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PNEUMOCOCCAL NEURAMINIDASE: PURIFICATION AND PROPERTIES

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SUMMARY

A relatively short purification scheme is described for pneumococcal neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18). The enzyme has been characterized using polyacrylamide gel electrophoresis and microisoelectric focusing on polyacrylamide gel. The purified enzyme has an estimated molecular weight of 69 800 and a pI of 4.90.

INTRODUCTION

In experiments performed to study the pathogenesis of cerebral dysfunction in experimental pneumococcal meningitis it became necessary to evaluate the effects of pneumococcal neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18) administered intrathecally to dogs (R. D. O'Toole and W. L. Stahl, unpublished data). Crude and purified neuraminidase was prepared by utilizing variations of published procedures. This study describes the purification scheme adapted and provides further insight into the molecular weight and isoelectric point of the enzyme.

MATERIALS AND METHODS

A Type I pneumococcus isolated from the cerebral spinal fluid of a patient with meningitis was employed in all experiments[1]. It was passaged in mice every six weeks, and stored in 50% defibrinated rabbit blood at −70 °C until used.

Enzymatic assays

Neuraminidase. 0.2 ml of enzyme preparation was incubated at 37 °C with

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Abbreviations: NANA, *N*-acetylneuraminic acid; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

2.5 mg of human glycoprotein (Fraction VI, CalBiochem) or 0.05–2 mg of *N*-acetylneuraminic acid (NANA) lactose (Sigma), which had been dissolved in 0.8 ml of 0.15 M NaCl–0.015 M sodium phosphate (pH 6.5) as substrate. After 60 min incubation (standard conditions), duplicate 0.4-ml samples were taken from the reaction mixture and were analyzed for free NANA by the thiobarbituric acid assay of Warren[2] with saline containing substrate as a control and crystalline NANA (Koch-Light Labs, Ltd, Colnbrook, England) as the standard. One unit of activity is defined as the amount of neuraminidase required to liberate 1 nmole of NANA per min under assay conditions. Released NANA was estimated using Eqn 2 of Warren[2].

NANA-lyase (N-acetylneuraminate pyruvate-lyase, EC 4.1.3.3) activity. This was measured by incubating enzyme with 100 mM potassium phosphate (pH 7.2) and 10 mM NANA in a final volume of 500 μl for 15 min at 37 °C. The reaction was stopped by heating to 100 °C for 2 min. The *N*-acetylmannosamine formed during this reaction was determined by the method of Reissig *et al.*[3]. After cooling, 0.1 ml of 0.8 M potassium tetraborate was added and the resulting solution was heated to 100 °C for 12 min. After cooling again, 3.0 ml of diluted *p*-dimethylaminobenzaldehyde reagent were added and the solution was incubated at 37 °C for 10 min. Absorbance was measured at 585 nm and the amount of *N*-acetylmannosamine was determined by comparison to a standard curve constructed using pure *N*-acetylmannosamine.

β-Galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23) enzyme activity. This was assayed[4] in a system consisting of 0.05 M Tris–HCl (pH 7.2), 5 mM *o*-nitrophenyl-β-D-galactopyranoside (Sigma); and 0.5 mg or less of enzyme in a final volume of 200 μl. This mixture was incubated for 30 min at 37 °C; the tubes were then chilled in ice and 200 μl of 0.2 M sodium carbonate–0.2 M sodium bicarbonate (pH 10.8) were added. After warming to room temperature the absorbance at 430 nm was read *vs* water.

Analytical methods

Protein. Protein was determined by the method of Lowry *et al.*[5] using bovine plasma albumin as standard.

