Characterization of an Endo-α-N-Acetyl Galactosaminidase from *Diplococcus Pneumoniae*

V. P. Bhavanandan, Junji Umemoto and E. A. Davidson

Department of Biological Chemistry
The Milton S. Hershey Medical Center
The Pennsylvania State University
Hershey, PA 17033

Received April 1,1976

SUMMARY: Evidence is presented for the presence in filtrates of *Diplococcus pneumoniae* of an endo-glycosidase capable of acting on the O-glycosidic linkage between N-acetyl galactosamine and serine or threonine residues. The glycosidase was partially purified by chromatography on Affi-Gel 202. The resulting preparation acted on glycopeptides from mouse melanoma, fetuin and pig submaxillary mucin to release a disaccharide characterized as galactosyl-N-acetyl galactosamine. The enzyme had no action on phenyl α-N-acetyl-D-galactosaminide, asialo ovine submaxillary mucin or monosialoganglioside. A similar activity was detected in a commercial preparation of *Clostridium perfringens* neuraminidase.

Endo-glycosidases would be extremely useful tools for structural studies on glycoproteins and for investigating the nature and function of cell surface complex carbohydrates. Endo-β-N-acetyl glucosaminidase activity, first demonstrated by Muramutsu (1) in *Diplococcus pneumoniae*, has since been isolated from various microorganisms (2-4). This enzyme hydrolyzes the di-N-acetyl-chitobiose linkages in glycoproteins having a glucosaminyl asparagine bond; the exact substrate specificity depends on the source of the enzyme (2-4). The presence of α-N-acetyl galactosaminyl oligosaccharase in a crude enzyme preparation from *Clostridium perfringens* has been reported (5). The present report describes some of the properties of such a glycosidase obtained from *D. pneumoniae*.

Chromatographic fractionation of glycosidases from *D. pneumoniae* gave a preparation of neuraminidase with very high specific activity. However, on digesting a labelled sialoglycopeptide from mouse melanoma cells with this enzyme fraction, it was discovered that in addition to sialic acid, an oligosaccharide was released. Structural analysis of this oligosaccharide

resulted in the identification of an endo-glycosidase which in contrast to the enzyme described by Muramutsu acts on the O-glycosidic linkage between α-N-acetyl galactosamine and serine or threonine in glycoproteins.

MATERIALS AND METHODS

The crude preparation of enzymes isolated from the culture filtrates of *Diplococcus pneumoniae* type 1 and having neuraminidase, β-N-acetyl hexosaminidase, β-galactosidase and endo-β-N-acetyl glucosaminidase activities but no proteases was generously provided by Dr. Gilbert Ashwell.

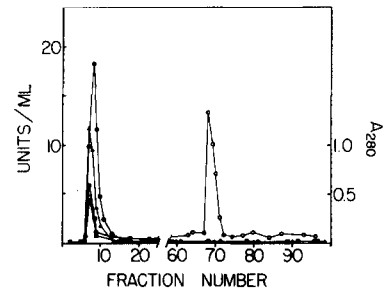
Mouse melanoma sialoglycopeptides (class I) were isolated from [^{3}H]-glucosamine labelled cells as described (6,7). The tritium label in this preparation was distributed between sialic acid (40%) and galactosamine (60%). Pig submaxillary mucin (PSM)[1] isolated from pooled glands was a gift from Dr. M. de Salegui. Glycopeptides from this glycoprotein were isolated as described (8), except that the pronase digest was ultrafiltered through a UM_2 membrane (Amicon) before chromatography on Sephadex G-50. The anthrone positive material consisting of the mixed glycopeptides was used in these studies. Fetuin was purchased from Gibco and the O-glycosidically linked glycopeptides from it were prepared as described by Spiro and Bhoyroo (9), with the following modification: the pronase digest was ultrafiltered using a $UM_{0.5}$ membrane (Amicon) and directly fractionated on a Sephadex G-25 (2.6 x 128 cm) column. The glycopeptides corresponding to Fraction C of Spiro and Bhoyroo were used in these studies; 36 mg was obtained from 1 g fetuin. Ovine submaxillary mucin and asialo ovine submaxillary glycopeptides were isolated as described previously (10). Neuramin-(2→3)-lactose was isolated from skimmed bovine colostrum as described previously (11).

