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Neuraminidase in *Bacteroides fragilis*

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A neuraminidase from *Bacteroides fragilis* was purified 542-fold by isoelectric focusing, adsorption chromatography on Affi-Gel 202, and gel filtration chromatography on Sephadex G-200. On isoelectric focusing the neuraminidase was resolved into three differently charged fractions with pI values of 6.8, 7.1, and 7.4. The major component of pI 7.1 was used for further purification. The purified enzyme had optimal activity at pH 6.4 with *N*-acetylneuraminlactose as the substrate. Its molecular weight, determined by Sephadex G-200 gel filtration chromatography, was 92,000. The neuraminidase hydrolyzed terminal neuraminic acid residues from *N*-acetylneuraminlactose, fetuin, bovine submaxillary mucin, and porcine stomach lining mucin. A new method for the detection of neuraminidase activity is described which is based on rocket affinoelectrophoresis. It utilizes the differences in the interaction of sialylated and desialylated mucin with *Helix pomatia* lectin, enzymatic activity being detected by formation of affinorockets after incubation of the neuraminidase with bovine submaxillary mucin.

Neuraminidases (EC 3.2.1.18) are produced by many bacterial species including pathogens, e.g., *Vibrio cholerae* (22) and *Clostridium perfringens* (9, 19) and indigenous bacteria such as a wide range of species within the family *Bacteroidaceae* (10). Neuraminidase has been considered to be a virulence factor by participating in the degradation of the protective mucin layer in the human colon (24) or by hydrolyzing neuraminic acid from human immunoglobulins (8).

Studies on the carbohydrate sources of human indigenous bacteria indicate that simple dietary sugars are not available to the colon microflora (23). Instead, the major sources may be either nondigestable mucopolysaccharides or secretorial glycoproteins (25), indicating an important role for various glycoside hydrolases, including neuraminidase, in bacterial nutrition. The *Bacteroides fragilis* group accounts for approximately 27% of the cultivable human microflora (12), and the species *B. fragilis* is frequently isolated from intraabdominal infections (21). *B. fragilis* has been shown to ferment bovine submaxillary mucin (24) and to produce a wide range of exoglycosidases (2, 10).

Assays for neuraminidase activity, using naturally occurring glycoproteins or mucopolysaccharides as substrates, have been based on the detection of free neuraminic acid by the thiobarbituric acid assay (1, 28). This method allows accurate determination of the amount of neuraminic acid hydrolyzed, but cannot be used when samples containing various other carbohydrates are to be analyzed. However, during the past 5 years, alternative electrophoretic techniques have been developed which permit detection of glycoproteins and desialylated glycoproteins based on their interactions with lectins (4, 16).

The aim of the present investigation was to purify and characterize neuraminidase from *B. fragilis*. Concomitantly, a new method for detection of neuraminidase activity was developed which is based on the interaction between *Helix pomatia* lectin and the α-*N*-acetylgalactosyl moieties of desialylated bovine submaxillary mucin.

MATERIALS AND METHODS

Chemicals. Ampholine carrier ampholytes were purchased from LKB-Produkter, Stockholm, Sweden; Affi-Gel 202 was from Bio-Rad Laboratories, Richmond, Calif. *H. pomatia* lectin, Sephadex G-200, and blue dextran were from Pharmacia Fine Chemicals, Uppsala, Sweden; agarose (Indubiose A37) was from L'Industrie Biologique Francaise, Gennevilliers, France; *N*-acetylneuraminic acid (NANA), bovine serum albumin, *p*-nitrophenyl glycosides, *N*-acetylneuraminlactose, bovine submaxillary mucin, fetuin, porcine stomach lining mucin, Azocasein, and molecular weight markers were from Sigma Chemical Co., St. Louis, Mo.; proteose peptone and yeast extract were from Difco Laboratories, Detroit, Mich.