Molecular weight determination. The polyacrylamide system described in detail by Weber and Osborn[6] was used. In brief, proteins were preincubated at 37 °C for 2 h in sodium phosphate buffer (pH 7.0) containing sodium dodecyl sulfate and β-mercaptoethanol. Bromophenol blue tracking dye and glycerol were then added and approximately 50 μl of sample were applied to the gels. Gels contained: acrylamide (10%, w/v), methylenebisacrylamide (0.27%, w/v), ammonium persulfate (0.075%, w/v), *N,N,N',N'*-tetramethylethylenediamine (TEMED) (0.0015%, v/v), sodium phosphate (0.10 M, pH 7.0) and sodium dodecyl sulfate (0.1%, w/v). These were cast in gel tubes 10 cm long with an inner diameter of 6 mm and the polymerized gels were 82–83 mm long.

Both chambers of the analytical electrophoresis apparatus were filled with sodium phosphate (0.10 M, pH 7.0) containing sodium dodecyl sulfate (0.1%, w/v), and electrophoresis was initiated at 8 mA per gel with migration of the enzyme toward the anode for 4 h. Temperature was maintained at 20 °C throughout a typical 4-h run. Gels were stained with Coumassie brilliant blue. Sources for the standards were: Mann Research Labs, Inc., New York (chymotrypsinogen, ovalbumin and bo-

vine serum albumin); and Boehringer Mannheim Corp., New York (phosphorylase a and glyceraldehyde phosphate dehydrogenase).

Isoelectric microfocusing and determination of isoelectric point of proteins. The method of Catsimpoolas[7,8] was used. Gels were cast in 7.5 cm tubes with an inner diameter of 6 mm. The lower gel was 50 mm long and consisted of 4.9% acrylamide, 0.19% bisacrylamide, 0.05% TEMED, 1% riboflavin and 4.9% ampholine (LKB, 40% solution, pH 3–10). After layering with water, photopolymerization was carried out at room temperature for 40 min. The sample (0.2 mg protein maximum) was added to a solution having a final volume of 100 μl and the following final concentrations: 5% acrylamide, 0.2% bisacrylamide, 0.05% TEMED, 1% riboflavin and 5% ampholine. After this solution was layered on the 50-mm polymerized gel another 100 μl of the first gel solution were applied. After photopolymerization for 40 min the gel tubes were positioned in the Buchler polyAnalyst apparatus with the sample end in the upper chamber, which contained 5% (v/v) H_3PO_4. The lower chamber contained 5% (v/v) ethylenediamine. Electrophoresis (6–8 gels) was performed at constant voltage (140 V). Current was initially adjusted to 5 mA per tube. After 1.5 h the current had declined and stabilized at about 0.6 mA per tube.

At the end of the electrophoresis run duplicate gels were removed and sliced with a lateral gel slicer (Canalco) into approx. 34 units (approx. 1.7 mm each). Duplicate slices were placed in 500 μl of deionized water and soaked overnight at 4–5 °C. The fluid was removed and the pH measured using a small glass combination electrode at room temperature. After recording the observed pH and when enzymic assays were to be performed, the solutions were adjusted to approximately pH 7.0–7.4 by the addition of 1 M Tris or 1 M HCl. Neuraminidase was then assayed in aliquots of these latter samples using 0.05 mg of NANA-lactose as substrate. Generally, additonal gels were stained with Amidoblack to determine the number and positions of protein bands[8]. This was always done with the albumin and ovalbumin standards and neuraminidase since a means for assessing protein band position was essential. After destaining by diffusion the gels were sliced and segments were placed in 500 μl of 1 M NaOH which solubilized the dye within a few h at 37 °C. The $A_{500\ nm}$ was then read and correlated with the pH profile from corresponding gels. When hemoglobin S was used, the $A_{550\ nm}$ was measured on the fluid from the same gel segments used for the pH determination.

Electrofocusing for longer than 1.5 h did not affect the positions of the protein bands.

