PRESENCE OF ENDO-β-N-ACETYLGLUCOSAMINIDASE AND PROTEASE ACTIVITIES IN THE COMMERCIAL NEURAMINIDASE PREPARATIONS ISOLATED FROM CLOSTRIDIUM PERFRINGENS[1]

Su-Fang Chien, Steven J. Yevich, Su-Chen Li[2] and Yu-Teh Li[3]

Dept. of Biochemistry and Delta Regional Primate Research Center
Tulane University, New Orleans, Louisiana 70112

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SUMMARY. Commercial neuraminidase preparations isolated from Clostridium perfringens were found to be contaminated with endoglycosidase and protease activities. The preparations release oligosaccharide fragment(s) with GlcNAc at the reducing end, from the intact ovalbumin. The enzymes also converted Asn-$(GlcNAc)_2(Man)_5$ isolated from ovalbumin into Asn-GlcNAc and an oligosaccharide with GlcNAc at the reducing end. Similar results were obtained when Taka-amylase A and its glycopeptide were used as substrates. These results identify the endoglycosidase activity as endo-β-N-acetylglucosaminidase. Furthermore, the commercial neuraminidase preparations contain a protease activity which hydrolyzes Azocoll, hemoglobin, casein, ovalbumin, human serum albumin, and α_1-acid glycoprotein.

INTRODUCTION. Although specific exoglycosidases are very useful in studying the anomeric configuration and sequential arrangement of saccharide units in heterosaccharide chains, contamination by other glycosidase activities in the enzyme preparation presents a serious shortcoming of this method. Since the exoglycosidases are often used for structural studies of saccharide chains in intact glycoproteins, a protease-contaminated preparation could further compound the severity of this shortcoming. While using commercial neuraminidases to cleave sialic acids from various glycoproteins, we found that products other than sialic acid were liberated. This communication reports that commercial neuraminidase preparations isolated from Clostridium perfringens contain protease and endo-β-N-acetylglucosaminidase activities.

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Abbreviations: SDS = sodium dodecyl sulfate; TCA = trichloroacetic acid.

MATERIALS AND METHODS. Neuraminidase type V Lot No. 103C-81501 (0.2 unit/mg protein[4]), and type VI Lot No. 63C-8211 (2.55 unit/mg protein[4]) were from Sigma Chemical. Ovalbumin 2X crystalline was from Nutritional Biochemicals. Azocoll was from Calbiochem. α-Mannosidase was isolated from jack bean meal (1). The glycopeptide Asn-$(GlcNAc)_2(Man)_5$ was prepared from ovalbumin according to the method of Huang et al (2). The following compounds were generous gifts: [^{14}C]-N-acetyl-Asn-$(GlcNAc)_2(Man)_5$ isolated from ovalbumin, Dr. A. Kobata, Kobe University, Japan; [^{3}H]-N-acetyl ovalbumin glycopeptide, Taka-amylase A glycopeptide and Asn-GlcNAc, Dr. I. Yamashina, Kyoto University, Japan; crystalline ovalbumin, Dr. R. Montgomery, University of Iowa; Asn-GlcNAc from Cyclo Chemical, courtesy of Dr. M. F. Lou, Alfred I. DuPont Institute, Wilmington, Delaware; α_1-acid glycoprotein, Dr. K. Schmid, Boston University; Taka-amylase A, Dr. Y. Matsushina, Osaka University, Japan, and Dr. Y. C. Lee, Johns Hopkins University. All other chemicals were obtained from commercial sources and were of the highest grade.

All incubations were done in 0.05 M sodium acetate, pH 5.5, at 37°.

To assay endoglycosidase activity, we incubated 5 mg of ovalbumin in 0.5 ml buffer containing 0.5% SDS with 20 µl of enzyme solution. After 1 to 2 hours, we added an equal volume of cold 12.5% TCA, held the tubes at 0° for 15 minutes, then centrifuged at 23,000 g for 30 minutes. We assayed 0.5 ml aliquot of the clear supernatant for total neutral sugar by the phenol-H_2SO_4 method (3). For the control, we added TCA to the ovalbumin before adding the enzyme.

