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Neuraminidase Production by a *Pasteurella haemolytica* A1 Strain Associated with Bovine Pneumonia

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The properties of an extracellular neuraminidase produced by a *Pasteurella haemolytica* A1 strain (isolated from a case of bovine pneumonia) during growth in a defined medium were examined in this investigation. This enzyme, isolated from concentrated culture supernatants of *P. haemolytica* A1, was active against *N*-acetylneuramin lactose, human alpha 1-acid glycoprotein, fetuin, and bovine submaxillary mucin. Neuraminidase production paralleled bacterial growth in a defined medium and was maximal in the stationary phase of growth. The enzyme was purified to homogeneity by a combination of salt fractionation, ion-exchange chromatography on DEAE-Sephacel, and gel filtration on Sephadex G-200. These procedures yielded an enzyme preparation that possessed a specific activity of 100.62 μmol of sialic acid released per min per mg of protein against human alpha 1-acid glycoprotein. The K_m value for this enzyme with human alpha 1-acid glycoprotein as the substrate was 1.1 mg/ml, and the enzyme possessed a pH optimum of 6.5. The *P. haemolytica* A1 neuraminidase had a molecular weight of approximately 150,000 as estimated by gel filtration and approximately 170,000 when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was stable at 4°C for 3 h. At 37°C for 3 h, 25% of enzymatic activity was lost. Approximately 55% of the enzyme activity was lost within 30 min at 50°C, with greater than 70% of the enzyme activity being destroyed within 10 min at temperatures of ≥65°C.

The elaboration of the enzyme neuraminidase has long been proposed as a virulence factor for several microorganisms. The first suggestion that this enzyme was involved in the infectious process of pathogens was by Hirst in 1943 (12). He proposed that the enzyme played an important role in the disease process caused by the influenza virus. Many years later, in 1967, Isacsen suggested the importance of this enzyme as the causative agent in autoimmune disease in which the myxoviruses were involved (13). Neuraminidase production by bacteria has also been well documented (18). Scharmann et al. (26) were the first to demonstrate neuraminidase production by *Pasteurella* spp. They found that 102 of 104 strains of *Pasteurella multocida* and 3 of 5 strains of *P. haemolytica* showed neuraminidase activity. Frank and Tabatabai (10) examined type isolates of the 12 established serotypes of *P. haemolytica* as well as bovine and ovine field isolates. These investigators reported that the type strains of serotypes 2, 3, 8, 10, and 11 did not produce neuraminidase while the other seven serotypes did. They also suggested that the neuraminidase activity levels among the field isolates studied were serotype associated. The same investigators examined a soluble extracellular neuraminidase produced by *P. haemolytica* A1. They demonstrated that antiserum against whole *P. haemolytica* A1 inhibited neuraminidase activity 2.0- to 2.7-fold (29). They suggested that the enzyme was part of the antigenic complex of the bacterium.

P. haemolytica A1 is involved with most acute fibrinohemorrhagic pneumonias that develop in market-stressed feeder/stocker calves after shipment (15). Because of the marked damage (23) that occurs in the lungs of animals infected with *P. haemolytica* A1, we sought to identify and characterize bacterial exoproducts that might account for this pathology. Because of the pathogenesis of pneumonia caused by *P. haemolytica* A1, we attempted to characterize neuraminidase and its production.

MATERIALS AND METHODS

Bacterium. *P. haemolytica* A1 was isolated from a confirmed case of acute bovine respiratory disease. Cultures were stored at −70°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) plus 20% glycerol. For routine use, frozen cultures were thawed and incubated for 24 h at 37°C on nutrient agar plus 5% goat or bovine erythrocytes. The stock cultures used to inoculate the blood agar plates were only one transfer away from the original frozen culture. 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-ethanesulfuric 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 a 2-liter baffled flask. 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 & Lomb, Inc., Rochester, N.Y.). When cultures were ready for harvest, they were immediately chilled in an ice bath and the culture volume was determined. Cells were removed by centrifugation at 17,700 × *g* in a J2-21 refrigerated centrifuge (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C for 30 min. The supernatants were then filtered to remove any remaining bacteria. Filtrates were then concentrated to dryness by lyophilization,

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and the powder was suspended in 200 ml of 10 mM sodium acetate buffer, pH 6.5, and dialyzed against 10 liters of the same buffer at 4°C for 48 h.

