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Extracellular Neuraminidase Production by a *Pasteurella multocida* A:3 Strain Associated with Bovine Pneumonia

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The properties of an extracellular neuraminidase produced by a *Pasteurella multocida* A:3 strain that was isolated in a case of bovine pneumonia were examined during growth in a defined medium. This enzyme (isolated from concentrated culture supernatants of *P. multocida* A:3) was active against *N*-acetylneuramin lactose, human α-1-acid glycoprotein, fetuin, colominic acid, and bovine submaxillary mucin. Enzyme elaboration was correlated with the growth of the organism in a defined medium, with maximum quantities produced in the stationary phase. The enzyme was purified by a combination of ammonium sulfate fractionation, ion exchange on DEAE-Sephacel, and gel filtration on Sephadex G-200. The purified neuraminidase possessed a specific activity of 9.36 μmol of sialic acid released per min per mg of protein against fetuin. The enzyme possessed a pH optimum of 6.0 and a K_m of 0.03 mg/ml. The *P. multocida* A:3 neuraminidase had a molecular weight of approximately 500,000 as estimated by gel filtration. The enzyme was stable at 4 and 37°C for 3 h. Approximately 75% of the neuraminidase activity was lost within 30 min at 50°C. Greater than 90% of the enzyme activity was destroyed within 10 min at temperatures of ≥65°C. The *P. multocida* neuraminidase does not appear to be serologically related to the *Pasteurella haemolytica* A1 neuraminidase since antiserum prepared against the purified *P. haemolytica* enzyme did not neutralize the *P. multocida* enzyme.

Several *Pasteurella* species have been shown to cause important domestic-animal diseases (4). These diseases include acute septicemia in chicken and turkeys and pneumonia in cattle and sheep. *Pasteurella multocida* has been shown to cause both of these widely differing disease states (4). There are 16 somatic serotypes of *P. multocida* (numbered 1 through 16), and these serotypes can occur in any of five separate capsular groups (lettered A, B, D, E, and F) (26). Capsular type A strains of *P. multocida* produce cholera in fowl, snuffles in rabbits, and pneumonia in pigs, cattle, and sheep (4, 13). Type D *P. multocida* strains produce atrophic rhinitis in swine and pneumonia in cattle (4, 20). *P. multocida* capsular types B and E have been shown to cause hemorrhagic septicemia in buffalo and cattle (18).

We have previously examined the neuraminidase of *Pasteurella haemolytica* A1. This enzyme was chosen because of our belief that *Pasteurella* extracellular products may play a role in the disease processes of these organisms (22, 25). We have recently demonstrated that *P. haemolytica* A1 neuraminidase was produced in vivo during a pneumonic infection (24).

Neuraminidase is produced by a variety of other bacteria besides *P. haemolytica* (16), including *P. multocida*. One of the first reports describing neuraminidase production by *P. multocida* was by Scharmann et al. (21). They demonstrated that 102 of 104 strains of *P. multocida* possessed neuraminidase activity. In a later study, Drzeniek et al. (7) demonstrated that nearly all of the *P. multocida* strains they examined produced this enzyme. These workers reported that this enzyme was bound to the bacterial cell wall and could be released by sonication or sodium chloride extraction. Most recently, Ifeanyi and Bailie (10) described the partial purification of a saline extract of *P. multocida* neuraminidase and the protective capability of anti-neuraminidase antibody in mice.

As a result of the fact that a wide variety of diseases can be caused by *P. multocida*, we sought to characterize and identify bacterial exoproducts that might play a role in the disease process. Because of the possible role of neuraminidase in these processes, we sought to characterize *P. multocida* neuraminidase and its production. Finally, in light of the possible role that the neuraminidase of *P. haemolytica* plays in the disease process, we attempted to determine if the two *Pasteurella* neuraminidases were related in any way.

MATERIALS AND METHODS

Bacterium. *P. multocida* A:3 was isolated in a confirmed case of acute bovine respiratory disease. Cultures were stored at −70°C in reconstituted double-strength powdered milk on filter paper. For routine use, frozen cultures were thawed and incubated for 24 h at 37°C on nutrient agar (Difco Laboratories, Detroit, Mich.) plus 5% bovine erythrocytes. The frozen stock cultures were transferred only once before use in experiments. Colonies from the blood agar were used to initiate cultures in defined medium.

Medium and growth conditions. Typical colonies were transferred to 10 ml of a chemically defined medium (RPMI 1640 buffered with 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.2]; Gibco, Grand Island, N.Y.). After 7 h of incubation at 37°C with shaking at 180 rpm, the entire contents of the tube were used to inoculate 1 liter of the chemically defined medium in 2-liter baffled flasks. The 2-liter flasks were incubated at 37°C with shaking (180 rpm) for the specified time. Growth was measured turbidimetrically at 540 nm in a Spectronic 20 colorimeter (Bausch and Lomb, Inc., Rochester, N.Y.). When cultures were ready for harvest, they were chilled immediately. Cells were removed by centrifugation at 17,700 × *g* in a J2-21 refrigerated centrifuge (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C for 45 min. The supernatants were then filtered to remove any remaining bacteria. Filtrates were then concentrated to dryness by lyophilization, and the powder was suspended in 500 ml of 10 mM sodium acetate buffer (pH 6.5) and dialyzed against 10 liters (twice) of the same buffer for 48 h.