To isolate the oligosaccharide(s) from the enzymic digest, we incubated 1 mg of neuraminidase with 200 mg of ovalbumin overnight in 20 ml of buffer containing 0.5% SDS. We lyophilized the mixture, dissolved it in 5 ml of H_2O, and applied it to a Sephadex G-50 column (2.5 x 90 cm) equilibrated with H_2O. We eluted the column with H_2O and detected the oligosaccharide(s) in the column effluents by phenol-H_2SO_4 (3).

When the glycopeptide Asn-$(GlcNAc)_2(Man)_5$ was used as substrate, we incubated 2 mg of glycopeptide and 0.5 mg of enzyme in 0.15 ml of buffer for 4 hours. The liberation of Asn-GlcNAc was detected by paper chromatography using 1-butanol-ethanol-water (2:1:1) as solvent (4). The Asn-GlcNAc was visualized with 0.5% nihydrin in acetone. For the detection of endo-β-N-acetyl-glucosaminidase activity using [^{14}C] or [^{3}H]-N-acetyl-Asn-$(GlcNAc)_2(Man)_5$, we used the procedure of Koide and Muramatsu (5), except that the reaction mixture was analyzed by paper chromatography (4).

Presence of N-acetylglucosamine at the reducing end of the saccharide chain was determined by treating the oligosaccharide with $NaBH_4$, and subsequently detecting glucosaminitol in the acid hydrolyzate by means of an amino acid analyzer (6).

We assayed for various exoglycosidase activities by the procedure previously described (1), and for sugar composition of oligosaccharides, glycoproteins, and glycopeptides by the procedure described by Spiro (6).

Proteolytic activity was measured by using insoluble azo-dye-bound collagen (Azocoll), casein, hemoglobin, human serum albumin, and ovalbumin as substrates (7).

RESULTS AND DISCUSSION. Fig. 1 shows the release of TCA-soluble oligosaccharide(s) from ovalbumin by neuraminidase type V. When the time interval was fixed, the release of oligosaccharide(s) from ovalbumin was proportional to the amount of enzyme added (Fig. 1A); with fixed enzyme concentration, the

[4]Using neuramin-lactose as substrate.

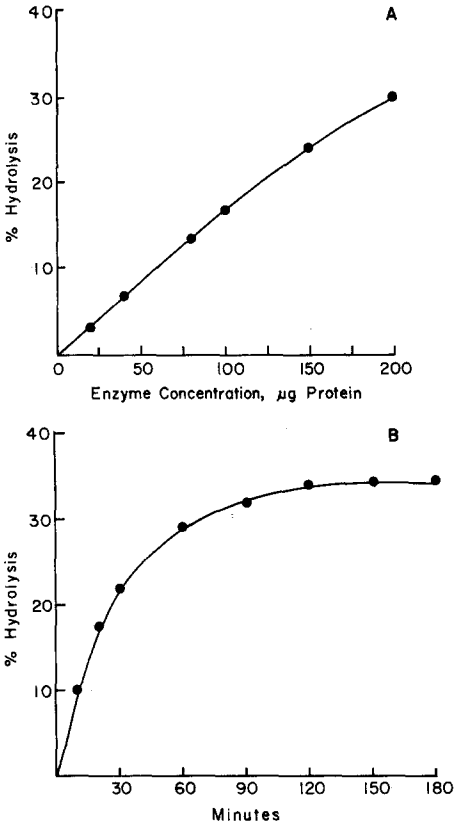

Fig. 1 Rate of release of TCA-soluble neutral sugar from ovalbumin as a function of protein (A) and time (B). (A) 5 mg each of ovalbumin in 0.5 ml 0.05 M sodium acetate, pH 5.5, containing 0.5% SDS was incubated with different amounts of neuraminidase type V at 37° for 1 hour. (B) 5 mg each of ovalbumin in 0.5 ml 0.05 M sodium acetate, pH 5.5, containing 0.5% SDS was incubated with 200 µg of neuraminidase type V at 37° for different periods of time. A detailed assay procedure is described in "Material and Methods."

