A COMPARISON OF THE SUBSTRATE SPECIFICITIES OF ENDO-β-N-ACETYLGLUCOSAMINIDASES FROM STREPTOMYCES GRISEUS AND DIPLOCOCCUS PNEUMONIAE

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Received September 16,1975

SUMMARY

The substrate specificities of the endo-β-N-acetylglucosaminidases from Diplococcus pneumoniae and Streptomyces griseus were compared and found to differ considerably. The enzyme from D. pneumoniae released Asn-GlcNAc-Fuc-containing glycopeptides from exoglycosidase-treated acidic IgM glycopeptides but was limited in its capacity to hydrolyze ovalbumin glycopeptides larger than $Asn(GlcNAc)_2(Man)_5$. In contrast, the enzyme from S. griseus hydrolyzed this and larger neutral oligosaccharides but could not hydrolyze the above fucose-containing IgM glycopeptides. Removal of the fucose residue, however, converted the latter to an active substrate for the S. griseus enzyme, thus broadening its substrate range to encompass most of those substrates hydrolyzed by the D. pneumoniae endoglycosidase.

INTRODUCTION

In addition to the exoglycosidases, another valuable tool for examining the structure of glycoproteins and for clarifying the role of carbohydrates in glycoproteins has been provided with the discovery of the endoglycosidases. One type purified to homogeneity from Streptomyces griseus (1), called endo-β-N-acetylglucosaminidase H, hydrolyzes the di-N-acetylchitobiosyl moiety in the core region of neutral glycopeptides and glycoproteins to release the following intact oligosaccharides:

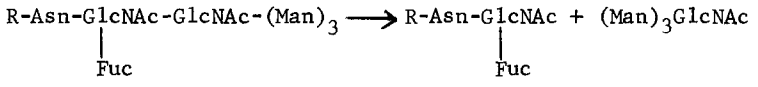
$$R\text{-}Asn\text{-}GlcNAc\text{-}GlcNAc\text{-}(Man)_X(GlcNAc)_Y \longrightarrow R\text{-}Asn\text{-}GlcNAc + (GlcNAc)_Y(Man)_XGlcNAc$$

R = intact protein or peptide chain

Through the use of this enzyme oligosaccharide chains ranging from 5 (ovalbumin) to 50 (invertase) mannosyl residues have been released, with no apparent effect on the structure or function of these proteins. Similar results were obtained with RNase B, DNase A, and the neutral oligosaccharides of thyroglobulin and IgM (2). In contrast, the "acidic" or complex oligosaccharides from

intact IgM, thyroglobulin, and α_1-acid glycoprotein, or their corresponding glycopeptides, were resistant to hydrolysis even after removal of the peripheral residues of sialic acid, galactose and N-acetylglucosamine.

However, another endoglycosidase from Diplococcus pneumoniae (3, 4) termed endo-β-N-acetylglucosaminidase D, could affect this release providing the peripheral sugar residues were removed first. This reaction proceeded as follows:



R = peptide chain

More recently a partial purified preparation of the D. pneumoniae enzyme was shown to release oligosaccharides of a more complex nature (5), but it is not possible to state at this time whether a single enzyme is involved.

Because of the importance of these enzymes and the lack of definitive data on their substrate specificities, a study of this nature was undertaken with substrates of known composition and structure.

MATERIALS AND METHODS

Pure endo-β-N-acetylglucosaminidase H (specific activity 20 units/mg protein) was isolated from cultural filtrates of Streptomyces griseus as described previously (1). Endo-β-N-acetylglucosaminidase D (specific activity 0.21 unit/mg protein) was partially purified from cultural filtrates of D. pneumoniae (6). The H and D enzymes were assayed at their respective pH optima of 5.0 and 6.5.