Substrate preparation. A solution of fetuin (Sigma Chemical Co., St. Louis, Mo.; 0.1 to 10 mg/ml; 8.75% bound sialic acid, type III fetal calf serum) dissolved in 10 mM sodium acetate (pH 6.5) was used as the substrate in most of these studies. Other substrates examined in this study included human α-1-acid glycoprotein (6.2% bound sialic acid; Sigma), bovine submaxillary mucin (8.0% bound sialic acid; Sigma), *N*-acetylneuramin lactose (48.9% bound sialic acid; Sigma)

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from bovine colostrum, and colominic acid (Sigma), the sodium salt from *Escherichia coli*.

Enzyme assays. The concentration of enzyme present in concentrated filtrates of *P. multocida* A:3 or in purified enzyme preparations was determined by measuring sialic acid amounts released from the substrates mentioned above. The various enzyme preparations were incubated at 37°C for 5 to 60 min, depending on the enzyme preparation. Most enzyme assay mixtures (except for those containing serum) contained the following components in a volume of 300 μl: 0.1 to 1.0 mg of substrate, 10 mM $CaCl_2$, 33.3 mM sodium acetate (pH 6.0 or 6.5), and *P. multocida* A:3 enzyme preparations (usually 100 μl). Each set of assays included a substrate blank, and reactions were initiated by the addition of enzyme to the other components. The enzyme substrate reactions were halted by adding 0.025 M periodic acid (in 0.125 M H_2SO_4) and placing them on ice. The amount of sialic acid released was measured by the thiobarbituric acid assay of Aminoff (1) with *N*-acetylneuraminic acid (Sigma) as the standard. Protein concentrations were determined in duplicate by the Folin phenol assay of Lowry et al. (12) with bovine serum albumin (BSA) as the standard. Specific activities were expressed as micromoles of sialic acid released per minute per milligram of protein. For pH optimum determinations for the purified neuraminidase, a pH range of 5.0 to 8.0 was examined with a 10 mM acetate buffer used to obtain all values. The neuraminidase assay employed was the same as that described above, except that the pH was changed accordingly. For the determination of protease activity, a modification of the assay of Rinderknecht et al. (19) was used. Briefly, scintillation vials containing 20 mg of the horsehide powder blue substrate, 10 mM sodium acetate (pH 6.5), and 100 μl of the enzyme in a final volume of 5 ml were incubated for 8 h at 37°C with shaking. Assays were performed in duplicate, and the reaction was terminated by filtration with a Millipore apparatus, removing any remaining insoluble substrate. The supernatant fluid was read at 595 nm with a model DU 70 spectrophotometer (Beckman Instruments, Inc., Irvine, Calif.).

Purification of the extracellular neuraminidase of *P. multocida* A:3. (i) Stage I: dried culture supernatant. The extracellular neuraminidase was obtained from an early-stationary-phase culture (18 h; final optical density at 540 nm, 0.96) of *P. multocida* A:3 grown in 10 liters of the chemically defined medium. Cells were harvested by centrifugation at 17,700 × *g* for 30 min at 4°C, and the supernatant fluid was filtered and concentrated to dryness by lyophilization (stage I).

(ii) Stage II: ammonium sulfate precipitate. Stage I concentrate was resuspended in approximately 500 ml of 10 mM sodium acetate (pH 6.5) and dialyzed against 20 liters of the same buffer for 48 h at 4°C. Stage I enzyme was then brought to 100% ammonium sulfate saturation, and after being stirred overnight, the suspension was centrifuged at 48,000 × *g* for 30 min and the precipitate was suspended in the smallest volume possible of 10 mM sodium acetate. This material (stage II) was utilized for further purification.

(iii) Stage III: DEAE-Sephacel eluate. A column (2.5 by 20 cm) of DEAE-Sephacel (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was poured and equilibrated overnight with 10 mM sodium phosphate buffer (pH 7.5). Stage II enzyme (after concentration by lyophilization and equilibration overnight with the starting buffer) was then placed on the DEAE-Sephacel column. The column was washed twice with the starting buffer, and the adsorbing material was eluted with a linear NaCl gradient (0 to 0.5 M) in the starting buffer. The eluting peaks were monitored at 280 nm with a flowthrough model 2138 Uvicord S monitor (LKB Instruments, Rockville, Md.) and recorded with a model 6520-S chopper-bar six-channel recorder (LKB). Fractions of 100 drops (ca. 4.5 ml) were collected. Every fifth tube was dialyzed against 10 mM sodium acetate (pH 6.5) and tested for neuraminidase activity by the procedure of Aminoff (1). All tubes containing neuraminidase activity were pooled, and this pool was designated stage III.

(iv) Stage IV: Sephadex G-200 eluate. Stage III enzyme was then applied to an ascending-flow column (2.6 by 90 cm) of Sephadex G-200 (Pharmacia) equilibrated with 10 mM sodium acetate (pH 6.5) at 4°C. Fractions of 100 drops (ca. 5.0 ml) were collected, and protein peaks were monitored and recorded at 280 nm. Every fifth tube was assayed for neuraminidase activity as described above (1). The resultant pool was designated stage IV.

