respectively, and at pH 9.0, 1.7, 0.9, and 0.5mM, respectively. Quantitatively identical results were obtained with the step-3 preparation. The relative V_{max} values of the step-3 preparation, assayed at pH 4.0 and 9.0, were approximately equal, but the relative V_{max} value of the step-5 preparation, assayed at pH 4.0, was 50–60% of the V_{max} value found at pH 9.0. Thus, the shift in pH profile already described was due to a change in the relative V_{max} value of the enzyme activity assayed at pH 4.0 and 9.0, and not to an alteration of the K_m value.

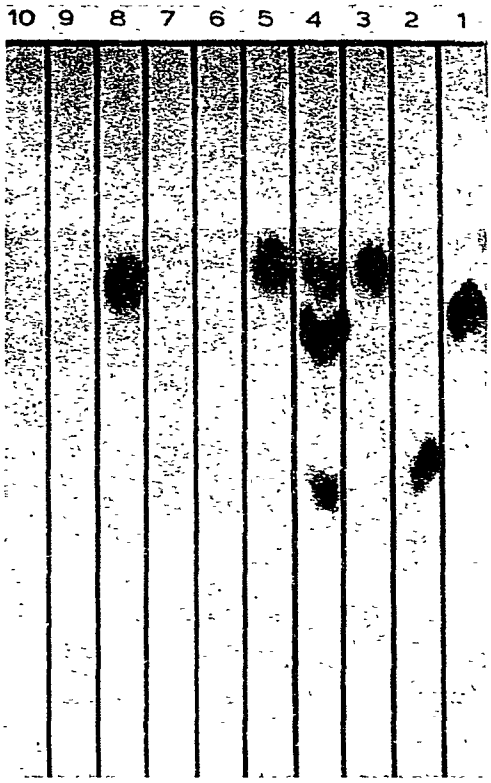

Fig. 2. Paper chromatography of enzymic hydrolyzate of β-Gal-(1→3)-α-GalNAc-1→OPh. [Chromatographic conditions are given in Materials and methods. Right to left: lanes 1, 2, and 3 contained, as reference compounds, D-galactose, 2-acetamido-2-deoxy-D-galactose, and β-Gal-(1→3)-GalNAc, respectively; lane 4 contained all three reference compounds; lanes 5 and 8 identify the carbohydrate product of the hydrolysis of substrate by the step-5 preparation of endo-*N*-acetyl-α-D-galactosaminidase assayed at pH 4.0 and 9.0, respectively; lanes 6 and 7, and 9 and 10, show controls of pH 4.0 and 9.0 assays from which substrate or enzyme were omitted. Identical results were obtained if step-1–4 enzymes were used, or if *p*- or *o*-nitrophenyl substrates were used.]

The β anomer of the *p*-nitrophenyl substrate at 6mM neither inhibited endo-*N*-acetyl-α-D-galactosaminidase activity at pH 4.0 or 9.0, nor was hydrolyzed, and thus was not a substrate. However, this compound inhibited this activity from culture fluids of *Diplococcus pneumoniae*[14].

The substrate specificity of exo-β-D-galactosidase from various sources was determined by using chromogenic substrates that are positional isomers (see Table II). The nitrophenyl group of these substrates was released through the sequential action of exo-β-D-galactosidase and exo-β-D-hexosaminidase. For example, neither highly purified *Escherichia coli* exo-β-D-galactosidase, which had no exo-*N*-acetyl-β-D-hexosamidase activity, nor *Aspergillus niger* exo-*N*-acetyl-β-D-hexosamidase, which had no exo-β-D-galactosidase activity, liberated any *p*-nitrophenol from the substrates listed in Table II (data not given). However, together they did (see Table II). Thus,

TABLE II

SUBSTRATE SPECIFICITY OF EXO-β-D-GALACTOSIDASES[a]

Exo-β-D-galactosidase source	*β-Gal-(1→3)-β-GlcNAc-1→OC$_6$H$_4$NO$_2$-p*	*β-Gal-(1→3)-β-GalNAc-1→OC$_6$H$_4$NO$_2$-p*	*β-Gal-(1→4)-β-GlcNAc-1→OC$_6$H$_4$NO$_2$-p*	*β-Gal-(1→6)-β-GlcNAc-1→OC$_6$H$_4$NO$_2$-p*
	p-*Nitrophenol released/h (nmol)*			
C. perfringens (step-2 enzyme)	0	0	83	9
C. perfringens (step-5 enzyme)	0	0	82	0
D. pneumoniae	0	0	138	5
E. coli	0.2	0.1	72	159
A. niger	0.2	0.1	111	73
Almond emulsin (assayed at pH 6.0)	0.3	0.1	10	26
Almond emulsin (assayed at pH 4.5)	0.6	0.2	39	24

[a]Assay mixtures contained the appropriate substrate (1.2 mM), 1 unit of purified, *A. niger* exo-β-*N*-acetylhexosaminidase (which was devoid of exo-β-D-galactosidase activity), and 0.05M cacodylate–HCl, pH 6.0, 0.05M cacodylate–HCl, pH 7.2 (*E. coli* enzyme), or 0.05M acetate, pH 4.5 (almond emulsin). Mixtures containing enzyme, along with controls without enzyme or substrate, were incubated (volume 50 μL) for various times to ensure a linear rate, and were processed as described in Materials and methods for routine assays. Addition of purified *A niger* exo-*N*-acetyl-β-hexosaminidase was not needed for assaying crude preparations from *C. perfringens*, *D. pneumoniae*, *A. niger*, and almond emulsin, because these sources contain considerable exo-*N*-acetyl-β-hexosaminidase activity. In all assays, exo-*N*-acetyl-β-hexosaminidase activity was present in sufficient excess that it was not rate-limiting. The identification of galactose and either 2-acetamido-2-deoxyglucose or 2-acetamido-2-deoxygalactose as the only products of positive reactions, and the absence of these compounds from negative reactions, was confirmed by subjecting parallel assays to paper chromatography as described in Materials and methods.

the order of the rate of hydrolysis of various linkages by *E. coli* exo-β-D-galactosidase is (1→6) > (1→4) > (1→3), which agrees with results obtained by another method[15].