Purification of neuraminidase

(1) Preparation of the crude enzyme. Thawed pneumococci were inoculated into Todd–Hewitt broth (Difco) and incubated at 37 °C for 48 h. Purity of growth was confirmed by culture onto rabbit blood agar plates. After centrifugation at 700 × *g* for 2 h at 4 °C pneumococci were discarded and protein was precipitated from the supernatant by addition of solid NH_4SO_4 to 75% saturation. After standing overnight at 4 °C the suspension was centrifuged at 700 × *g* for 1 h at 4 °C, the supernatant was discarded, and the precipitate was taken up in 0.9% NaCl and stored at −20 °C. When sufficient precipitate was prepared, all samples were pooled, dialyzed against repeated changes of deionized water, then against 0.9% NaCl (adjusted to pH 7.3 with $NaHCO_3$), and then centrifuged at 95 000 × *g* for 60 min in the cold. The super-

natant was then concentrated through an XM-50 Diaflo membrane filter at 4 °C, filtered through a Millipore filter (pore size 0.45 μm), cultured to confirm sterility, and stored at −20 °C.

(II) DEAE-Sephadex column chromatography. DEAE-Sephadex (A-50, Pharmacia) was washed overnight by stirring in 1 M sodium acetate (pH 7.0) at room temperature. The supernatant was removed by decantation and washing was continued for several days in 0.1 M sodium acetate (pH 7.0) until the supernatant from the washes was chloride free. Columns were packed at room temperature and all subsequent operations were carried out at 4 °C. Enzyme was dialyzed overnight *vs* 0.1 M sodium acetate (pH 7.0) before application to columns. Buffers consisted of 0.1, 0.2, 0.3 and 0.4 M NaCl in 0.1 M sodium acetate (pH 7.0). Fractions were dialyzed *vs* deionized water (pH 6.5–7) overnight prior to enzyme and analytical determinations. Pooled fractions were concentrated by pressure dialysis *vs* deionized water (pH 6.5–7) and were stored at −20 °C or at 4 °C with little change in activity over a period of several months.

(III) G-100 Sephadex chromatography. G-100 Sephadex (particle size 40–120 μm) was swollen by heating with stirring at 90 °C for 5 h and then permitted to stand at room temperature overnight. The columns were equilibrated with 0.10 M sodium acetate (pH 7.0) in the coldroom and the void volume was determined using Blue Dextran. Fraction II (Table I) was dialyzed *vs* the buffer overnight. Fractions were assayed for enzyme activity without further treatment. Fractions having the highest specific activity of neuraminidase were pooled and concentrated by pressure dialysis *vs* deionized water at pH 6.5–7 at 4 °C. Samples were stored at 4 °C.

RESULTS AND DISCUSSION

Purification of neuraminidase

The procedure described here is a modification of the method of Tanenbaum *et al.*[9] in which several of their purification steps have been deleted or modified. Preliminary experiments using CM-Sephadex columns proved unsatisfactory in that enzyme was eluted with the void volume of the column under all conditions attempted. DEAE-Sephadex chromatography, using discontinuous elution provided a relatively simple method for separating neuraminidase of rather high purity from contaminating proteins. In preliminary experiments using small columns the neuraminidase was recovered as a single peak with no indication of multimolecular forms whereas Tanenbaum *et al.*[9] using the same bacterial strain observed multiple profiles of pneumococcal neuraminidase activity with a purification scheme employing DEAE-cellulose and CM-cellulose column chromatography. On larger DEAE-Sephadex columns (Fig. 1) employing lengthy elution times two additional peaks (A and B) were observed which represented approximately 28% of the total enzymic activity recovered. These three major peaks of neuraminidase activity were a consistent observation on the larger columns and supports the findings of Tanenbaum *et al.*[9] on the presence of several neuraminidase isoenzymes in pneumococci. This purification procedure was also successful in removing NANA-lyase which eluted after Peak C at 0.3–0.4 M Na^+.