The asparagine-containing oligosaccharides, designated Asn-$(GlcNAc)_2$-$(Man)_5$, Asn-$(GlcNAc)_2(Man)_6$, and Asn-$(GlcNAc)_4(Man)_6$ were isolated from pronase-digested ovalbumin by the procedure of Huang et al. (7) and N-acetylated with [2-^{14}C]-acetic anhydride (Amersham-Searle, 29.7 mc/mmole). [^{3}H]-$(Man)_5$-GlcNAc-GlcNAc-ol was prepared by removing the asparagine moiety of Asn-$(GlcNAc)_2$-$(Man)_5$ with glycosyl asparaginase (8), and then reducing with sodium borotritide (New England Nuclear, 142 mc/mmole). Reducing sugars were liberated from the glycopeptides and oligosaccharides by hydrolysis at 100° for 2.5 hr with 2N HCl in evacuated, nitrogen-flushed tubes and quantitated on a Technicon carbohydrate analyzer (9). The glycopeptide designated $(aa)_X$-Asn-$(GlcNAc)_2(Man)_3$-$(Fuc)_1$ was kindly provided by Dr. Thomas H. Plummer, Jr., New York State Department of Health, and was prepared from a sialoglycopeptide fraction of human IgM by enzymic removal of peripheral sialic acid, galactose and N-acetylglucosamine residues. The molar ratios of the residual amino acids relative to glycosidically bound asparagine were: Asp, 0.62; Thr, 0.28; Ser, 0.20; and Glu, 0.36. Fucose was removed from this glycopeptide to the extent of 92% with a highly purified hen oviduct α-L-fucosidase (6). The [^{14}C]- and [^{3}H]-labeled substrates were purified by chromatography on a 0.9 x 200-cm column of Sephadex G-25 and their specific activities (cpm/μmole) were determined relative to asparagine or to hexose content (10).

The hydrolysis of the [^{14}C]- and [^{3}H]-labeled substrates by the endoglycosidases was determined as follows: Radioactive substrate (5 nmoles in 5 μl) was added to the tip of a 1.5-ml conical disposable microtube, followed by 5 μl of either 0.5 M sodium citrate, pH 5.0, or 0.5 M potassium phosphate, pH 6.5, and 5 μl of enzyme. The reaction vessels were capped and incubated at 37° for a specified time, at the end of which the contents of each were pipetted onto a 60-cm long strip of Whatman 3MM within a 0.5-cm disc. The [^{14}C]-labeled glycopeptides were electrophoresed in pyridine-acetic acid-water (3:1:387), pH 5.5, for 4 hr at 17 v/cm. The [^{3}H]-labeled oligosaccharides were chromatographed descendingly for 8 hr in ethyl acetate-pyridine-water (12:5:4). Radioactive areas were located on the paper strips with a Nuclear Chicago 4 π strip scanner. These regions were excised, eluted with water and the eluate counted in a Packard liquid scintillation counter.

RESULTS AND DISCUSSION

Since endoglycosidase hydrolysis of the di-N-acetylchitobiosyl moiety of the IgM glycopeptide $(aa)_X$-Asn$(GlcNAc)_2(Man)_3$ yields $(Man)_3GlcNAc$ (4, 6), the reduction of this tetrasaccharide to $(Man)_3GlcNAc$-ol by sodium borotritide, followed by gel filtration, can be used to measure enzyme activity. As indicated in Fig. 1A, little incorporation of tritium into the glycopeptide area (tubes 18-24) occurred in the absence of added enzyme. However, in the presence of either the Streptomyces (Fig. 1B) or the Diplococcus enzyme (Fig. 1C), $(aa)_X$-Asn$(GlcNAc)_2(Man)_3$ was almost completely hydrolyzed, as evidenced by a shift in the phenol-sulfuric acid-positive material in tubes 18-24 to a radioactive phenol-sulfuric acid-positive product in tubes 22-26 (see legend to Fig. 1). The tritiated product in Fig. 1B and 1C was determined by analysis to be [^{3}H]-$(Man)_3GlcNAc$-ol and co-migrated with a known sample of this tetrasaccharide in two solvent systems. On treatment with jack bean meal α-D-mannosidase, the tritiated tetrasaccharide was converted to [^{3}H]-βMan(1$\rightarrow$4)GlcNAc-ol.

One difference between the two enzymes was immediately apparent on comparing their activities towards $(aa)_X$-Asn$(GlcNAc)_2(Man)_3(Fuc)_1$: The Streptomyces enzyme hydrolyzed only the fucose-depleted glycopeptide, while the Diplococcus enzyme (data not shown) hydrolyzed both the fucose-containing and depleted glycopeptides at approximately the same rate (Fig. 2). It has been reported (11) that a fucose-depleted mouse myeloma IgG glycopeptide, with the same composition and sequence as the IgM glycopeptide studied here,