PAGE. All neuraminidase preparations were subjected to polyacrylamide gel electrophoresis (PAGE) in slab gels (6.0% separating gel) by the procedure of Davis (5). Samples containing 10 to 30 μl (5% glycerol) were applied to each well. Bromophenol blue (0.2%) was used as the tracking dye. The gels were run at 150 V (for approximately 4 h) until the tracking dye reached the bottom of the gel. The gels were run at 0 to 4°C. The gels were fixed immediately and stained with 0.1% Coomassie brilliant blue R (50% methanol, 10% glacial acetic acid) overnight or with silver stain (9). For localization of the neuraminidase activity, a lane was cut from the gel and immediately sliced into 1.0-cm pieces after electrophoresis, and each fraction was mashed, placed in dialysis bags, eluted at 4°C for 72 h with 0.5 ml of 10 mM sodium acetate (pH 6.5), and dialyzed against 1 liter of the same buffer. The gel residue was then removed, and the eluate was examined for neuraminidase activity by the procedure of Aminoff (1).

Molecular weight determination: gel filtration on Sephadex G-200 and sodium dodecyl sulfate (SDS)-PAGE. The Sephadex G-200 gel filtration column used to purify extracellular *P. multocida* A:3 neuraminidase was calibrated with proteins of known molecular weights (Sigma). Elution volumes were determined for the following (molecular weights shown in parentheses): blue dextran (2×10^6), thyroglobulin (669,000), sweet potato β-amylase (200,000), alcohol dehydrogenase (150,000), ovalbumin (43,000), chymotrypsinogen (25,000), and RNase (13,700), by recording the volumes at which these proteins or dextrans displayed maximal A_{280} values. The molecular weight of the purified neuraminidase was then estimated by interpolation from a graph of partition coefficients between the liquid phase and the gel phase (K_{av}) versus the $\log_{10}$ molecular weights, utilizing the K_{av} values of the standard proteins and the peaks containing neuraminidase activity (8). Purified neuraminidase was also run on 6.0% reducing gels (with 3% stacking gels) in concentrations ranging from 0.1 to 25 μg of protein per lane. The gels were silver stained for visualization of proteins. Molecular mass standards consisted of high molecular masses (18 to 330 kDa) (Pierce) as well as broad-range (14.4 to 200 kDa) molecular masses (Bio-Rad) and were stained with Coomassie brilliant blue R. The molecular weight was determined by interpolation from a graph of the standards.

Attempted neutralization of heterologous *P. multocida* A:3 neuraminidase. New Zealand White rabbits were injected subcutaneously with 1 ml (200 μg) of purified *P. haemolytica* A1 neuraminidase (25), which was emulsified with an equal volume of Freund's complete adjuvant (Difco). Two weeks after the first subcutaneous injection, the rabbits were injected again with the same preparation in Freund's incomplete adjuvant. One week later, blood was obtained from these rabbits by cardiac puncture, and serum was separated from clotted blood by a method described previously (3). The presence or absence of neutralizing antibody to the *P. multocida* A:3 neuraminidase was determined by the neuraminidase assay (25). Purified *P. multocida* A:3 or *P. haemolytica* A1 neuraminidases were incubated with either 0.1 ml of preimmune serum or 0.1 ml of anti-*P. haemolytica* A1 neuraminidase in the presence of 0.1 mg of fetuin, 10 mM $CaCl_2$, and 33.33 mM sodium acetate (pH 6.0) in a final volume of 0.4 ml. Each set of assays included a substrate blank, and enzyme reactions were initiated by the addition of the enzyme to the remaining components. The enzyme and serum preparations were incubated together for 90 min at 4°C before the assay was begun, to allow the antibody to react with the enzyme. After incubation, the percent reduction in neuraminidase activity was determined by incubating the neuraminidase mixture (1) for 60 min at 37°C and comparing the differences in activity between the preimmune and immune sera.

Determination of cell-bound enzyme activity. Typical colonies were transferred to 10-ml tubes containing RPMI 1640-HEPES and incubated overnight at 37°C to the stationary phase. The entire contents were dialyzed overnight at 4°C against 4 liters of 10 mM sodium acetate buffer (pH 6.0). The next day, the entire contents of the dialysis bag were centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was then filtered through a 0.45-μm syringe filter, lyophilized to dryness, and resuspended in 1.0 ml of deionized water. This fraction was designated the supernatant. The pellet was resuspended in 1.0 ml of 10 mM sodium acetate buffer (pH 6.0), containing 4.0% sodium azide, and stored overnight at 4°C. The following day, this pellet resuspension was centrifuged at 12,000 × *g* for 5 min in a microcentrifuge (model 235V; Fisher Scientific, Pittsburgh, Pa.). The pellet was washed three times in 10 mM sodium acetate (pH 6.0), with a final resuspension in 500 μl of the same buffer. This was designated the whole-cell fraction. We determined previously (data not shown) that there is no inhibition of enzyme activity in assays containing up to 1.0% NaN_3. The supernatant and the whole-cell fractions were both plated overnight on blood agar plates to ensure a cell-free supernatant as well as a sterile whole-cell population. Assays were performed as described by Aminoff (1) with a total volume of 300 μl as described previously; 100-μl volumes of both the supernatant and the whole-cell fractions were used. In addition, Lowry protein assays (12) were performed with appropriate volumes of each of the fractions.