In Fraction III, a 9-fold purification was observed over the starting material with a net recovery of 22%. The specific activity of Fraction III was comparable to that of the partially purified preparation described by Hughes and Jeanloz[4]. How-

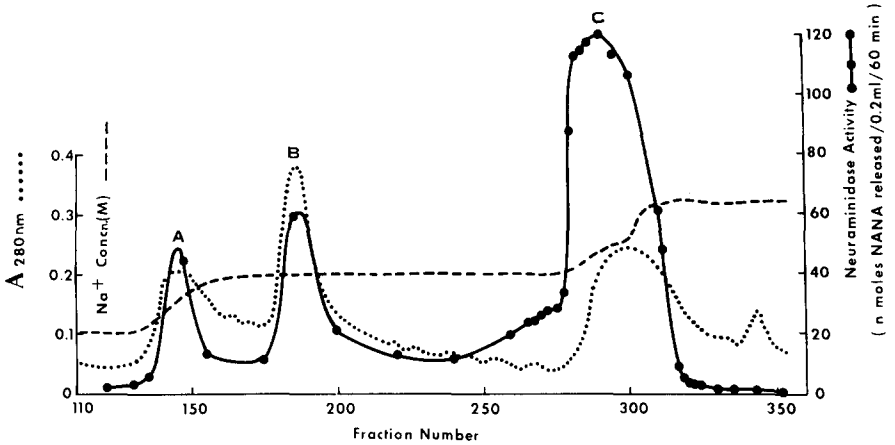


Fig. 1. Purification on DEAE-Sephadex Column. A portion of Fraction I was dialyzed *vs* 0.1 M sodium acetate at 4 °C overnight and 302.5 mg of protein were applied to a DEAE-Sephadex column (9 cm × 13 cm; flow rate: 0.7 ml/min) in the cold room (4 °C). The column was eluted initially with 0.1 M sodium acetate (pH 7.0) and then with 0.1–0.4 M NaCl in 0.1 M sodium acetate (pH 7.0) as indicated and 10-ml aliquots were collected. Enzyme activity was determined on fractions dialyzed *vs* deionized water (pH 6.5–7). Neuraminidase activity on the dialyzed column effluent was measured using human glycoprotein as substrate as described in the text.

ever, the K_m, using NANA-lactose as substrate, was about 3-fold lower than observed by these authors. A comparison to Tanenbaum *et al.*[9] cannot readily be made since different substrates were utilized.

In this study Peaks A and B (Fig. 1) were not characterized. Peak C was further purified by passage through G-100 Sephadex. As observed by Hughes and Jeanloz[4] the enzymic activity appeared immediately after the void volume of the column and a substantial increase in purification of the enzyme was achieved (Table 1). After elution from DEAE-Sephadex columns the fractions containing neuraminidase activity were pooled and concentrated. They were found to be devoid of β-galactosidase and NANA-lyase activities. The purity of these preparations is discussed below.

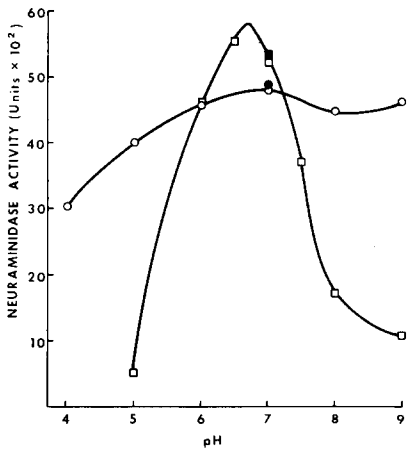
pH optimum and stability. The effect of variation of pH on stability and optimum activity is shown in Fig. 2. Neuraminidase activity reached an optimum at about pH 6.7 which closely agrees with the findings of Hughes and Jeanloz[4]. Little variation in pH stability was observed between pH 6–9 after incubation at 37 °C for 1 h in various buffers at different ionic strengths.

TABLE I

PURIFICATION OF NEURAMINIDASE

Fractions were assayed for 60 min using 2 mg NANA-lactose per assay tube as substrate.