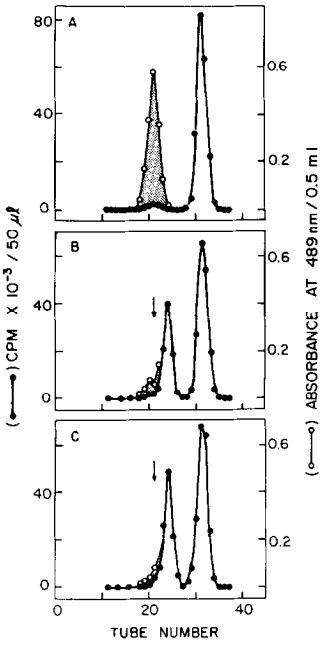


Figure 1. The hydrolysis of defucosylated $(aa)_X$-Asn$(GlcNAc)_2(Man)_3$ by endo-β-N-acetylglucosaminidases H and D. The fucose-depleted glycopeptide (0.62 µmole) was incubated at 37° for 24 hr under the following conditions: (A) 1.0 ml of 0.1 M sodium citrate, pH 5.0; (B) 1.0 ml of 0.1 M sodium citrate, pH 5.0, containing two units of endo-β-N-acetylglucosaminidase H (100 µg); (C) 1.0 ml of 0.1 M potassium phosphate, pH 6.5, containing 0.2 unit of endo-β-N-acetylglucosaminidase D (1 mg total protein). The reactions were adjusted to pH 9 with several drops of 0.7 M sodium borate, cooled to 0°, and reduced with about 1 mg of sodium borotritide for 24 hr. The pH was then lowered to 4 with glacial acetic acid and each sample was passed through a column of Sephadex G-25 (0.9 x 200 cm) with 0.1 M acetic acid as the elutant. Fractions of 3 ml were collected at a flow rate of 9.6 ml/hr; the indicated aliquots were analyzed for both radioactivity (●—●) and hexose content (O—O). To conserve material, the latter was determined only on the front part of the region just preceding the radioactive peak, but on pooling tubes 22-26 all of the carbohydrate was recovered. The darkened area represents the elution volume of the glycopeptide prior to treatment; the (↓) shows where the compound would elute if unaffected by the enzyme.

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was resistant to hydrolysis by the endo-β-N-acetylglucosaminidase H. The apparent difference between these results and ours is not clear, but it should be noted that the α-fucosidase treated IgG glycopeptide was neither isolated nor shown to be fucose-depleted prior to treatment with endoglycosidase H (11). In contrast, the results in Fig. 2 indicate that the Streptomyces enzyme can hydrolyze core glycopeptides of the above indicated

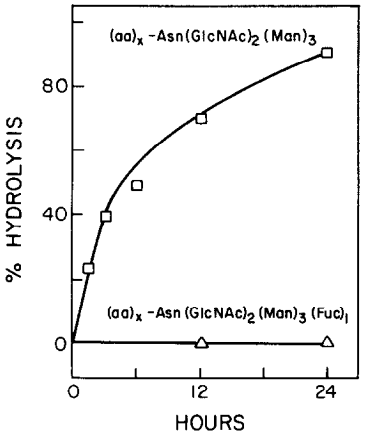

Figure 2. The rate of hydrolysis of $(aa)_X$-Asn$(GlcNAc)_2(Man)_3(Fuc)_1$ and its defucosylated derivative by endo-β-N-acetylglucosaminidase H. The [^{14}C]-acetyl glycopeptides were incubated with 5.0 μg of pure endo-β-N-acetylglucosaminidase H and assayed as described in Materials and Methods.

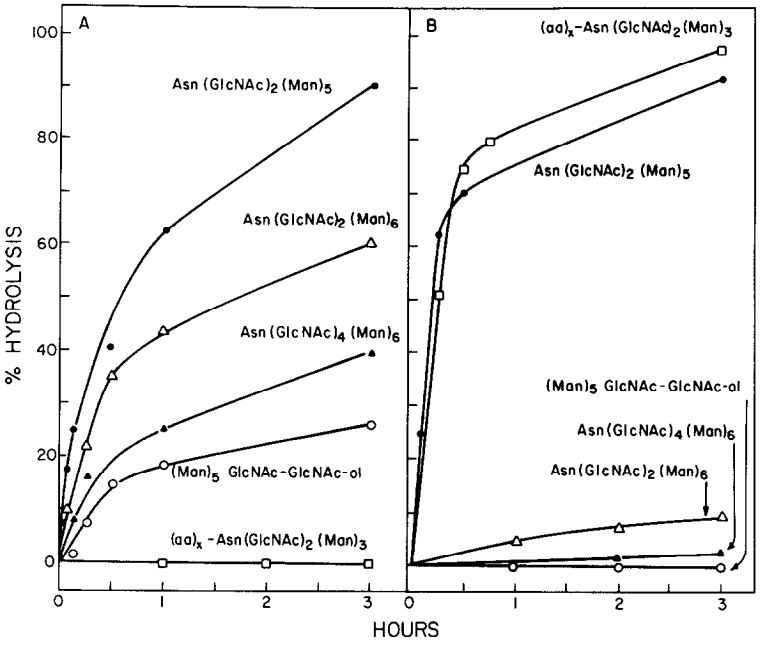

Figure 3. A comparison of the rates of hydrolysis of various oligosaccharide derivatives by endo-β-N-acetylglucosaminidases H and D. The designated substrates were incubated for the indicated times with (A) 2.5 ng of pure endo-β-N-acetylglucosaminidase H or (B) 0.5 μg of protein containing endo-β-N-acetylglucosaminidase D. The assays were conducted as described in Materials and Methods.

composition providing fucose is first removed.