Attempted neutralization of homologous extracellular *P. multocida* A:3 and cell-bound neuraminidase. New Zealand White rabbits were injected subcutaneously with 1 ml (200 μg) of purified *P. multocida* A:3 neuraminidase which was emulsified with an equal volume of Freund's complete adjuvant (Difco). Two and 4 weeks after the first subcutaneous injection, the rabbits were injected with the same preparation of Freund's incomplete adjuvant. One week later, blood was obtained from these rabbits, and the serum was separated. The presence or absence of neutralizing antibody to the extracellular and the cell-bound neuraminidases was determined by the neuraminidase assay. Isolation of whole cells and supernatant was the same as that described for determining cell-bound enzyme activity. Supernatant fractions and whole-cell fractions were incubated with 0.1 ml of preimmune serum or 0.1 ml of anti-*P. multocida* A:3 neuraminidase in the presence of 0.1 mg of fetuin, 10 mM $CaCl_2$, and 33.33 mM sodium acetate (pH 6.0) in a final volume of 0.4 ml. Assays were conducted in the same fashion described above for attempting neutralization of *P. multocida* A:3 neuraminidase except that the percent reduction in neuraminidase activity between preimmune and immune sera was not determined.

RESULTS

Relationship between *P. multocida* A:3 growth curve and neuraminidase production. Studies were undertaken to determine the optimal phase of the growth curve for maximal yield of extracellular neuraminidase produced by *P. multocida* A:3. Figure 1 shows the neuraminidase activities of enzyme present in supernatants at various stages of the growth curve with

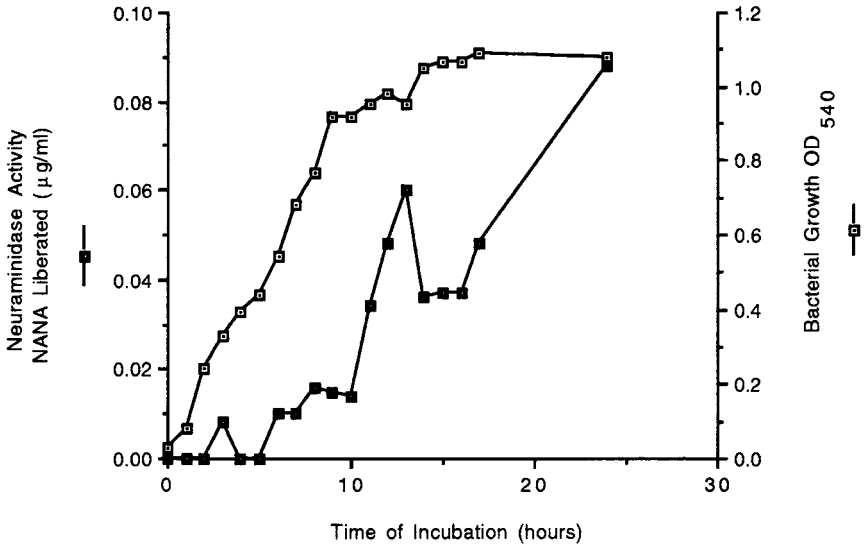

FIG. 1. Relationship between extracellular neuraminidase production and bacterial growth for *P. multocida* A:3 cultured at 37°C in RPMI 1640 plus 25 mM HEPES. The substrate was fetuin. NANA, *N*-acetylneuraminic acid; OD, optical density.

fetuin as the substrate. The pattern observed for production of the enzyme in the defined medium at 37°C paralleled the growth curve of the organism. The bacterium exhibited a biphasic growth with two exponential phases. Enzyme production increased gradually throughout the first 10 hours of growth but increased dramatically as the organisms entered the first stationary phase and continued throughout the second stationary phase.

Substrate specificity of *P. multocida* A:3 neuraminidase and enzyme kinetics. To study the substrate specificity of the *P. multocida* A:3 neuraminidase, we examined the ability of this enzyme to cleave sialic acid from a variety of compounds. In each case, 1 mg of the various glycoprotein preparations was treated with stage I *P. multocida* A:3 neuraminidase, and specific activity was calculated for each substrate. The *P. multocida* neuraminidase was able to remove sialic acid from all five substrates studied. The specific activities of the *P. multocida* A:3 neuraminidase (stage I) against these five substrates were 97.7 (*N*-acetylneuramin lactose), 44.0 (human α-1-acid glycoprotein), 39.9 (fetuin), 25.4 (colominic acid), and 19.6 (bovine submaxillary mucin) μmol of sialic acid released per min per mg of protein. The absorption spectra of complexes obtained from the *P. multocida* A:3 neuraminidase reaction products and *N*-acetylneuraminic acid were essentially identical (data not shown).

The kinetics of the *P. multocida* A:3 enzyme were examined with fetuin as the substrate. As determined from the Lineweaver-Burk plot (11), the K_m value of this neuraminidase was 0.03 mg/ml and the V_{max} value was 0.0004 μmol/min (data not shown).