Fraction	*Protein (mg)*	*Specific activity (units/mg)*	*Total activity (units)*	*Recovery (%)*
I. Initial $(NH_4)_2SO_4$ ppt. (dialyzed)	302.5	37.5	11344	100
II. DEAE-Sephadex Peak C	27.8	193.2	5371	47.3
III. Sephadex G-100 peak	7.3	341.8	2495	21.9



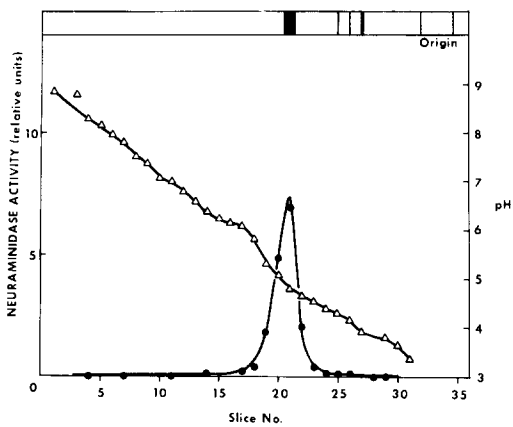


Fig. 2. pH optimum and stability of neuraminidase. To test for pH stability (○) 1.6 μg of enzyme (Fraction II) in 25 μl of water were mixed with 25 μl of buffer. After 1 h at 37 °C, 20-μl aliquots were removed and assayed under standard conditions described in the text with 0.5 mg NANA-lactose as substrate. Buffers used for pH stability were: pH 4 and 5, 0.1 M citric acid mixed with 0.2 M Na_2HPO_4 to the desired pH; pH 6 and 7, 0.2 M Na_2HPO_4 mixed with 0.2 M Na_2HPO_4; pH 7 (●) 0.075 M Na_2HPO_4–0.075 M NaH_2PO_4–0.43% NaCl; pH 8, 0.1 M Tris with 0.1 M HCl; pH 9, 0.2 M glycine with 0.2 M NaOH. The final pH after dilution in standard buffer and during enzyme assay was 6.5 in each case. For the pH optimum (□) 0.64 μg of enzyme in 10 μl was added to 5 μl of substrate (0.5 mg NANA-lactose) and 885 μl of buffer at the desired pH (as used in the stability study). Assay was for 1 h at 37 °C. ■, assayed with 0.075 M Na_2HPO_4–0.075 M NaH_2PO_4–0.43% NaCl (pH 7.0) as buffer.

Fig. 3. Isoelectric point of neuraminidase. This is a representative isoelectric microfocusing experiment of Fraction II (200 μg protein per gel) as described in the text. Two gels were sliced and corresponding slices were combined; these were used for pH and enzymic determinations. A third gel was washed for several days with 12% trichloroacetic acid, rinsed in 7% acetic acid and stained with 0.5% amidoblack in 7% acetic acid and it was destained by diffusion. △—△, pH; ●—●, neuraminidase activity.

Time course of neuraminidase assay. Utilizing NANA-lactose as substrate it was shown that the assay was linear with time for at least 45 min with a small apparent decrease in activity between 45 and 60 min. Assaying for 60 min and calculating on a nmole/min basis may therefore slightly underestimate enzymic activity.

Kinetic paremeters. Hughes and Jeanloz[4] showed that the pneumococcal neuraminidase reaction rate with NANA-lactose was apparently about three times greater than with human glycoprotein. They observed a K_m of $18 \cdot 10^{-4}$ M and V of 3.1 μmoles/min per mg protein using NANA-lactose as substrate. Using NANA-lactose as substrate the velocity of release of NANA was ascertained for Fractions II and III. Lineweaver–Burk plots were constructed and indicated a K_m value of $6.25 \cdot 10^{-4}$ M with V values of 1.47 and 2.49 μmoles/min per mg protein for Fractions II and III, respectively.

Isoelectric point and purity of preparation

All fractions were analyzed by microisoelectric focusing on polyacrylamide gel. The original Fraction I showed many protein bands (data not shown), but after DEAE-Sephadex treatment Fraction II showed only one major protein band (Fig. 3).

TABLE II

ISOELECTRIC POINT OF NEURAMINIDASE AND STANDARD PROTEINS

Means ± S.E. are reported for duplicate runs with the number of separate experiments in parenthesis. For the neuraminidase experiments 2 preparations of Fraction II and one of Fraction III were used.