Bacterial strain. *B. fragilis* strain B70 was obtained from the Department of Bacteriology, National Bacteriological Laboratory, Stockholm, Sweden. The strain was isolated from a patient with septicemia and identified by the method of Holdeman et al. (13).

Culture conditions. *B. fragilis* B70 was grown under anaerobic conditions, at pH 7.0 ± 0.05 in a 1-liter fermentor (FL 101; Biotec, Stockholm, Sweden). The

culture was stirred at 200 rpm, and the temperature was 37 ± 0.1°C. The medium contained proteose peptone, yeast extract, glucose, cysteine hydrochloride, and resazurin (2). The cultures were harvested in the late-logarithmic phase of growth.

Neuraminidase assays. (i) Determination by release of sialic acid. Neuraminidase activity was measured by incubating 20 μl of enzyme with 180 μl of 1 mM *N*-acetylneuraminlactose in 0.1 M sodium acetate buffer (pH 6.0). The reaction was stopped by the addition of 0.1 ml of 0.2 M sodium periodate in 9 M *ortho*-phosphoric acid. Enzymatically released NANA was determined by the method of Warren (28). Concentrations of NANA were obtained from a standard curve of absorbance versus the amount (nanomoles) of NANA. Enzymatic activity was expressed in katals. Bovine submaxillary mucin, porcine stomach lining mucin, and fetuin were used as substrates in a similar manner, except that, before the periodate oxidation, the reaction was stopped by the addition of an equal volume of 5% phosphotungstic acid in 2 M hydrochloric acid. The tubes were centrifuged at 2,500 × *g* for 20 min to remove precipitated proteins, and 0.2 ml of the supernatant fraction was used in the thiobarbituric acid assay.

(ii) Detection of neuraminidase activity by rocket affinoelectrophoresis. The reaction mixture consisted of 0.2 ml of bovine submaxillary mucin (2 mg/ml) in 0.1 M sodium acetate buffer (pH 6.0) and 0.1 ml of the enzyme solution. The reaction was stopped by the addition of 2.1 ml of Tris-barbiturate buffer, pH 8.6 (73 mM Tris, 24 mM barbituric acid), containing calcium lactate (0.36 mM) and sodium azide (3 mM) and immediately immersing the tubes into a boiling water bath for 5 min. Samples (5 μl) of the reaction mixture were applied into wells (2.5-mm diameter) in agarose gel containing 5 μg of *H. pomatia* lectin per ml. Electrophoresis was carried out at 10°C for 2 h (10 V/cm) in Tris-barbiturate buffer (30) with Multiphor electrophoresis equipment (LKB-Produkter). After electrophoresis, the gels were dried and stained with Coomassie brilliant blue R-250 (29).

Other enzyme assays. α-L-Fucosidase, α- and β-galactosidase, α- and β-*N*-acetylglucosaminidase, and α-*N*-acetylgalactosaminidase were assayed with *p*-nitrophenyl glycosides (5 mM in 0.05 M sodium phosphate buffer, pH 6.2) as substrates (20). Protease was assayed with Azocasein as the substrate (7).

Protein determination. Elution of protein was monitored by the measurement of absorbancy at 280 nm. Quantitative determination of protein was by the method of Lowry et al. (17) with bovine serum albumin as the standard.

Purification procedure. (i) Step 1. A 1,000-ml culture of *B. fragilis*, grown to the late-logarithmic phase, was centrifuged at 13,700 × *g* for 15 min. The supernatant fluid was discarded, and the cells were washed three times in 0.9% sodium chloride and centrifuged at 13,700 × *g*. The cells were suspended in 90 ml of a 1% (wt/vol) Ampholine solution (pH range, 3.5 to 10) and disrupted with a W-375 sonicator (Heat Systems-Ultrasonic, Inc., Plainview, N.Y.). The suspension of disrupted cells was centrifuged in two steps, at 10,000 × *g* for 30 min to remove whole cells and cell walls, and at 67,000 × *g* for 40 min to remove membrane fragments.