Activities of β-galactosidase and β-N-acetyl hexosaminidase were measured using O-nitrophenyl-β-D-galactopyranoside and p-nitrophenyl-N-acetyl-β-D-glucosaminide (both from Calbiochem) respectively, as described (12). α-N-acetyl-D-galactosaminidase was assayed using either phenyl-N-acetyl-α-D-galactosaminide (Nakarai Chemicals, Japan) or asialo ovine submaxillary glycopeptides as substrate. The incubation mixtures were assayed for N-acetyl galactosamine by the Morgan-Elson reaction (13) and in the case of the former substrate also by Folin-Ciocalteu reaction for phenol. The incubation mixture for the neuraminidase assay consisted of 25 µl of 0.1 M phosphate-citrate buffer, pH 6.5 (12), 50 µl of a 5 mM solution of Neuramin-(2→3)-lactose and enzyme in a final volume of 200 µl. One unit of neuraminidase activity is defined as the amount of enzyme that causes the release of 1 µmole of NANA per 30 min. After incubation at 37°C for 30 min, the released sialic acid was measured by a modification of the thiobarbituric acid method (14). Incubation with the endo-α-N-acetyl galactosaminidase was done under the same conditions as for neuraminidase using the appropriate substrates. After the incubation, aliquots of the mixture were assayed for Morgan-Elson positive material. The product was further identified by gel filtration on BioGel P2 <400 mesh columns (0.9 x 64 cm) and by paper chromatography.

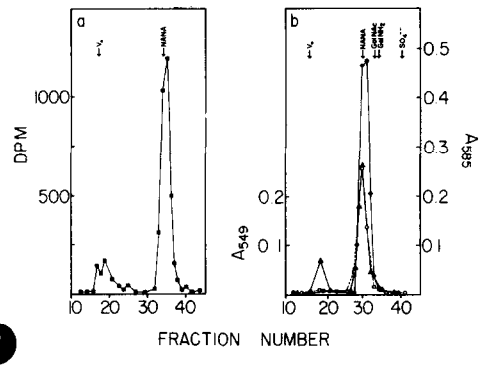
Paper chromatography was done on Whatman No. 1 paper using the following solvents: A, N-butyl acetate:glacial acetic acid:water (3:2:1); B,N-butyl alcohol:pyridine:water (6:4:3). Radioactivity in column fractions and on paper chromatograms was determined as described previously (6,7).

---

[1] Abbreviations used: PSM, pig submaxillary mucin; GalNAc, N-acetyl galactosamine, NANA, N-acetyl neuraminic acid.



1



2

Figure 1. Chromatography of *Diplococcus pneumoniae* crude enzyme preparation on Affi-Gel 202 at 4°C. The column (1.2 x 26 cm) was eluted with 0.02 M Tris-HCl buffer, pH 7.0 and fractions of 1.5 ml were collected. The eluant was changed to 0.5 M NaCl in the same buffer at fraction 62. The fractions were analyzed for ultraviolet absorbance at 280 (—■—■—), β-N-acetyl-D-hexosaminidase (—●—●—), β-galactosidase (—▲—▲—) and neuraminidase (—○—○—) as described under "Methods". The scale for neuraminidase activity is 1/20 of that of the other enzymes.

Figure 2. Fractionation of enzyme fraction II digests of a) [^{3}H]-asialoglycopeptides from B16 mouse melanoma; b) fetuin glycopeptide fraction C on BioGel P2 <400 mesh columns. The columns were eluted with 0.1 N pyridine acetate at the rate of 30 ml per hr and fractions of 1 ml were collected. Aliquots were analyzed for tritium radioactivity (—■—■—), Morgan-Elson reacting material (—●—●—), free sialic acid (—○—○—) and total sialic acid after acid hydrolysis (—▲—▲—). Void volume (Vo) and peak elution positions of standard sugars and inorganic sulfate are indicated by arrows.