Substrate preparation. A solution of human alpha 1-acid glycoprotein (0.1 to 10.0 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) dissolved in 10 mM sodium acetate (pH 6.5) and containing 6.2% bound sialic acid was used as the substrate for most of the experiments in this study. Other substrates examined for their susceptibility to the *P. haemolytica* A1 neuraminidase included bovine submaxillary mucin (8.0% bound sialic acid; Sigma), fetuin (8.75% bound sialic acid; type III, fetal calf serum; Sigma), and *N*-acetylneuramin lactose (48.9% bound sialic acid) from bovine colostrum (Sigma).

Enzyme assays. The amount of neuraminidase present in concentrated filtrates of *P. haemolytica* A1 or in purified enzyme preparations was determined by measuring the amount of sialic acid released from the substrates mentioned above. The various preparations were incubated at 37°C for time periods from 1 to 60 min depending on the enzyme preparation. Each assay mixture contained the following components in a volume of 0.3 ml: 0.1 to 1.0 mg of substrate, 10 mM $CaCl_2$, 33.3 mM sodium acetate (pH 6.5), and *P. haemolytica* A1 enzyme preparations (usually 10 to 100 μl). Each set of assays included a substrate blank, and enzyme reactions were initiated by addition of the enzyme to the remaining components. The enzyme-substrate reaction was terminated by adding 0.025 M periodic acid (in 0.125 N H_2SO_4) and placing the reaction mixture on ice. The amount of released sialic acid was quantitated by the colorimetric 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. (17) with bovine serum albumin (BSA) as the standard, and specific activities were expressed as micromoles of sialic acid activity 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 100 mM acetate buffer used to obtain all pH values. The neuraminidase assay employed was essentially that described above except that the pH was changed appropriately. For the quantitation of protease activity, a modification of the assay of Rinderknecht et al. (25) was employed. 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 sample 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.

Purification of the extracellular neuraminidase of *P. haemolytica* A1. (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 [OD_{540}] = 0.75) of *P. haemolytica* A1 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 200 ml of 10 mM sodium acetate, pH 6.5, and dialyzed against 10 liters of the same buffer for 48 h at 4°C. Stage I enzyme was then brought to 20% 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 as small a volume as possible of 10 mM sodium acetate (pH 6.5). The resuspended precipitate was dialyzed and saved, whereas the supernatant fluid was brought to 60% ammonium sulfate saturation and treated as described above. The majority of the neuraminidase activity was in the region of 20 to 60% saturation, and this precipitate (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., Pistcataway, 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 21128 Uvicord S monitor (LKB Instruments, Rockville, Md.) and recorded by a 6520-S Chopper-Bar six-channel recorder (LKB). Fractions of 100 drops (ca. 4.2 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.

(v) Stage V: Sephadex G-200 dialyzed eluate. Stage IV enzyme was concentrated to dryness by lyophilization and then dialyzed against 10 mM sodium acetate, (pH 6.5). It was then reapplied to a Sephadex G-200 column as described above. Every fifth tube was assayed for neuraminidase activity (1). The resultant pool was designated stage V.

PAGE. All neuraminidase preparations were subjected to polyacrylamide gel electrophoresis (PAGE) in slab gels (7.5% separating gel) by the procedure of Davis (4). Samples containing 10 to 30 μg of protein in a maximum volume of 100 μl (5% glycerol) were applied to each well. Bromphenol blue (0.2%) was used as a 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 immediately fixed and stained in 0.1% Coomassie brillant blue R (50% methanol, 10% glacial acetic acid) overnight. The gels were destained by diffusion the next day in a solution containing 5% (vol/vol) methanol and 7.5% (vol/vol) acetic acid. 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 5°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 estimation. (i) SDS-PAGE. Stage V enzyme and reference proteins were subjected to sodium dodecyl sulfate (SDS)-PAGE by the discontinuous slab gel system described by Laemmli (14). Samples were dissociated and reduced in 0.01 M Tris-hydrochloride buffer (pH 8.0) containing 1% 2-mercaptoethanol and 1% SDS for 2 min