Temperature stability of the enzyme. The stability of the enzyme in 10 mM sodium acetate after incubation for various periods of time and at various temperatures was examined. Preincubation of the enzyme at 4°C for 3 h before assay did not significantly alter its activity. The same was true of enzyme activity when the neuraminidase was incubated for 3 h at 37°C in 10 mM sodium acetate (pH 6.5). A loss of enzyme activity of approximately 75% occurred after incubation of the enzyme in 10 mM sodium acetate (pH 6.5) for 30 min at 50°C, but greater than 90% of the enzyme activity was lost after 10 min of incubation at 65°C (data not shown).

Enzyme purification. The stage I preparation had a specific activity of 3.25 μmol/min/mg of protein. No protease activity was detected in stage I *P. multocida* A:3 neuraminidase (data not shown). After the stage II precipitate was dialyzed against 2 liters of 10 mM sodium acetate, the specific activity of this preparation was 0.52 μmol of sialic acid released per min per mg of protein (Table 1). Figure 2 shows the elution profile of stage II *P. multocida* A:3 neuraminidase on DEAE-Sephacel. All of the neuraminidase activity eluted from the column at NaCl concentrations of 0.075 to 0.175 M. This material, which had a specific activity of 6.26 μmol of sialic acid released per min per mg of protein (Table 1), was then applied to a Sephadex G-200 column. The resultant elution profile is shown in Fig. 3. All of the neuraminidase activity eluted from the G-200 column in one peak (stage IV) and possessed a specific activity

TABLE 1. Purification of *P. multocida* A:3 extracellular neuraminidase

Stage	Enzyme prepn	Total protein (mg)	Neuraminidase sp act (μmol/min/mg of protein)	Total neuraminidase activity (μmol/min)
I	Lyophilized supernatant fluid (10 liters, 100-fold concentrated)	296.35	3.25	963.14
II	$(NH_4)_2SO_4$ precipitate (100%)	168	0.52	87.36
III	DEAE-Sephacel eluate	31.82	6.26	199.19
IV	Sephadex G-200 eluate	1.42	9.36	13.35

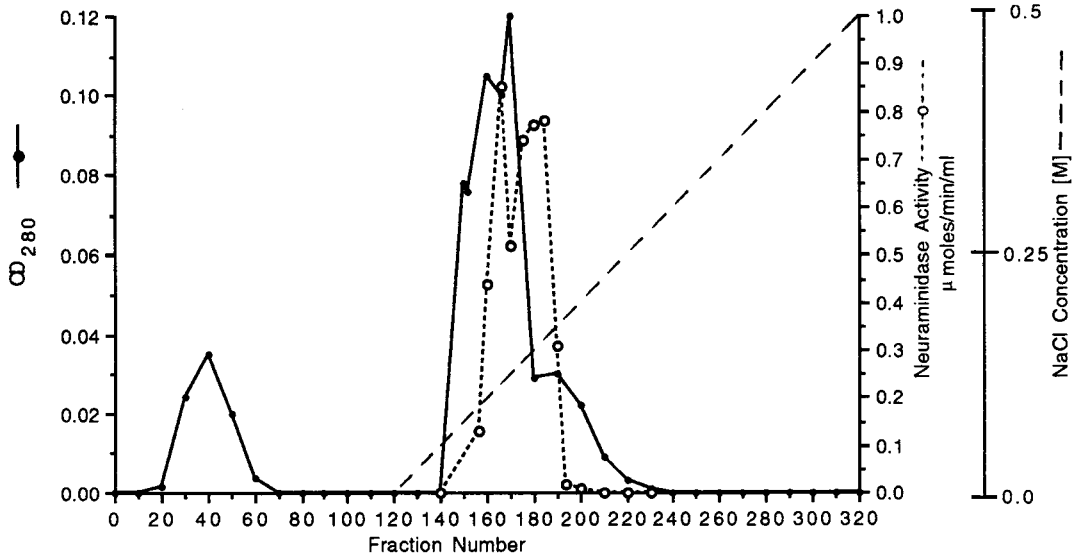

FIG. 2. Chromatography of stage II *P. multocida* neuraminidase on DEAE-Sephacel. The peaks were monitored at 280 nm (optical density [OD] at 280 nm) and eluted with an increasing NaCl gradient in 0.01 M sodium phosphate buffer (pH 7.5) at 4°C. Every fifth tube was assayed for neuraminidase activity by the method of Aminoff (1).

of 9.36 μmol of sialic acid released per min per mg of protein (Table 1). PAGE of the stage IV *P. multocida* A:3 neuraminidase preparation showed that the enzyme migrated as a broad band near the top of the gel. When a 7.5% polyacrylamide concentration was used in the separating gel, the enzyme would not enter the gel. Only when a 6.0% polyacrylamide separating gel was employed were we able to get the *P. multocida* A:3 neuraminidase to enter the gel. Neuraminidase activity was always found in the gel slices containing this broad band (Fig. 4). When the gels were stained with Coomassie