release of oligosaccharide(s) was proportional to the incubation time for the first 30 minutes (Fig. 1B). It should be noted that under these conditions, the enzyme could only liberate about 34% of the total protein-bound neutral sugar from the intact ovalbumin after prolonged incubation. With ovalbumin, and using 0.05 M acetate buffer, the optimal pH for endoglycosidase activity was found to be 5.5, which is within the optimal pH range of the neuraminidase (8). We found that the presence of 0.5% SDS greatly facilitated the enzymic

release of the oligosaccharide(s) from the intact ovalbumin; without SDS, the hydrolysis was only 20% of that with SDS. Although it has been reported that heat denaturation (9) or sulfitolysis (10) of ovalbumin facilitates the action of endoglycosidases, we found that the addition of SDS was simpler and the results more reproducible.

The possibility that the TCA-soluble, phenol-H_2SO_4-positive material released from ovalbumin may be mannose due to the action of contaminating α-mannosidase was considered. However, first, no free mannose could be detected in the reaction mixture by an automated sugar analyzer (11). Secondly, no hydrolysis of the following p-nitrophenylglycoside occurred upon incubation

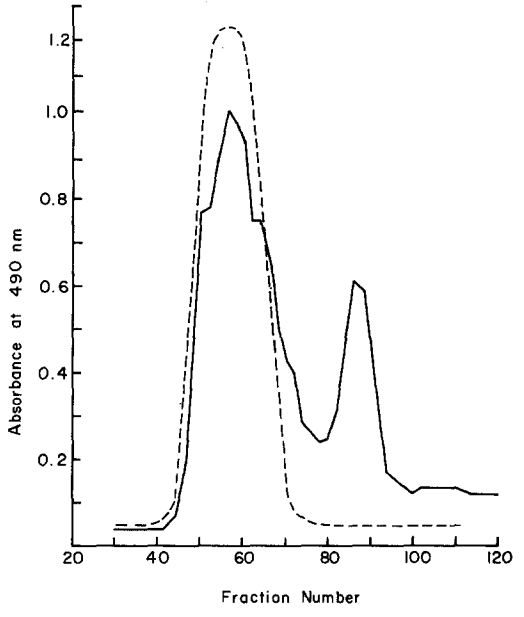

Fig. 2 Sephadex G-50 chromatography of the products formed on incubation of ovalbumin with neuraminidase type V. Ovalbumin (200 mg) in 20 ml 0.05 M sodium acetate, pH 5.5, was incubated at 37° for 16 hours with 1 mg neuraminidase type V (_____), and without enzyme (-----). The reaction mixture was freeze-dried, dissolved in 5 ml H_2O, and applied to a Sephadex G-50 (2.5 x 80 cm) column previously equilibrated with H_2O. Fractions of 5 ml were collected at a flow rate of 15 ml per hour. Aliquots of 0.5 ml were analyzed for neutral sugar. Fractions 82 to 93 were pooled, lyophilized, and designated the oligosaccharide fraction.

with 500 μg neuraminidase types V and VI for 16 hours (1): α- and β-D-galactopyranosides, α- and β-D-mannopyranosides, α- and β-D-glucopyranosides, α- and β-2-acetamido-2-deoxy-D-glucopyranosides, and α-2-acetamido-2-deoxy-D-galactopyranoside.

