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SUBSTRATE SPECIFICITY OF *DIPLOCOCCAL* β-*N*-ACETYLHEXOSAMINIDASE, A USEFUL ENZYME FOR THE STRUCTURAL STUDIES OF COMPLEX TYPE ASPARAGINE-LINKED SUGAR CHAINS

by

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SYMMARY: The substrate specificity of *diplococcal* β-*N*-acetylhexosaminidase was studied in detail by using oligosaccharides of known structure. The enzyme cannot cleave GlcNAcβ1→4Man and GlcNAcβ1→6Man linkages although it readily hydrolyzes GlcNAcβ1→2Man, GlcNAcβ1→3Gal and GlcNAcβ1→6Gal linkages. The GlcNAcβ1→2Man linkage in GlcNAcβ1→4(GlcNAcβ1→2)Man group is cleaved by the enzyme but the linkage in GlcNAcβ1→6(GlcNAcβ1→2)Man group is not, probably because of the steric effect of GlcNAcβ1→6Man residue on GlcNAcβ1→2Man linkage. Similar steric effect is also observed in the case of GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)(GlcNAcβ1→4)Manβ1→4 $GlcNAc_{OT}$. The enzyme can cleave only one of the two GlcNAcβ1→2Man linkages of the heptaitol and produces GlcNAcβ1→2Manα1→6(Manα1→3)(GlcNAcβ1→4)Manβ1→4$GlcNAc_{OT}$. The substrate specificity of *diplococcal* β-*N*-acetylhexosaminidase can be used effectively for the structural studies of asparagine-linked sugar chains.

β-*N*-Acetylglucosaminidase in the culture medium of *Diplococcus pneumoniae* was first purified by Hughes and Jeanloz (1) and has been used widely for the studies of glycoproteins because it acts on intact glycoproteins as well as oligosaccharides. During the structural study of the asparagine-linked sugar chains of plasma membrane glycoproteins of calf thymocytes (2), we noticed that some of the β-*N*-acetylglucosamine residues located at the non-reducing termini of the sugar chains could not be removed by *diplococcal* β-*N*-acetylglucosaminidase, although they were readily removed by jack bean β-*N*-acetylhexosaminidase. Since exoglycosidases with narrow substrate specificities were useful in discriminating positional isomers in sugar chain structures (3-9), the substrate specificity of *diplococcal* β-*N*-acetylglucosaminidase was studied by using various oligosaccharides currently available.

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MATERIALS AND METHODS

Oligosaccharides -- Manα1→6(Manα1→3)Manα1→6(GlcNAcβ1→2Manα1→3)(GlcNAcβ1→4)Manβ1→4GlcNAc$_{OT}$[1] and Manα1→3Manα1→6[GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3](GlcNAcβ1→4)Manβ1→4GlcNAc$_{OT}$ were prepared from ovalbumin glycopeptides as reported previously (10). GlcNAcβ1→2Manα1→3Manβ1→4GlcNAc$_{OT}$, GlcNAcβ1→2Manα1→6Manβ1→4GlcNAc$_{OT}$, GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAc$_{OT}$, GlcNAcβ1→2Manα1→6[GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAc$_{OT}$, GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAc$_{OT}$ and GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6[GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAc$_{OT}$ were prepared by jack bean β-galactosidase digestion of the urinary oligosaccharides obtained from G_{M1}-gangliosidosis patient (11). GlcNAcβ1→4Manα1→3Manβ1→4GlcNAc$_{OT}$, GlcNAcβ1→6Manα1→6Manβ1→4GlcNAc$_{OT}$ and GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)(GlcNAcβ1→4)Manβ1→4GlcNAc$_{OT}$ were purified from oligosaccharides excreted in the urine of Sandhoff disease patient[2]. GlcNAcβ1→3Galβ1→4GlcNAc$_{OT}$ and GlcNAcβ1→6Galβ1→4GlcNAc$_{OT}$ were obtained by partial acid hydrolysis from lacto-N-neohexaitol (12). In this paper these oligosaccharides will be called by alphabet as shown in Table I.

Chemicals and Enzymes -- NaB[^{3}H]$_4$ (154 mCi/nmol) was purchased from New England Nuclear, Boston, Mass. Jack bean α-mannosidase was prepared according to the method of Li and Li (13). *Diplococcal* β-*N*-acetylglucosaminidase was purified by the method of Glasgow et al (14). One unit of glycosidase was defined as the amount of enzyme required to hydrolyze 1 μmol of p-nitrophenyl-glycosides per min.