(ii) Step 2. The clear ultracentrifugation supernatant fraction was used for density gradient isoelectric focusing in a 440-ml column (8100; LKB-Produkter). Carrier Ampholines pH 3.5 to 10 and pH 6 to 8 in equal parts were used in a final concentration of 2% (wt/vol) to establish the pH gradient. The isoelectric focusing was performed with the anode at the bottom of the column, which resulted in considerable precipitation of acidic proteins and nucleic acids in the lower pH ranges. To avoid contamination of harvested fractions with these precipitates, the column was eluted upward, i.e., drained from the top by pumping a dense sucrose solution in at the bottom. The electrofocusing was run for 48 h at 10°C with a final potential of 500 V. Fractions (10 ml each) containing neuraminidase activity were pooled and dialyzed against 0.02 M Tris-hydrochloride (pH 7.0).

(iii) Step 3. The dialyzed fractions were applied onto a gel bed (1.6 by 20 cm) of Affi-Gel 202 equilibrated with 0.02 M Tris-hydrochloride buffer (pH 7.0). The column was washed with the same buffer until no further proteins were eluted. The neuraminidase was eluted by the Tris-hydrochloride buffer containing 0.5 M sodium chloride and concentrated to 2 ml by ultrafiltration (Pellicon Membrane Disc; nominal molecular weight exclusion limit, 10,000; Millipore Corp., Bedford, Mass.).

(iv) Step 4. The neuraminidase pool from step 3 was applied to a gel bed (1.6 by 70 cm) of Sephadex G-200 equilibrated and eluted with 0.1 M sodium acetate

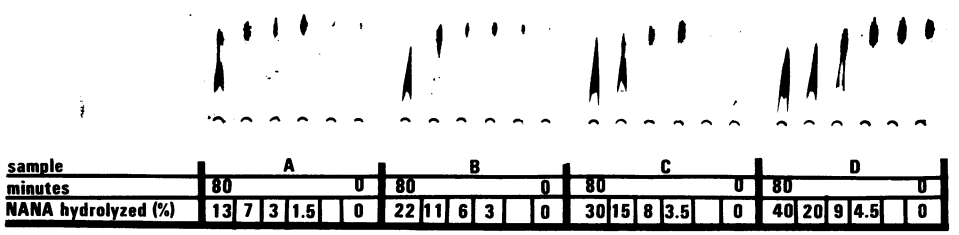

FIG. 1. Affinorocket formation after 80, 40, 20, 10, 5, and 0 min. The purified neuraminidase fraction from step 4 was incubated with bovine submaxillary mucin as a substrate in the following concentrations (milligrams per milliliter): 10 (A), 5 (B), 2.5 (C), and 1.25 (D). Before the electrophoresis, the samples were diluted by the addition of the electrophoresis buffer solution to a final concentration of 0.15 mg of bovine submaxillary mucin per ml.

TABLE 1. Purification of neuraminidase from *B. fragilis*

Step	Procedure or fraction	Vol (ml)	Total protein (mg)	Total activity (nkat)	Sp act (nkat/mg)	Yield (%)	Purification factor
1	Ultracentrifugation supernatant fluid	90	1.597	27.9	0.0175	100	1
2	Isoelectric focusing	10	35.0	5.9	0.17	21	10
3	Affi-Gel 202	8	1.6	3.8	2.38	13	135
4	Sephadex G-200	2	0.2	1.9	9.5	6.8	542

buffer (pH 6.0). Neuraminidase-active fractions were pooled and used in the characterization experiments.