Isolation of the endo-enzyme: The crude enzyme was placed on a column of Affi-Gel 202 (BioRad) and the column eluted with 0.02 M Tris-HCl buffer, pH 7.0 followed by 0.5 M NaCl in the same buffer in the cold room, 4°C. The result of the fractionation is illustrated in Figure 1.

RESULTS AND DISCUSSION

The designated fractions in Figure 1 were combined and concentrated by ultrafiltration. The concentrated enzyme fractions I and II were tested for glycosidases and proteases. Fraction I had β-galactosidase, β-N-acetyl hexosaminidase and neuraminidase activities. The only detectable exo-glycosidase activity in fraction II was neuraminidase; the protein in this fraction was very low and there was no protease activity when tested against Azocoll (Calbiochem).

Treatment of mouse melanoma class I [^{3}H]-sialoglycopeptide with enzyme fraction II followed by chromatography on a BioGel P2 column released

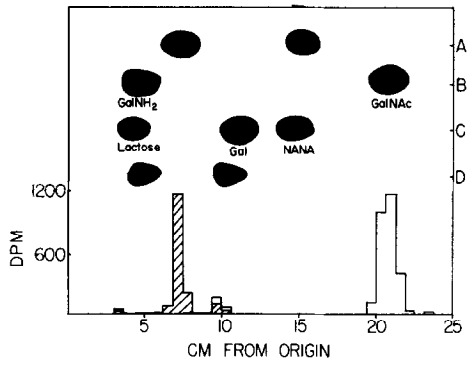


Figure 3. Paper chromatography of low molecular weight material isolated by BioGel P2 column chromatography of enzyme fraction II digests. The position of the labelled oligosaccharide from mouse melanoma asialoglycopeptides (fractions 33-37, Figure 2a) is indicated by the shaded area. After treatment with Jack bean β-galactosidase the labelled material (unshaded area) has the same mobility as GalNAc. The low molecular weight material from fetuin glycopeptide (fractions 29-33, Figure 2b) before (lane A) and after (lane D) acid hydrolysis (3N HCl, 100°C, 6 hr). Lanes B and C are standards.

about 90% of the radioactivity as low molecular weight material. This was unexpected since only about 40% of the radioactivity was in sialic acid was determined by mild acid hydrolysis (0.1 N H_2SO_4, 80°, 1 hr). Treatment of [^{3}H]-asialoglycopeptide, prepared by *Vibrio cholerae* neuraminidase digestion, with fraction II for 24 hrs, released 73% of the radioactivity (Figure 2). Paper chromatography in solvent A of the low molecular weight material released from the sialoglycopeptide showed two spots, one with the mobility of N-acetyl neuraminic acid and the other with a lower R_F was apparently an oligosaccharide (Figure 3). The material released from the asialoglycopeptide showed only the oligosaccharide on paper chromatography. On rechromatography of the product from asialoglycopeptide, after treatment with Jack bean β-galactosidase, a labelled spot with the mobility of N-acetyl galactosamine was detected (Figure 3). These results indicated that an enzyme in fraction II was releasing an oligosaccharide with the structure $(Gal)_x 1 \xrightarrow{\beta} GalNAc$. The class I mouse melanoma glycopeptide has a mucin type structure in which the prosthetic group is linked to serine

and/or threonine through α-N-acetyl galactosaminyl residues (7); therefore, it appeared that an endo-glycosidase in fraction II was cleaving this linkage. In order to confirm this finding the action of this enzyme on O-glycosidically linked glycopeptides from fetuin and PSM was tested.