Enzyme Assay -- The standard assay mixture for *diplococcal* β-*N*-acetylglucosaminidase activity contained 2 nmol (approximately 2 x 10^4 cpm) of radioactive oligosaccharide and appropriate amount of *diplococcal* β-*N*-acetylglucosaminidase in 50 μl of 0.05 M sodium citrate buffer, pH 6.0. The mixture was incubated at 37°C for 18 h. One drop of toluene was added to all reaction mixtures to inhibit bacterial growth. Reaction was terminated by heating the reaction mixture in a boiling water bath for 2 min. The solution was passed through a mixed bed column (0.5 x 3 cm) of Bio-Rad AG-50 (H^+-form) and AG-3 (OH^--form) and the column was washed with 3 bed volumes of distilled water. The eluate and washing were combined and evaporated to dryness. The residue was dissolved in 0.4 ml of water containing 4.0 mg dextran hydrolysate. The solution was analyzed by Bio-Gel P-4 column chromatography.

General Methods -- Radioactivity was determined by Aloka liquid scintillation spectrometer model LSC-700. Bio-Gel P-4 (under 400 mesh) column chromatography was performed using columns (2 m x 2 cm i.d.) equipped with a water jacket. During operation the column was kept at 55°C by circulating warm water in the jacket. Sugars were eluted from the column with distilled water at a flow rate of 1.0 ml/min using model 6000A solvent delivery system (Waters Associates, Inc., Milford). A differential refractometer, Shodex RI model SE-11 (Showa Denko Ltd., Tokyo), was used for monitoring dextran hydrolysate eluted from the column, and eluate was fractionated 3.0 ml per tube. Methylation analysis of oligosaccharides was performed as described in the previous paper (15) except the times of hydrolysis were prolonged to 5 h. Analysis of partially-*O*-methylated hexitols and *N*-acetylglucosaminitols were performed with a gas chromatography-mass spectrometer Shimadzu-LKB model 9,000 (Shimadzu Corporation) using either a glass column (2.5 mm x 1 m) of 5% OV-17 on Gas-chrom Q (60 to 80 mesh) or that (3 mm x 2 m) of 3% OV-210 coated on Gas-chrom Q (100 to 120 mesh). The column temperature was programmed from 140°C to 220°C at a rate of 2°C/min.

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1 Substript OT is used in this paper to indicate NaB^{3}H$_4$ reduced oligosaccharides. All sugar mention in this paper are of D configuration except for fucose.

2 Yamashita, K., Tachibana, Y., Nishigaki, M., Suzuki, Y., and Kobata, A. m.s. in preparation.

Table I. Structures and effective sizes of substrates and their hydrolysis products with *diplococcal* β-*N*-acetylhexosaminidase in Bio-Gel P-4 column chromatography.

Substrates		Effective size	Hydrolysis Products		Effective size
Name	Structures	(glucose units)	Name	Structures	(glucose units)
a.	Gβ1→2Mα1→3Mβ1→4G$_{OT}$	6.2		Mα1→3Mβ1→4G$_{OT}$	4.2
b.	Gβ1→2Mα1→6Mβ1→4G$_{OT}$	5.8		Mα1→6Mβ1→4G$_{OT}$	3.9
c.	Gβ1→4Mα1→3Mβ1→4G$_{OT}$	6.1		non hydrolyzed	6.1
d.	Gβ1→6Mα1→6Mβ1→4G$_{OT}$	5.7		non hydrolyzed	5.7
e.	Gβ1→2Mα1→6 Gβ1→2Mα1→3Mβ1→4G$_{OT}$	8.9		Mα1→6 Mα1→3Mβ1→4G$_{OT}$	5.0
f.	Gβ1→2Mα1→6 Gβ1→4 Gβ1→2Mα1→3Mβ1→4G$_{OT}$	10.8	f'.	Mα1→6 Gβ1→4Mα1→3Mβ1→4G$_{OT}$	6.8
g.	Gβ1→6 Gβ1→2Mα1→6 Gβ1→2Mα1→3Mβ1→4G$_{OT}$	10.4	g'.	Gβ1→6 Gβ1→2Mα1→6 Mα1→3Mβ1→4G$_{OT}$	8.3
h.	Gβ1→6 Gβ1→2Mα1→6 Gβ1→4Mα1→3Mβ1→4G$_{OT}$ Gβ1→2	12.2	h'.	Gβ1→6 Gβ1→2Mα1→6 Gβ1→4Mα1→3Mβ1→4G$_{OT}$	10.4
i.	Gβ1→6 Gβ1→2Mα1→6Mβ1→4G$_{OT}$	7.4		non hydrolyzed	7.4
j.	Gβ1 ↓ Mα1→6Mα1→6 Mα1→3 4 Gβ1→2Mα1→3Mβ1→4G$_{OT}$	9.3	j'.	Gβ1 ↓ Mα1→6Mα1→6 Mα1→3 4 Mα1→3Mβ1→4G$_{OT}$	7.6
k.	Gβ1 ↓ Mα1→3Mα1→6 4 Gβ1→4Mα1→3Mβ1→4G$_{OT}$ Gβ1→2	10.2	k'.	Gβ1 ↓ Mα1→3Mα1→6 4 Gβ1→4Mα1→3Mβ1→4G$_{OT}$	8.6
l.	Gβ1 ↓ Gβ1→2Mα1→6 4 Gβ1→2Mα1→3Mβ1→4G$_{OT}$	9.3	l'.	Gβ1 ↓ Gβ1→2Mα1→6 4 Mα1→3Mβ1→4G$_{OT}$	7.8