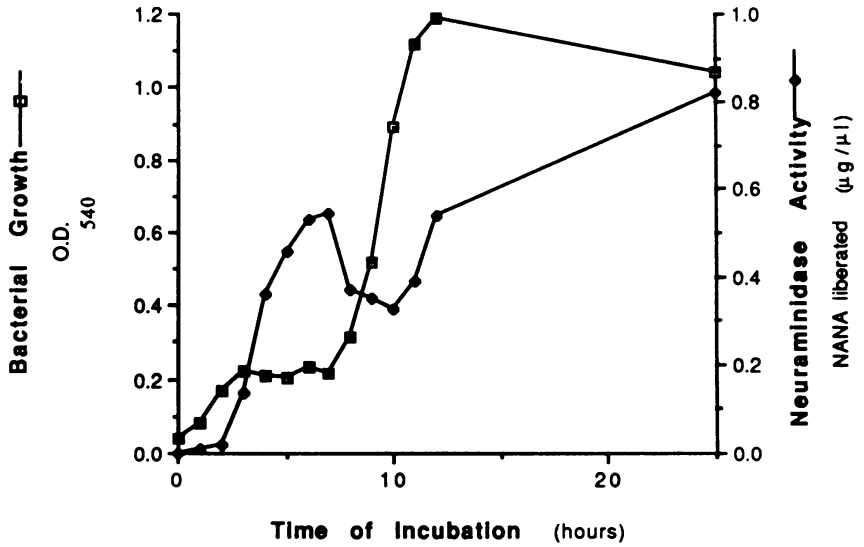

FIG. 1. Correlation of extracellular neuraminidase production with bacterial growth for *P. haemolytica* A1 cultured at 37°C in RPMI 1640 plus 25 mM HEPES. The substrate was human alpha 1-acid glycoprotein. NANA, *N*-acetylneuraminic acid.

at 100°C and directly loaded onto a 10% acrylamide separation gel with a stacking gel composed of 5% acrylamide. Standard proteins with molecular weights ranging from 14,400 to 212,000 (Pharmacia) were run simultaneously with the purified neuraminidase preparation. Plots of percent migration of standards and unknown protein samples versus $\log_{10}$ molecular weights were prepared, and unknown molecular weights were estimated by interpolation (30).

(ii) Gel filtration on Sephadex G-200. The Sephadex G-200 gel filtration column used to purify extracellular *P. haemolytica* A1 neuraminidase was calibrated with proteins of known molecular weights (Sigma). Elution volumes were determined for blue dextran (molecular weight, 2×10^6, sweet potato β-amylase (200,000), alcohol dehydrogenase (150,000), BSA (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and RNase (13,700) by recording the volume at which these proteins or dextrans displayed maximal A_{280}. 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 (7).

RESULTS

Relationship between *P. haemolytica* A1 growth curve and neuraminidase production. Experiments were performed to determine the optimal phase of the growth curve for maximum yield of extracellular neuraminidase produced by *P. haemolytica* A1. Cells were grown in RPMI 1640-HEPES as described previously, and at various times, 20-ml samples were withdrawn and the cell-free supernatant fluids were concentrated by lyophilization. Figure 1 shows the neuraminidase activities of enzyme present in supernatants at various stages of the growth curve with alpha 1-acid glycoprotein as the substrate. The pattern observed for production of the enzyme in the defined medium at 37°C closely paralleled the growth curve of the organism. The bacterium possessed a biphasic growth curve with two exponential phases. Enzyme production increased dramatically as the organisms entered the first stationary phase and continued during this phase. The neuraminidase production declined during the second log phase but increased as the organisms entered the second stationary phase. There was an increase in enzyme activity when incubation was continued after completion of the second log phase of growth.

Substrate specificity of *P. haemolytica* A1 neuraminidase and enzyme kinetics. To examine the specificity of the *P. haemolytica* A1 neuraminidase, we compared the ability of this enzyme to cleave sialic acid from a variety of glycoprotein substrates. In each case 1 mg of glycoprotein preparation was treated with a lyophilized supernatant fluid from an early-stationary-phase culture of *P. haemolytica* A1 and specific activity was calculated for each preparation. The *P. haemolytica* A1 neuraminidase was able to hydrolyze sialic acid from all four substrates examined. The enzyme was active against *N*-acetylneuramin lactose, human alpha 1-acid glycoprotein, and fetuin but was only slightly active against bovine submaxillary mucin. The specific activities of the *P. haemolytica* A1 neuraminidase (stage I) against these four substrates were 80.91, 31.38, 13.62, and 0.32 μmol of sialic acid released per min per mg of protein, respectively.