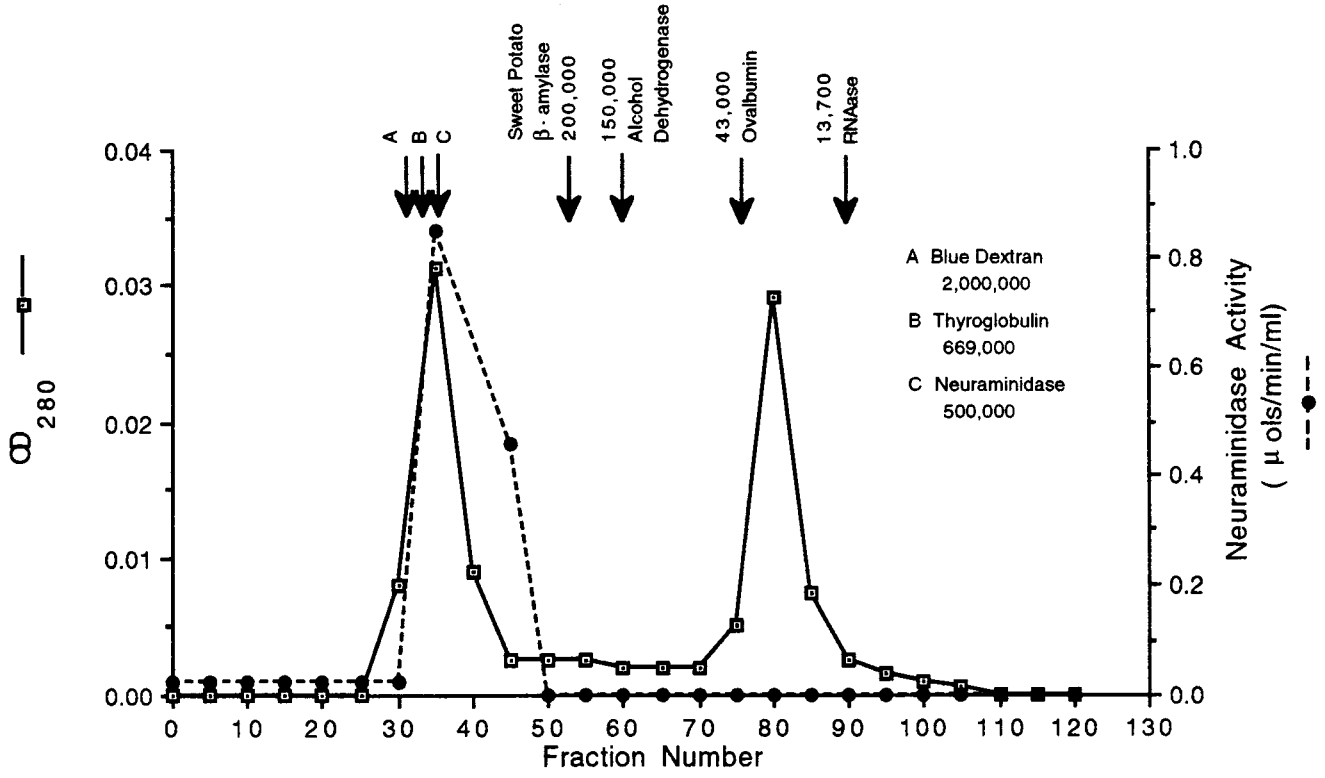

FIG. 3. Chromatography of stage III neuraminidase on a Sephadex G-200 column (2.5 by 90 cm). The peaks were monitored at 280 nm (optical density [OD] at 280 nm) and eluted with 10 mM sodium acetate buffer (pH 6.5) at 4°C. Every fifth tube was assayed for neuraminidase activity by the procedure of Aminoff (1). Calibration of the column with blue dextran and proteins of known molecular weights was performed with the same buffer.

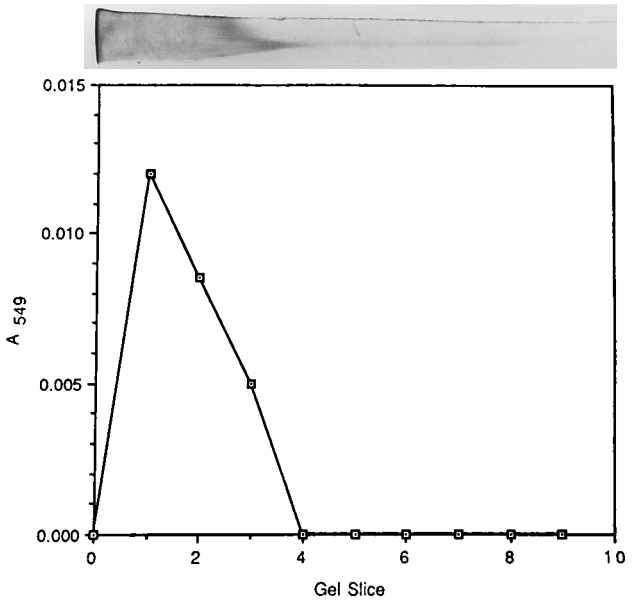

FIG. 4. PAGE of stage IV *P. multocida* A:3 neuraminidase in 6.0% polyacrylamide. Ten micrograms of protein was placed on the gel and silver stained. One lane was cut into 12 1-cm sections, which were placed in dialysis bags and dialyzed at 4°C against 0.01 M sodium acetate for 72 h. After dialysis, the contents of each bag were analyzed for neuraminidase activity, with fetuin as the substrate, by the procedure of Aminoff (1).