Digestion of fetuin glycopeptide fraction C with this enzyme resulted in the production of a Morgan-Elson positive material. In order to identify this product the incubation mixture was chromatographed on a Bio-Gel P2 column (Figure 2). Two peaks were obtained; fractions in the second peak reacted with Morgan-Elson and thiobarbituric acid reagents, whereas fractions in the first peak reacted with neither but reacted with the latter reagent after acid hydrolysis (0.1 N H_2SO_4, 80°C, 1 hr). Chromatography of a control digest, without enzyme, on the same column gave only the first peak. The material eluting in the second peak was isolated and on paper chromatography (solvents A and B) showed two silver nitrate staining components with the same mobilities as N-acetyl neuraminic acid and the oligosaccharide from mouse melanoma glycopeptide (Figure 3). The oligosaccharide was separated from sialic acid by chromatography on an AG1 (formate) resin column. Acid hydrolysis (3 N HCl, 100°, 6 hr) of the oligosaccharide gave galactose and galactosamine, identified by paper chromatography (Figure 3). Quantitative analysis indicated the presence of equimolar amounts of these sugars in the hydrolysate; galactose was determined by anthrone reaction (15) and galactosamine by the Elson-Morgan reaction (16). Reduction of the oligosaccharide with sodium borohydride followed by acid hydrolysis gave galactose and galactosaminitol as identified by chromatography on borate treated paper in solvent A. These results prove that an enzyme activity in fraction II is capable of releasing the disaccharide galactosyl-(1→3)N-acetyl galactosamine from fetuin glycopeptide fraction C.

Submaxillary glycopeptides on digestion with the enzyme fraction II gave a Morgan-Elson positive product which was isolated by BioGel P2

column chromatography and identified as the disaccharide, galactosyl-N-acetyl-galactosamine, as described above. The same product was obtained when PSM glycopeptides from which fucose and sialic acid had been removed by mild acid hydrolysis (1 M HCOOH, 100°C, 1 hr) and the galactosamine re-N-acetylated (17) was used as substrate. The PSM preparation was from pooled glands and thus probably has both blood group A^+ and A^- active components. The isolation of only a disaccharide and not higher oligosaccharides from digests of the PSM glycopeptides indicates that the enzyme might require an unsubstituted galactose for activity. It will be interesting to determine whether this enzyme is capable of releasing sialo-oligosaccharides, such as sialyl-N-acetyl galactosamine from ovine submaxillary glycopeptides. This can be determined once the enzyme is purified free of neuraminidase.

The enzyme also released the same disaccharide from intact PSM and PSM from which fucose and sialic acid had been removed and the galactosamine re-N-acetylated. It is thus capable of acting on intact glycoproteins, a property which will be very valuable in structural studies on glycoproteins. The enzyme fraction II did not have any action on the following: phenyl-α-N-acetyl galactosaminide, asialo ovine submaxillary glycoprotein and its glycopeptides, monosialoganglioside (GM_1) and asialo GM_1 prepared by acid treatment (1 M HCOOH, 100°, 1 hr). It is thus apparent that it has no exo-N-acetyl galactosaminidase activity and the endo activity is specific for the α-linkage, since in GM_1 (Gal→GalNAc) is linked β to galactose.

Using class I [^{3}H]-sialoglycopeptides from mouse melanoma we were able to detect the same endo-glycosidase activity in a commercial preparation (Worthington) of *Clostridium perfringens* neuraminidase.

The evidence reported in this communication firmly establishes the presence of an endo-glycosidase, identified as endo-α-N-acetyl galactosaminidase, in a fraction obtained from *D. pneumoniae* culture filtrates. The only other detectable enzyme activity in this fraction is neuraminidase.

The binding of neuraminidase and the endo-enzyme to Affi-Gel 202 followed by their elution with NaCl is of interest. Affi-Gel 202 is an agarose gel bead to which the spacer arm $-\overset{\overset{NH}{|}}{C}O\,NH(CH_2)_3NH(CH_2)_3NHCO(CH_2)_2COOH$ is attached. This support is used for immobilizing ligands for affinity chromatography. *D. pneumoniae* neuraminidase also bound to Affi-Gel 202 to which p-aminobenzyl-1-thio-2-acetamido-2-deoxy-β-D-glucopyranoside was coupled (J. Umemoto, V. P. Bhavanandan and E. A. Davidson, unpublished results). This indicates that the neuraminidase is bound to the Affi-Gel 202 by non-specific (hydrophobic) interaction with the spacer arm, and not to the carboxyl group.