RESULTS

Affinoelectrophoretic assay. By utilizing the differences in precipitates formed by the interaction of *H. pomatia* lectin with mucin before and after desialylation to various degrees, a screening assay for neuraminidase activity was developed. The extent of hydrolysis of NANA from mucin was determined by the thiobarbituric acid assay and compared with the formation of affinity rockets after electrophoresis (Fig. 1). Upon electrophoresis into agarose gels containing the lectin of *H. pomatia*, the desialylated bovine submaxillary mucin gave distinct affinity rockets of reproducible height. When 8% of the total NANA content was hydrolyzed, precipitate formation started. With 20% of the NANA released, precipitates, easily distinguished as affinorockets, were formed at a distance from the sample wells, whereas upon 30% hydrolysis the affinity precipitate extended from the edge of the sample well.

Purification. A summary of the purification procedure is given in Table 1. The accuracy of the affinoelectrophoretic assay is demonstrated in Fig. 2 by comparison of the affinity rocket electrophoresis assay with the Warren method for determination of free sialic acid in a screening of neuraminidase activity in step 2 from the purification procedure. The isoelectric focusing step resolved neuraminidase into three differently charged fractions. One major fraction, almost free from α-L-fucosidase, but containing large amounts of β-galactosidase, eluted at pH 7.1, and two minor fractions eluted at pH 7.4 and 6.8 together with α-L-fucosidase and β-galactosidase.

The pI 7.1 fraction was used for Affi-Gel 202 adsorption. By this chromatographic procedure, neuraminidase was eluted free from α-L-fucosidase. The subsequent Sephadex G-200 gel filtration chromatography separated the neuraminidase from β-galactosidase (Fig. 3).

Properties. The activity of partially purified neuraminidase from the isoelectric focusing step was retained to 95% after storing at −18°C for 1 month. In contrast, the enzyme, once purified 540-fold, was unstable and lost about 25% of its activity when stored at −18°C for 24 h.

Neuraminidase activity at various pH values in three different buffers was measured with *N*-acetylneuraminlactose as substrate. Optimal activity was achieved at pH 6.4. The hydrolysis

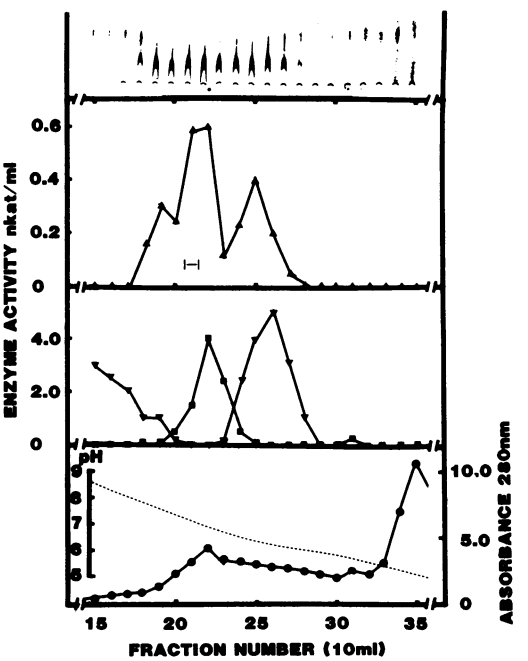

FIG. 2. Isoelectric focusing of neuraminidase from *B. fragilis*. The ultracentrifugation supernatant fluid from step 1 (90 ml) was applied to the column in a 2% Ampholine solution stabilized with a sucrose gradient. After 48 h at 400 to 500 V, the column was drained from the top, and 10-ml fractions were collected. Neuraminidase activity was assayed by the thiobarbituric acid assay after incubation of the dialyzed fractions with *N*-acetylneuraminlactose (▲) or by rocket affinoelectrophoresis of bovine submaxillary mucin, after incubation with the fractions, in agarose gel containing *H. pomatia* lectin. The fraction (21) indicated by the bar was further purified. Symbols: (●) absorbance at 280 nm, (.....) pH, (■) β-galactosidase, (▼) α-L-fucosidase.