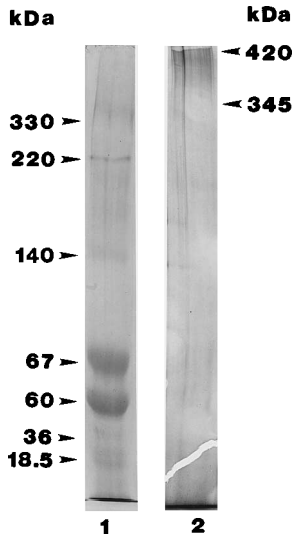

FIG. 5. SDS-PAGE of stage IV *P. multocida* A:3 neuraminidase in 6% acrylamide. Ten micrograms of protein was placed on the gel (lane 2) and silver stained. The molecular weight standards (lane 1) employed are described in Materials and Methods. The molecular weight standards were stained with Coomassie brilliant blue R.

blue, no protein bands were visible. Only when the gels were silver stained was the broad band containing neuraminidase activity observed.

Molecular weight and pH optimum of *P. multocida* A:3 neuraminidase. The molecular weight of the *P. multocida* A:3 neuraminidase was calculated by its elution profile on Sephadex G-200 and by SDS-PAGE. The *P. multocida* A:3 neuraminidase had an apparent molecular weight of approximately 500,000 by chromatography on Sephadex G-200 (Fig. 3) and a range of 345,000 to 420,000 by SDS-PAGE (Fig. 5). The pH optimum was determined for the enzyme with fetuin as the substrate. A peak of maximal activity occurred at pH 6.0 in 10 mM sodium acetate containing 10 mM $CaCl_2$ (Fig. 6).

Neutralization of neuraminidases produced by *P. haemolytica* A1 and *P. multocida* A:3 with anti-*P. haemolytica* A1 neuraminidase. In the presence of immune serum, there was a 50% reduction in activity of the homologous stage I *P. haemolytica* A1 enzyme preparation (data not shown). The stage I *P. multocida* A:3 neuraminidase was also incubated with either preimmune or immune serum to purified *P. haemolytica* A1 neuraminidase and then assayed for activity. In the presence of preimmune or immune serum, there was no reduction in the activity of the *P. multocida* A:3 neuraminidase.

Presence of cell-bound neuraminidase activity of *P. multocida* A:3. The supernatant fraction had a specific activity of 0.509 μmol of sialic acid released per min per mg of protein and a total activity of 0.450 μmol of sialic acid released per min and constituted 47% of total enzyme, while the whole-cell fraction had a specific activity of 0.287 μmol of sialic acid released per min per mg and a total activity of 0.516 μmol/min and constituted 53% of total enzyme.

Neutralization of extracellular and cell-bound neuraminidases produced by *P. multocida* with anti-*P. multocida* A:3 neuraminidase. In the presence of immune sera diluted to 1:8, the enzyme activity of the extracellular as well as the cell-bound enzyme was reduced by 45%. Undiluted preimmune sera possessed no neutralizing activity on either of the two enzyme sources. Dilutions of immune sera beyond 1:8 showed no reduction in enzyme activity.

DISCUSSION

There have been several studies describing neuraminidase production by *P. multocida*. Scharmann et al. (21), in 1970, first examined *P. multocida* strains for neuraminidase production. They demonstrated that 102 of the 104 strains they examined possessed neuraminidase activity. In 1971, Müller published two reports examining the in vitro and in vivo effects of *P. multocida* neuraminidase. In one paper, he reported that the neuraminidase of *P. multocida* was a high-molecular-weight, heat-labile enzyme (14). In the second paper, he reported that the *P. multocida* neuraminidase was active in vivo on the serum glycoproteins of guinea pigs and one human patient infected

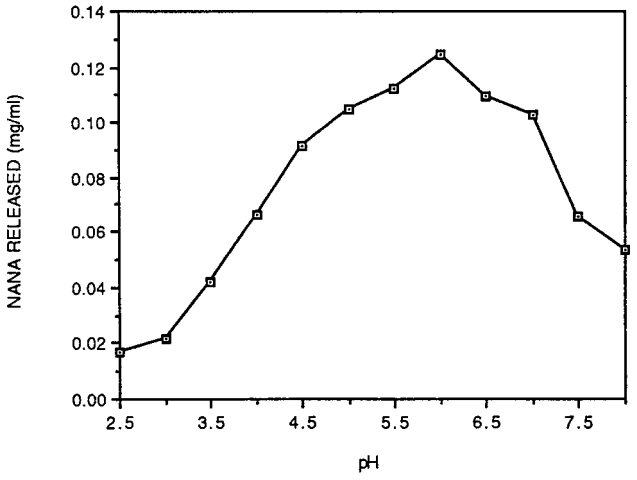

FIG. 6. pH optimum of *P. haemolyticia* A1 neuraminidase in 0.01 M sodium acetate buffer. The substrate was fetuin. NANA, *N*-acetylneuraminic acid.

with this organism (15). In 1972, Drzeniek et al. (7) reported that a neuraminidase was produced by nearly all *P. multocida* strains examined, and these authors partially purified the enzyme. They reported that the enzyme had a molecular weight of 250,000. In 1974, Müller and Krasemann (17) examined the virulence in mice of 25 *P. multocida* strains, isolated from the mouths of dogs and cats, in relation to their production of neuraminidase. They found that the *P. multocida* strains that produced high levels of neuraminidase were more virulent in mice than those producing low enzyme levels. Finally, in 1992, Ifeanyi and Bailie (10) examined the role of antibody to *P. multocida* neuraminidase in passive protection of mice against the homologous *P. multocida* strain. These authors demonstrated passive protection of mice with antiserum to neuraminidase from *P. multocida* serotype A:3. All of the previous studies examined a *P. multocida* neuraminidase that was cell bound and extracted by sonication or NaCl. We have isolated and purified an extracellular neuraminidase from *P. multocida*. In this study, we have purified this enzyme to homogeneity, characterized it as to its substrate specificity, pH optimum, molecular weight, and enzyme kinetics, and determined when it is produced in the *P. multocida* growth phase. Finally, we have shown that the neuraminidases of *P. haemolytica* and *P. multocida* are distinct immunologically.