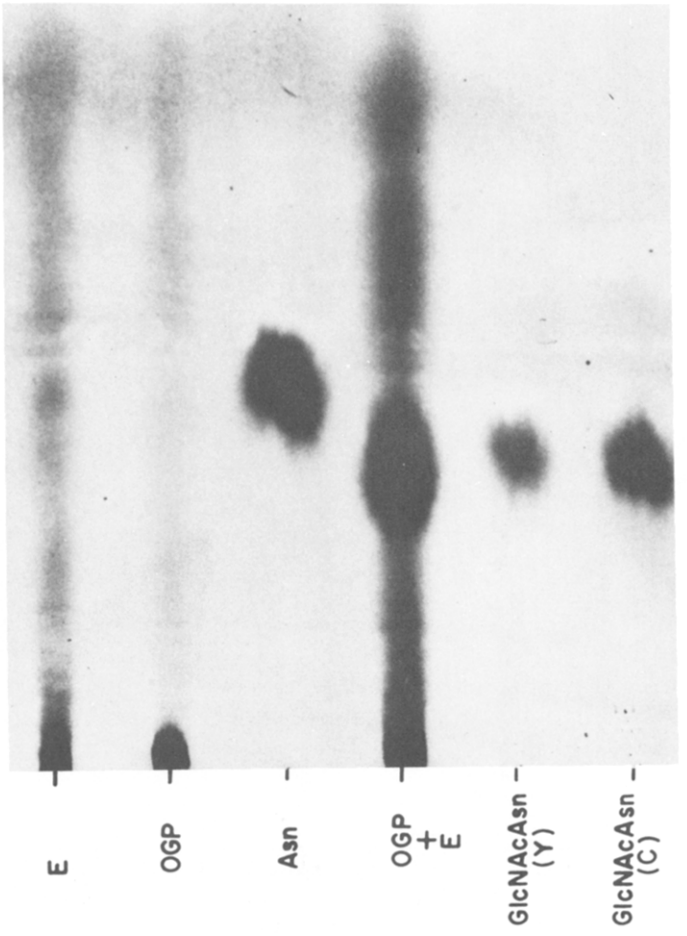

Fig. 3 Chromatographic detection of the liberation of Asn-GlcNAc from Asn-$(GlcNAc)_2(Man)_5$ by neuraminidase type V. OGP, the ovalbumin glycopeptide Asn-$(GlcNAc)_2(Man)_5$; E, neuraminidase type V; Asn, asparagine; Y, sample provided by Dr. I. Yamashina; C, product of Cyclo Chemical. Detailed assay conditions are described in "Materials and Methods."

Fig. 2 shows the enzymic liberation of oligosaccharide(s) from ovalbumin by Sephadex G-50 filtration. The oligosaccharide(s) peak (fractions 82 to 93) was pooled, lyophilized, and designated as the oligosaccharide fraction. Sugar analysis of this fraction revealed that it contained mannose and glucosamine in a ratio of 5.5:1. Reduction of the oligosaccharide fraction by $NaBH_4$ followed by acid hydrolysis (6) resulted in the detection of glucosaminitol.

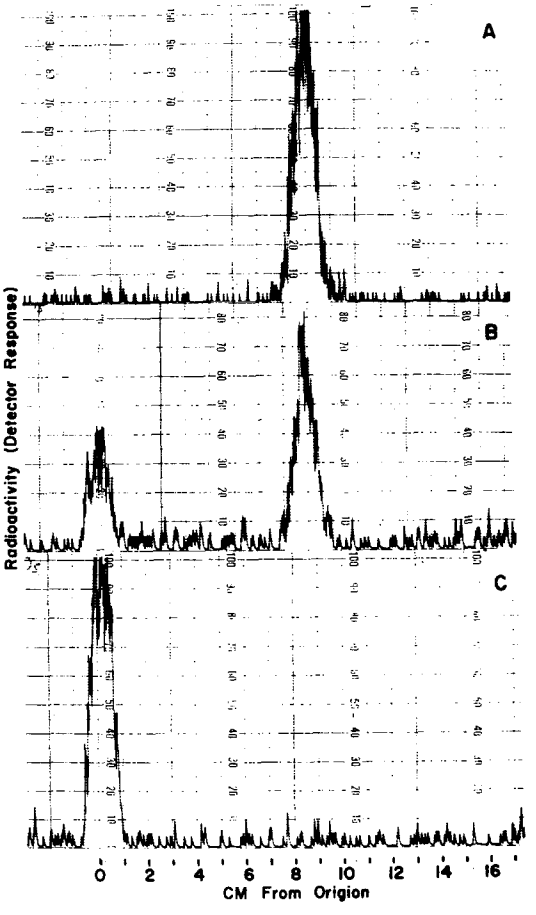

Fig. 4 Hydrolysis of [^{14}C]-N-acetyl-Asn-$(GlcNAc)_2(Man)_5$ by neuraminidase type V.