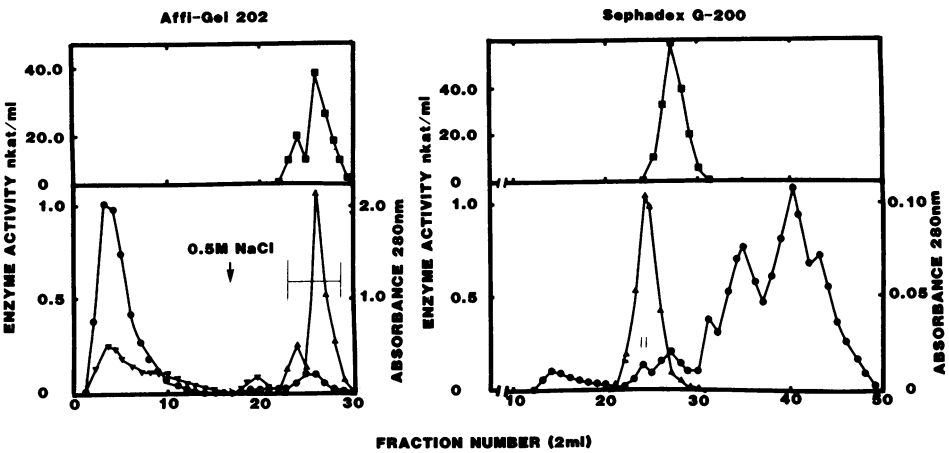

FIG. 3. Further purification of neuraminidase by Affi-Gel 202 adsorption and Sephadex G-200 gel chromatography. The pooled enzyme from step 2 was dialyzed and applied onto a gel bed (1.6 by 20 cm) of Affi-Gel 202 equilibrated with 0.02 M Tris-hydrochloride buffer and washed with the same buffer. Neuraminidase was eluted by the addition of 0.5 M NaCl into the buffer. The pooled enzyme was concentrated and applied onto a gel bed (1.6 by 70 cm) of Sephadex G-200 equilibrated and eluted with 0.1 M sodium acetate buffer (pH 6.0). The fractions indicated by the bar were pooled. Symbols: (●) absorbance at 280 nm, (▲) neuraminidase, (■) β-galactosidase, (▼) α-L-fucosidase.

rate was 52% less when 0.1 M sodium phosphate buffer was used instead of 0.1 M sodium acetate buffer at optimal pH.

Cations and other reagents were examined for their effects on the neuraminidase activity (Table 2). The enzyme activity was increased when organic acids were included in the assay or when the assay was performed in sodium acetate buffer which is a common property. When Tris-hydrochloride buffer was used, Ca^{2+}, Mg^{2+}, and Mn^{2+} increased the activity, whereas Zn^{2+} and EDTA decreased the hydrolysis.

The rate of release of NANA from different substrates was determined at different substrate concentrations. The results were plotted as double reciprocals to obtain K_m values. The K_m values for neuraminlactose, fetuin, and bovine submaxillary mucin were 0.51 mM, 6.0 mg/ml, and 2.0 mg/ml, respectively.

Neuraminic acid was also released from porcine stomach lining mucin. The total amount of NANA in the commercial preparation of this mucin was, however, too low to permit determination of the K_m value for this substrate.

The molecular weight of the neuraminidase was determined by gel filtration chromatography from its elution volume relative to void volume on a gel bed of Sephadex G-200 (1.6 by 40 cm). From a plot of K_{av} against $\log_{10}$ molecular weight for the standard markers blue dextran, bovine gamma-globulin, transferrin, bovine albumin, ovalbumin, β-chymotrypsinogen, myoglobin, RNase, and 2,4-dinitrophenylalanine, a molecular weight of 92,000 ± 5,000 was obtained.