It appears that the cell-bound *P. multocida* neuraminidase and the extracellular enzyme from the same organism are similar. Drzeniek et al. reported that the neuraminidase of *P. multocida* is bound to the bacterial cell (7). They demonstrated that after solubilization, it had a molecular weight of 250,000 and a pH optimum of 6.0. In this report, we have shown that the extracellular neuraminidase of *P. multocida* has a molecular weight of 345,000 to 500,000 and a pH optimum of 6.0. Our data indicate that the best time to harvest *P. multocida* A:3 cells for maximal extracellular enzyme yield is the stationary phase (Fig. 1). This is because this strain of *P. multocida* does not appear to produce a protease that destroys the neuraminidase in the stationary phase, as has been reported for at least two other neuraminidases (6, 23). Extracellular neuraminidase production paralleled the growth of *P. multocida* A:3, as has been reported for group A streptococci (6), *Streptococcus sanquis* (23), and *P. haemolytica* (25).

Purification of the extracellular *P. multocida* A:3 neuraminidase was facilitated by the use of a completely defined medium in which the enzyme appeared to be stable up to and including the stationary phase of growth. The stage IV enzyme preparation eluted from Sephadex G-200 in one sharp peak and ran as a broad band in PAGE. The entire broad band was shown to possess neuraminidase activity (Fig. 4). This purified enzyme possessed a specific activity of 9.36 μmol of sialic acid released per min per mg of protein when assayed with fetuin as the substrate. We could find no other reports in the literature for the specific activity of a purified neuraminidase from *P. multocida*.

The molecular weight estimate for the extracellular *P. multocida* A:3 on gel filtration (Fig. 3) was 500,000. By SDS-PAGE, the protein migrates as a broad band with a midpoint of 380,000 and a range of 345,000 to 420,000 (Fig. 5). These estimates indicate a decidedly larger molecular weight for the *P. multocida* neuraminidase than for any other neuraminidase examined to date. This figure is somewhat greater than molecular weight determinations of cell-bound *P. multocida* neuraminidase reported by other investigators. Balke et al. (2) reported the molecular mass of the cell-bound *P. multocida* to be between 200,000 and 300,000 Da when measured by gel filtration. The molecular mass of most bacterial neuraminidases is considerably smaller. For example, the molecular masses of the neuraminidases of *Clostridium perfringens*, *Vibrio cholerae*, and *Streptococcus pneumoniae* are 61,000, 68,000, and 81,000 Da, respectively (2). Interestingly, the only other bacterial neuraminidase with a molecular weight similar to that of *P. multocida* is that produced by another *Pasteurella* species. The molecular weight of the extracellular neuraminidase produced by *P. haemolytica* has been reported to be between 150,000 and 200,000 (22, 25).

The extracellular neuraminidase of *P. multocida* A:3 had a K_m value of 0.03 mg/ml. The K_m for a neuraminidase produced by another *P. multocida* strain was 2×10^{-4} M when 3′-sialyllactose was used as a substrate (7). Because different substrates were used, it is impossible to compare the two values. We also found a V_{max} value for this enzyme of 4×10^{-4} μmol/min when fetuin was used as the substrate. There are no other V_{max} values for this enzyme reported in the literature.

The role of the neuraminidase of *P. multocida* in the disease processes of this organism remains unknown. However, a review of the literature does offer some interesting insights. Ifeanyi and Bailie (10) suggested that neuraminidase may be one of the immunogenic protective proteins present in extracts of *P. multocida*. They based this assumption on the observed passive protection of mice with rabbit antiserum to neuraminidase from *P. multocida* A:3. In addition, Müller and Krasemann (17) concluded that neuraminidase production by *P. multocida* is associated with the virulence of these strains because high producers were more virulent in mice (50% lethal dose, <100 CFU) than were low producers. Certain *P. multocida* serogroups (A and D) have been shown to cause pneumonia in cattle more often than others. However, it is not known whether there is a relationship between the ability of *P. multocida* to produce this enzyme and its ability to cause pneumonia in cattle.

Finally, we were able to demonstrate that *P. multocida* A:3 excretes a significant amount of neuraminidase into its external environment and retains the rest on its cell surface. This demonstration of cell-bound activity supports the findings of Drzeniek et al. (7), who showed a large amount of cell-bound enzyme. In this study, we were able to show that approximately 47% of the total neuraminidase activity is released as extracellular enzyme during in vitro growth. By utilizing antisera produced against the extracellular neuraminidase, we have shown that these two enzymes are related antigenically.

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