In preliminary experiments the crude enzyme preparation was chromatographed on a Sepharosyl-glycyltyrosyl-[N-(p-aminophenyl)]oxamic acid column (0.8 x 2.8 cm) as described by Den *et al.* (18). The neuraminidase activity was separated from β-galactosidase and β-N-acetyl hexosaminidase but the endo-enzyme was detected in the neuraminidase containing fraction. It was noticed that on storage, the activity of the neuraminidase in the fraction obtained from Affi-Gel 202 column decreased more rapidly than that of the endo-enzyme. Partial separation of the neuraminidase and endoenzyme activities was also obtained when the crude preparation was chromatographed on Affi-Gel 202 using a NaCl gradient for elution. These results indicate that neuraminidase and the endoenzyme activities are two separate enzymes. The possibility of using these properties to separate the two enzyme activities is being explored. Purification of the endoenzyme as well as the properties of this enzyme, such as pH optimum and the exact substrate specificity, will be reported in a subsequent publication.

ACKNOWLEDGEMENTS: We are most grateful to Dr. Gilbert Ashwell for providing generous quantities of the crude enzyme preparation used in these studies. We also thank Dr. M. de Salegui for pig submaxillary mucin, Dr. Y-T. Li for Jack bean β-galactosidase, Dr. H. Den for the affinity column of N-(p-aminophenyl)oxamic acid, Dr. C.-L. Schengrund for monosialoganglioside and Mr. Jeffery Kemper for technical assistance. These studies were supported in part by grant CA 15483 from the U.S. Public Health Service.

REFERENCES

1. Muramatsu, T. (1971) J. Biol. Chem. 246, 5535-5537.
2. Tarentino, A. L., and Maley, F. (1974) J. Biol. Chem. 249, 811-817.
3. Ito, S., Muramatsu, T., and Kobata, A. (1975) Arch. Biochem. Biophys. 171, 78-86.
4. Tarentino, A. L., and Maley, F. (1975) Biochem. Biophys. Res. Comm. 67, 455-462.
5. Huang, C. C., and Aminoff, D. (1972) J. Biol. Chem. 247, 6737-6742.
6. Satoh, C., Banks, J., Horst, P., Kreider, J. W., and Davidson, E. A. (1974) Biochemistry 13, 1233-1241.
7. Bhavanandan, V. P., and Davidson, E. A. (1976) Biochem. Biophys. Res. Comm., in press.
8. Irimura, T., Kawaguchi, T., Terao, G., and Osawa, T. (1975) Carb. Res. 39, 317-327.
9. Spiro, R. G., and Bhoyroo, V. D. (1974) J. Biol. Chem. 249, 5704-5717.
10. Bhavanandan, V. P., Buddecke, E., Carubelli, R., and Gottschalk, A. (1964) Biochem. Biophys. Res. Comm. 16, 353-357.
11. Bhavanandan, V. P., Yeh, A. K., and Carubelli, R. (1975) Anal. Biochem. 69, 385-394.
12. Hughes, R. C., and Jeanloz, R. W. (1964) Biochemistry 3, 1535-1548.
13. Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955) J. Biol. Chem. 217, 959-966.
14. Yeh, A. K., Tulsiani, D. R. P., and Carubelli, R. (1971) J. Lab. Clin. Med. 78, 771-778.
15. Seifter, S., Dayton, S., Novic, B., and Muntwyler, E. (1950) Arch. Biochem. 25, 191-200.
16. Good, T. A., and Bessman, S. P. (1964) Anal. Biochem. 9, 253.
17. Roseman, S., and Ludowieg, T. (1954) J. Amer. Chem. Soc. 76, 301-302.
18. Den, H., Malinzak, D. A., and Rosenberg, A. (1975) J. Chromatography 111, 217-222.