DISCUSSION

On density gradient isoelectric focusing, neuraminidase was recovered in three differently charged fractions. A neuraminidase from *C. perfringens* has also been reported to resolve into multiple charged forms (19), and similar microheterogeneities have been found during the purification of a neuraminidase from *Coryne-*

TABLE 2. Effects of metal ions, EDTA, and organic acids on neuraminidase activity with *N*-acetylneuraminlactose as substrate[a]

Additive	Relative activity (%) with additive at indicated concentration:	
	1 mM	10 mM
Ca^{2+}	115	120
Mg^{2+}	120	107
Mn^{2+}	110	110
Zn^{2+}	58	52
EDTA	61	78
Acetic acid	125	116
Lactic acid	125	120
Propionic acid	125	120

[a] The assays were performed in 0.1 M Tris-hydrochloride (pH 7.0) with cations or EDTA as additives and in 0.1 M sodium phosphate buffer (pH 6.5) with the organic acids. A purified neuraminidase fraction from step 4 (0.5 nkat/ml) was used in the experiment.

bacterium diphtheriae (18) and during the purification of glycosidases from *B. fragilis* (2). For the purposes of further purification of neuraminidase in the present investigation, the minor fractions and a part of the pI 7.1 fraction were excluded due to high levels of α-L-fucosidase or β-galactosidase or both. The subsequent Affi-Gel 202 chromatography did not separate the neuraminidase and the β-galactosidase as has been reported for other, similar bacterial enzyme mixtures (3). Both enzymes were adsorbed and were not separated by elution with a sodium chloride gradient. However, in the final Sephadex G-200 gel filtration chromatography step, the neuraminidase and the β-galactosidase were separated.

The properties of the *B. fragilis* neuraminidase were not altogether in accordance with those reported for other bacterial neuraminidases. In contrast to our results, Fraser and Brown (10) reported almost no effect of Ca^{2+} when a *B. fragilis* neuraminidase was incubated with a high-molecular-weight substrate in sodium acetate buffer.

Although the neuraminidases from *Pasteurella haemolytica* (26), *C. perfringens* (6), and the group A streptococcus (8) also showed enhanced activity upon the addition of Ca^{2+} and Mg^{2+}, the activity of the *P. haemolytica* neuraminidase was increased by the addition of EDTA. The difference regarding the *B. fragilis* enzymes may be explained by isolation of neuraminidases from different subspecies, by the use of different growth media for neuraminidase production, and by the use of different neuraminidase substrates. The assay buffers were also different, and it might be possible that the effect of Ca^{2+} was undetectable in the sodium acetate buffer system used by Fraser and Brown.

The difference in pH optimum observed in the present investigation and that obtained by Fraser and Brown (10) for a neuraminidase from *B. fragilis* was 1.9 pH units. Our pH optimum with *N*-acetylneuraminlactose as substrate and that obtained for a neuraminidase from *Streptococcus pneumoniae* (14) are among the highest reported so far for bacterial neuraminidases. However, in a short communication, von Nicolai et al. (27) reported optimal activity at pH 6.0 for a neuraminidase from *B. fragilis*, probably with *N*-acetylneuraminlactose as a substrate, which also differs considerably from that reported by Fraser and Brown (10) of pH 4.5.

Bovine submaxillary mucin is frequently used as a substrate for neuraminidase (8). The predominant carbohydrate chain found in most submaxillary mucins is the sialyl-α-2,6-*N*-acetylgalactosamine disaccharide (5, 15). The carbohydrate binding of the lectin from *H. pomatia* is highly specific for *N*-acetylglycopyranosides with the highest affinity toward terminal nonreducing *N*-acetyl-α-D-galactosamine (11). By utilizing the differences in precipitates formed between lectin and mucin after varied extents of NANA hydrolysis, detection and limited quantitation of neuraminidase activity were possible. The assay procedure was not affected by the presence of sucrose in the reaction mixture. This finding permitted immediate screening of the fractions from the isoelectric focusing step for neuraminidase activity in spite of the presence of the sucrose used to stabilize the pH gradient. The method is, however, not as accurate for quantification of enzymatic activity as colorimetric methods, but can be valuable for localization of neuraminidase in fractions obtained on separation and purification.

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