The enzymes also differed in their rates of hydrolysis of substrates of varying oligosaccharide chain length. We demonstrated previously (1) that the asparagine-oligosaccharides, $Asn(GlcNAc)_2(Man)_1$ and $Asn(GlcNAc)_2(Man)_2$, were not hydrolyzed by the Streptomyces enzyme but that $Asn(GlcNAc)_2(Man)_6$ was an effective substrate. Somewhat similar results were also obtained by Arakawa and Muramatsu (11). The minimum requirement for hydrolysis, as indicated in Fig. 2, appears to be the presence of at least three mannosyl residues in the oligosaccharide chain, and while relatively large amounts of enzyme (5 μg) are required, complete hydrolysis can be achieved in about 24 hr. However, with ng quantities of enzyme and $(aa)_X$-$Asn(GlcNAc)_2(Man)_3$ as substrate, little or no hydrolysis could be detected, although $Asn(GlcNAc)_2(Man)_5$ was hydrolyzed in 3 hr (Fig. 3A). The relatively low rate of reaction with $(aa)_X$-$Asn(GlcNAc)_2(Man)_3$ does not appear to be due to the presence of the peptide chain since ovalbumin glycopeptides were hydrolyzed at approximately the same rate as their corresponding glycosyl asparagine derivatives.

A further increase in the oligosaccharide chain length, as illustrated with $Asn(GlcNAc)_2(Man)_6$ and $Asn(GlcNAc)_4(Man)_6$, yielded progressively slower rates of hydrolysis although, as indicated earlier, mannosyl oligosaccharides containing as many as 54 residues can be hydrolyzed (2). The chemical state of the proximal N-acetylglucosamine residue also contributes to the substrate specificity for as shown earlier (1), $(Man)_5GlcNAc$-GlcNAc is hydrolyzed as rapidly as $Asn(GlcNAc)_2$-$(Man)_5$ but reduction of the former compound to $(Man)_5$-GlcNAc-GlcNAc-ol impairs the rate of hydrolysis (Fig. 3A). For comparative purposes the concentration of the enzyme used in Fig. 3A was the same for all of the substrates, but when the amount of enzyme was increased 4-fold, all of the substrates with the exception of $(aa)_X$-$Asn(GlcNAc)_2(Man)_3$ were hydrolyzed completely within 6 hr.

In sharp contrast to the endoglycosidase from Streptomyces, that from D. pneumoniae appears more restricted in its substrate specificity (Fig. 3B).

As indicated, maximal rates were obtained with $(aa)_X$-Asn$(GlcNAc)_2(Man)_3$ and Asn$(GlcNAc)_2(Man)_5$, but the activity decreased sharply with longer oligosaccharides. In addition, little or no hydrolysis was obtained with $(Man)_5$-GlcNAc-GlcNAc-ol. This association of chain length with activity probably explains why the neutral unit A thyroglobulin glycopeptide, which contains an average of 8 mannosyl residues, is hydrolyzed by the Streptomyces enzyme but not by the Diplococcus enzyme (11).

The above comparative analysis indicates that structural features common to many of the acidic glycopeptides, namely, a fucose-containing core associated with at least three mannosyl residues, are characteristics well suited to the specificity requirements of the Diplococcus endoglycosidase. On the other hand, the absence of fucose and the presence of relatively greater amounts of mannose, features common to most neutral glycopeptides, make these compounds effective substrates for the Streptomyces endoglycosidase. If fucose is present, its removal may convert an inert substance to an active substrate, as shown in Fig. 2. It appears therefore that the removal of fucose extends the range of substrate specificity of this enzyme to encompass not only the longer chain mannosyl glycopeptides but also those substrates ordinarily hydrolyzed by the Diplococcus endoglycosidase. Another important feature of the Streptomyces enzyme is its capacity to remove oligosaccharides directly from many intact proteins (2), a property apparently not shared by the Diplococcus endoglycosidase.

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