G=GlcNAc, M=Man

RESULTS AND DISCUSSION

Since the exact glycon specificity of *diplococcal* β-*N*-acetylglucosaminidase has never been studied, an experiment was performed to see if the enzyme hydrolyzes

β-*N*-acetylgalactosamine linkage. When 200 μg of p-nitrophenyl β-*N*-acetylgalactosaminide was incubated with 10 munits of *diplococcal* β-*N*-acetylglucosaminidase in 220 μl of 0.05 M sodium citrate buffer, pH 6.0 at 37°C, p-nitrophenol was released at a rate of 1/6.7 of that from p-nitrophenyl β-*N*-acetylglucosaminide. Therefore, *diplococcal* β-*N*-acetylglucosaminidase hydrolyzes β-*N*-acetylgalactosaminyl linkage like enzymes from other sources. Accordingly, the *diplococcal* enzyme will be called as β-*N*-acetylhexosaminidase in the following part of this paper.

One *N*-acetylglucosamine residue was released from oligosaccharides *a* and *b* by incubation with 2 munit of *diplococcal* β-*N*-acetylhexosaminidase. On the other hand, oligosaccharides *c* and *d* were totally resistant to incubation even with 50 munit of the enzyme. These results indicate that *diplococcal* β-*N*-acetylhexosaminidase hydrolyzes GlcNAcβ1→2Man linkage but not GlcNAcβ1→4Man and GlcNAcβ1→6Man linkages. In order to obtain more information about the substrate specificity of the enzyme, oligosaccharides with more complicated structures were incubated with 10 munit of the enzyme. Oligosaccharide *e* was completely converted to a radioactive tetrasaccharide releasing 2 mol *N*-acetylglucosamine residues. The structure of the tetrasaccharide was confirmed as shown in Table I by methylation analysis (data not shown). Oligosaccharide *f* also released 2 *N*-acetylglucosamine residues and was converted to a radioactive pentasaccharide, the structure of which was confirmed by methylation analysis (Table II). Therefore, *diplococcal* β-*N*-acetylhexosaminidase can readily hydrolyze all GlcNAcβ1→2Man linkages in these oligosaccharides and cannot cleave GlcNAcβ1→4Man linkage in oligosaccharide *f*. The results with oligosaccharides *g* and *h* were not that simple: only 1 *N*-acetylglucosamine residue was removed from both oligosaccharides though they have two GlcNAcβ1→2Man groups. No more *N*-acetylglucosamine residue was released from the radioactive products by the second incubation with 50 munit of the enzyme. In order to identify the *N*-acetylglucosamine residue removed from the two oligosaccharides by the enzyme action, the products (*g'* and *h'*) were subjected to methylation analysis. The data shown in Table II indicated

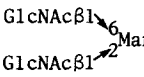
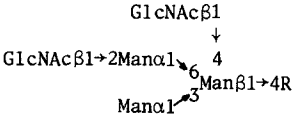
Table II. Methylation analysis of the products obtained by *diplococcal* β-*N*-acetylhexosaminidase digestion.