Protein	*Isoelectric point*		*Literature conditions*
	Observed	*Literature*	
Hemoglobin S	7.26 ± 0.01 (3)	7.23	Isoelectric focusing, polyacrylamide[11]
Serum albumin (bovine)	4.57 ± 0.04 (4)	4.71 (I = 0.1) 4.89 (I = 0.02)	Moving boundary electrophoresis[10]
Ovalbumin	5.07 ± 0.02 (3)	4.59 (I = 0.1) 4.71 (I = 0.01)	Moving boundary electrophoresis[10]
Neuraminidase	4.90 ± 0.02 (3)	—	—

This major band coincided with the peak in neuraminidase activity which had an apparent isoelectric point of 4.90 in this system (Table II). It is of course well known that pI varies with ionic strength as shown in experiments by moving-boundary electrophoresis (*cf.* Table II). In such experiments interpolation is used to estimate the pH that gives zero mobility. With isoelectric focusing a stable pH gradient is formed from low molecular weight synthetic carrier ampholytes. In this system proteins are focused at their isoelectric points and since salts are absent the isoelectric points determined should approach the value at zero ionic strength. Relatively good agreement was observed between the values observed in Table II and literature values for bovine serum albumin[10], ovalbumin[10] and hemoglobin S[11]. A range of values are reported in the literature and vary considerably with ionic strength and the type of buffer utilized. It is of interest, that the microfocusing system permits rapid determination of pI with a minimum amount of sample required, and at the same time permits some estimate of the purity of the preparation under study. This information can be of value in designing further purification steps using electrophoretic or isoelectric focusing procedures. The apparent pI of even relatively impure preparations may be assessed using an analysis of the type shown in Fig. 3. No differences in pI of the neuraminidase were observed between Fractions II and III. Isoelectric focusing of Fraction III yielded a single major protein component with a minor band having a pI of about 3.9 which was devoid of neuraminidase activity. A sizeable portion routinely remained at the origin in these experiments (Fig. 3) and although this area was not assayed in Fig. 3, in other experiments the origin always contained neuraminidase activity. Even more highly purified proteins left some residual material at the original application point and may be due to overloading the gel. Purified Fractions II and III are, therefore, still heterogeneous and will require further purification depending on specific needs. Attempts were made to purify further Fractions II and III by preparative isoelectric focusing on polyacrylamide gels. A single highly purified protein component was obtained but with large losses in enzymic activity. This approach is currently under study.

Molecular weight

Weber and Osborn[6] have shown that polyacrylamide gel electrophoresis in the

presence of sodium dodecyl sulfate can be used with great confidence to determine the molecular weights of a large variety of proteins. This technique was used with Fractions II and III. A straight line was obtained when the electrophoretic mobilities of a number of well-characterized proteins were plotted *versus* the logarithm of the known polypeptide chain molecular weights. Purified neuraminidase from Fractions II and III had an observed electrophoretic mobility of 0.177 ± 0.002 (S.E.) providing an estimated molecular weight of 69 800. This is in agreement with the value of 70 600 ± 1100 estimated by Tanenbaum and Sun[12] by gel filtration for the same pneumococcal enzyme used here but isolated by a somewhat lengthier procedure and differs from the value of 56 000 for the enzyme isolated from *Clostridium perfringens*[13]. These values for the pneumococcal enzyme are somehwat anamalous in that the enzyme has been reported as being excluded from Sephadex G-100 columns (Table 1 and ref. 4). Tanenbaum and Sun[12], however, have been able to obtain a molecular weight for the purified enzyme using G-100 Sephadex. This suggests that in certain experimental situations the enzyme may be aggregated into polymeric forms or perhaps associated with other cellular components such as RNA. Indeed, we estimate that Fraction III contains about 2% nucleic acid based on the observed $A_{280\ nm}/A_{260\ nm}$ ratio. Electrophoresis in the presence of sodium dodecyl sulfate would appear to obviate this difficulty and permits a facile estimation of molecular weight.

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