(A) Standard [^{14}C]-N-acetyl-Asn-GlcNAc; (B) incubation of [^{14}C]-N acetyl-Asn-$(GlcNAc)_2(Man)_5$ with neuraminidase type V; (C), incubation of [^{14}C]-N-acetyl-Asn-$(GlcNAc)_2(Man)_5$ in the absence of enzyme. The incubation mixtures were analyzed by paper chromatography as described in "Material and Methods."

This suggests that the oligosaccharide contains GlcNAc at its reducing terminal Work is in progress to determine whether the oligosaccharide fraction contains a single oligosaccharide chain or a mixture of several oligosaccharide chains.

As shown in Fig. 3, incubation of Asn-$(GlcNAc)_2(Man)_5$ with neuraminidase type V resulted in the detection of a ninhydrin-positive compound with the chromatographic mobility identical to that of Asn-GlcNAc. Treatment of Asn-$(GlcNAc)_2(Man)_5$ with neuraminidase type V also resulted in the detection of glucosaminitol. A similar result was obtained with Taka-amylase A glycopeptide. The liberation of [^{14}C]-N-acetyl-Asn-GlcNAc from [^{14}C]-N-acetyl-Asn-$(GlcNAc)_2(Man)_5$ is shown in Fig. 4.

The presence of proteolytic activity in neuraminidase type V was

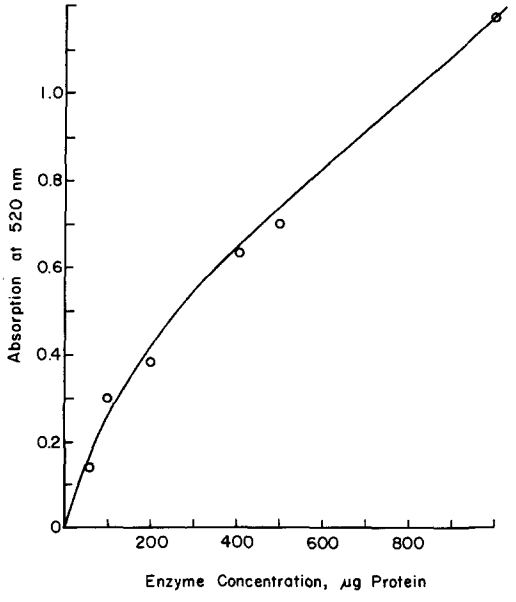

Fig. 5 Hydrolysis of Azocoll by neuraminidase type V. Varying amounts of enzymes were incubated with 2.25 mg Azocoll in 0.6 ml of 0.05 M Sodium Acetate, pH 5.5, at 37° for 90 minutes. A Millipore Swinnex 13 filter head with 14µ filter was used to filter the reaction mixture. Detailed assay conditions using Azocoll as substrate have been described by Moore (7).

demonstrated by using Azocoll as substrate (Fig. 5). Optimal proteolytic activity using Azocoll was found to be about pH 6.2. Besides Azocoll, the enzyme also hydrolyzed casein, hemoglobin, ovalbumin, human serum albumin, and α_1-acid glycoprotein.

The results documented above clearly indicate that neuraminidase type V contains endoglycosidase and protease activities. The specificity of endoglycosidase is concluded to be endo-β-N-acetylglucosaminidase. Endo-β-N-acetylglucosaminidase has been isolated from Streptomyces griseus (4, 10) and Diplococcus peneumoniae (5, 12). Generally the endo-β-N-acetylglucosaminidase and protease activities in neuraminidase type VI are approximately 10 to 15% of that in type V. It should be pointed out that these two activities in the commercial neuraminidase preparations varied from lot to lot. However, we have never obtained a preparation which is completely free from endo-β-N-acetylglucosaminidase and protease activities.

Many studies of the structure and function of glycoconjugates are being made by using exoglycosidases. We cannot overemphasize the serious consequences of interpreting the results of such studies using neuraminidase contaminated with endo-β-N-acetylglucosaminidase and protease.

Work is in progress to separate and purify the protease, endo-β-N-acetylglucosaminidase, and neuraminidase activities from the commercial preparations.

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