Almond emulsin was assayed at pH 4.5 and 6.0, because this source had been shown to contain two exo-β-D-galactosidases, having pH optima at 4.5 and 6.0, respectively[16]. Interestingly, almond emulsin showed different substrate-specificities at these pH values, indicating that the pH-4.5 enzyme preferentially hydrolyzes (1→4)-linkages, and the pH-6.0 enzyme, (1→6)-linkages (see Table II). Using a different method, it had also been found that an exo-β-D-galactosidase in almond emulsin hydrolyzes (1→4)-linkages faster than (1→3)-linkages[17]. Highly purified exo-β-D-galactosidases from *Diplococcus pneumoniae* and *Clostridium perfringens* digest only (1→4)-linkages[10] (see Table II). However, crude preparations from these sources also digest (1→6)-linkages slowly, suggesting that the (1→6) activity is separated from the (1→4) activity by purification procedures.

DISCUSSION

Using synthetic chromogenic substrates, we have identified, and partially purified, an endo-*N*-acetyl-α-D-galactosaminidase activity from the culture fluid of *C. perfringens*. It had been reported that an enzyme activity of a different strain of *C. perfringens* liberates the disaccharide β-Gal-(1→3)-GalNAc from various glycoproteins[18]; however, problems were encountered in the accurate quantitation of this activity. Our synthetic substrates avert these difficulties, and also facilitate the screening of large numbers of samples, such as fractions from column chromatography. The endo-*N*-acetyl-α-D-galactosaminidase had activity over a broad range of pH, making this enzyme useful for studies on the structure and function of glycoproteins under a wide variety of chemical and physiological conditions. By using conventional, chromatographic techniques, we were unable to separate, completely, endo-*N*-acetyl-α-D-galactosaminidase activity from exoglycosidases also present in the culture fluid. Nonetheless, a substantial degree of purification of the endoglycosidase was achieved. The major problems in separating the endoglycosidase from exoglycosidases are that (*a*) exo-β-D-galactosidase and exo-*N*-acetyl-β-D-glucosaminidase are present in 20-fold excess over the endoglycosidase in culture fluids, and (*b*) these enzymes behave similarly on gel-filtration and ion-exchange columns.

We also found that chromogenic substrates, which comprise a series of positional isomers in the linkage of D-galactose to 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose, can be used to identify the substrate specificity of exo-β-D-galactosidases in both crude and purified preparations of enzyme. These compounds will facilitate the screening of new sources for specific exo-β-D-galactosidases. In addition, these compounds can be used for the detection and purification of exo-β-D-galactosidases, which do not use the widely employed aryl β-D-galactosides as substrates, but require a glycosidic bond between galactose and another sugar[19]. Furthermore, these compounds may be useful for identifying fluctuations of specific exo-β-D-galactosidases in the genetic diseases of exo-β-D-galactosidase deficiency or in neoplasias.

REFERENCES

1 M. I. HOROWITZ AND W. PIGMAN, *The Glycoconjugates*, Vol. I, Academic Press, New York, 1977.
2 M. I. HOROWITZ AND W. PIGMAN, *The Glycoconjugates*, Vol. II, Academic Press, New York, 1978.
3 K. L. MATTA AND J. J. BARLOW, *Carbohydr. Res.*, 48 (1976) 51–56.
4 K. L. MATTA, in preparation.
5 K. L. MATTA AND J. J. BARLOW, *Carbohydr. Res.*, 43 (1975) 299–304.
6 B. WEISMAN, *J. Org. Chem.*, 35 (1970) 1690–1691.
7 H. M. FLOWERS AND D. SHAPIRO, *J. Org. Chem.*, 30 (1965) 2041–2043.
8 R. G. SPIRO, *J. Biol. Chem.*, 239 (1964) 567–573.
9 O. P. BAHL AND K. M. L. AGRAWAL, *J. Biol. Chem.*, 244 (1969) 2970–2978.
10 L. R. GLASGOW, J. C. PAULSON, AND R. L. HILL, *J. Biol. Chem.*, 252 (1977) 8615–8623.
11 L. HOUGH AND J. K. N. JONES, *Methods Carbohydr. Chem.*, 1 (1962) 21–31.
12 E. J. M. MCGUIRE, S. CHIPOWSKY, AND S. ROSEMAN, *Methods Enzymol.*, 28 (1972) 755–763.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.

14 J. UMEMOTO, K. L. MATTA, J. J. BARLOW, AND V. P. BHAVANANDON, *Anal. Biochem.*, 91 (1978) 186–193.
15 K. WALLENFELS, J. LEHMANN, AND O. P. MALHOTRA, *Biochem. Z.*, 333 (1960) 209–225.
16 Y. C. LEE, *Methods Enzymol.*, 28 (1972) 699–702.
17 M. ARAKAWA, S. OGATA, T. MURAMATSU, AND A. KOBATA, *J. Biochem. (Tokyo)*, 75 (1974) 707–714.
18 C. C. HUANG AND D. AMINOFF, *J. Biol. Chem.*, 247 (1972) 6737–6742.
19 T. LANGLEY, *Arch. Biochem. Biophys.*, 128 (1968) 304–311.