Partially methylated sugar	Molar ratio[a)]					
	f'	g'	h'	j'	k'	l'
Mannitol						
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	1.1	1.1	-	3.3	1.0	1.0
3,4,6-Tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	-	-	-	-	-	1.0
2,4,6-Tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	-	-	-	-	0.9	-
2,3,6-Tri-*O*-methyl (1,4,5-tri-*O*-acetyl)	0.9	-	1.1	-	1.1	-
3,4-Di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	-	0.9	0.9	-	-	-
2,4-Di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.0	1.0	1.0	1.0	-	-
2-Mono-*O*-methyl (1,3,4,5,6-di-*O*-acetyl)	-	-	-	0.9	1.1	0.9
2-N-Methylacetamido-2-deoxyglucitol						
1,3,5,6-Tetra-*O*-methyl (4-mono-*O*-acetyl)	0.8	0.7	0.9	0.7	0.7	0.9
3,4,6-Tri-*O*-methyl (1,5-di-*O*-acetyl)	1.1	2.3	3.2	1.2	2.2	2.3

a) Numbers in the table were calculated by making the value of the underlined partially methylated alditol acetate as 1.0.

that they have structures as shown in Table I. These results indicate that GlcNAcβ1→2Man linkage in GlcNAcβ1→6(GlcNAcβ1→2)Man group is not hydrolyzed by *diplococcal* β-*N*-acetylhexosaminidase, possibly by the steric effect of the C-6 linked *N*-acetylglucosamine residue. That the neighbouring Manα1→3 residue plays no inhibitory role was confirmed by the following experiment. Radioactive oligosaccharide *g'* was converted to radioactive oligosaccharide *i* as shown in Table I by incubation with jack bean α-mannosidase. The radioactive pentasaccharide was completely resistant to *diplococcal* β-*N*-acetylhexosaminidase action. Some of the asparagine-linked sugar chains of the plasma membrane glycoproteins of calf thymocytes contain GlcNAcβ1→4(GlcNAcβ1→2)Manα1→6 and GlcNAcβ1→6(GlcNAcβ1→2)Manα1→3 groups (2). Since the former group was converted to GlcNAcβ1→4Manα1→6 and the latter was not hydrolyzed by *diplococcal* β-*N*-acetylhexosaminidase treatment (2), the aglycon specificity does not include the α-mannosyl linkage.

Table III. Structures resistant to *diplococcal* β-*N*-acetylhexosaminidase digestion

GlcNAcβ1→4Manα1→3 and 6Manβ1→4R[a)]
GlcNAcβ1→6Manα1→3 and 6Manβ1→4R



a) R indicates $GlcNAc_{OT}$, GlcNAcβ1→4$GlcNAc_{OT}$ and GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$

That *diplococcal* β-*N*-acetylhexosaminidase cannot remove *N*-acetylglucosamine residue linked at the C-4 position of the β-mannosyl residue of the trimannosyl core portion was confirmed by using oligosaccharides *j* and *k*. By the β-*N*-acetylhexosaminidase treatment, they were converted to *j'* and *k'* in Table I, the structures of which were confirmed by methylation study (Table II).

An interesting observation is that *diplococcal* β-*N*-acetylhexosaminidase removed only 1 *N*-acetylglucosamine residue from oligosaccharide *l*. Methylation analysis of the product (*l'*) shown in Table II indicated that 1 of the 2 GlcNAcβ1→2Man linkages in oligosaccharide *l* was hydrolyzed. This linkage should be exclusively located on Manα1→3 side because GlcNAcβ1→4 residue in oligosaccharide *j* did not inhibit the hydrolysis of its GlcNAcβ1→2Man linkage by β-*N*-acetylhexosaminidase. Therefore, the structure of oligosaccharide *l'* should be as shown in Table I. These results may indicate that a GlcNAcβ1→4 residue in GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)Man group sterically inhibits the hydrolysis of the neighbouring GlcNAcβ1→2Man linkage by *diplococcal* β-*N*-acetylhexosaminidase.

Table III summarizes the aglycon specificity of *diplococcal* β-*N*-acetylhexosaminidase so far described. It must be stressed that the aglycon specificity can be applied regardless of the structure of "R" moiety. Although

no data was shown, we have confirmed that oligosaccharides with $GlcNAc_{OT}$, GlcNAcβ1→4$GlcNAc_{OT}$ and GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ as their "R" portions gave the same results by *diplococcal* β-*N*-acetylhexosaminidase digestion. Since the oligosaccharides a~*l* are all structurally related to asparagine-linked sugar chains, the specificity might be used effectively in the study of the sugar chains of glycoproteins. The enzyme, however, may not be useful for the study of mucin-type sugar chains, because it released an *N*-acetylglucosamine residue from GlcNAcβ1→3Galβ1→4Glc_{OT} and GlcNAcβ1→6Galβ1→4Glc_{OT} (data not shown). GlcNAcβ1→3Gal and GlcNAcβ1→6Gal linkages represent most of the *N*-acetylglucosaminyl linkages in mucin-type sugar chains so far known.

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