Although the actual identity of the reaction product of the *P. haemolytica* A1 neuraminidase acting on any of the substrates has not yet been established, it is most probably a member of the sialic acid family of compounds. Data to support this hypothesis come from absorption spectra of the complexes obtained when purified *P. haemolytica* A1 neuraminidase was allowed to react at 37°C with human alpha 1-acid glycoprotein. The absorption spectra of complexes obtained from the A1 neuraminidase reaction product and *N*-acetylneuraminic acid were essentially identical (data not shown).

The kinetics of the enzyme were studied with human alpha 1-acid glycoprotein as the substrate. As determined from the Lineweaver-Burk plot (16), the K_m value of this bacterial neuraminidase was 1.1 mg/ml and the V_{max} value was 1.81 μmol/min (data not shown).

Temperature stability of the enzyme. The stability of the enzyme in 10 mM sodium acetate (pH 6.5) after incubation for various periods of time and at various temperatures was

TABLE 1. Purification of *P. haemolytica* A1 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)	2,112.00	0.81	1,710.72
II	$(NH_4)_2SO_4$ precipitate (20 to 60%)	80.00	2.10 (2.59)[a]	168.00
III	DEAE-Sephacel eluate	25.48	2.74 (3.38)	69.81
IV	Sephadex G-200 eluate	1.00	40.23 (49.66)	40.23
V	Sephadex G-200 dialyzed eluate	0.40	100.62 (124.22)	40.25

[a] Numbers in parentheses are ratios of specific activity to the specific activity of stage I.

examined. Preincubation of the enzyme at 4°C for 3 h before assay did not significantly alter the activity of the enzyme. Twenty-five percent of the enzyme activity was lost when the enzyme was incubated for 3 h at 37°C in 10 mM sodium acetate, pH 6.5. A loss of enzyme activity of approximately 55% occurred after incubation of the enzyme in 10 mM sodium acetate (pH 6.5) for 30 min at 50°C, but greater than 70% of the enzyme activity was lost after enzyme incubation for ≥10 min at ≥65°C (data not shown).

Enzyme purification. The stage I preparation had a specific activity of 0.81 μmol/min/mg of protein. No protease activity was detected in this preparation (data not shown). The majority of this activity resided in the 20 to 60% $(NH_4)_2SO_4$-saturated fraction (stage II) and possessed a specific activity of 2.10 μmol/min/mg of protein (Table 1). Figure 2 shows the elution profile of stage II *P. haemolytica* A1 neuraminidase on DEAE-Sephacel. Some neuraminidase activity (fractions 5 to 25) did not stick to the column. This was shown to be a result of overloading the column because when these fractions were reapplied to the column, they bound to the DEAE-Sephacel and eluted with the majority of the neuraminidase activity. All of the neuraminidase activity eluted from the column at NaCl concentrations from 0.1 to 0.25 M. This material, which had a specific activity of 2.74 μmol/min/mg of protein (Table 1), was then applied to a Sephadex G-200 column; the resultant profile is shown in Fig. 3. All of the neuraminidase activity eluted from the Sephadex G-200 column in one peak (stage IV) and possessed a specific activity of 40.23 μmol/min/mg of protein (Table 1). This material was then reapplied to the same Sephadex G-200 column. The absorption profile at 280 nm was slightly different from that of stage IV, but the neuraminidase profile was essentially the same (stage V; data not shown). The final (stage V) neuraminidase preparation had a specific activity of 100.62 μmol/min/mg of protein (Table 1). PAGE of the stage V neuraminidase preparation showed one band migrating near the top of the gel, and neuraminidase activity was always found in the gel slice containing this band (Fig. 4).

Molecular weight and pH optimum of *P. haemolytica* A1 neuraminidase. The molecular weight of the *P. haemolytica* A1 neuraminidase was calculated by its elution profile on the Sephadex G-200 column. The A1 neuraminidase had an apparent molecular weight of approximately 150,000 by chromatography on Sephadex G-200 (Fig. 3). When the purified A1 neuraminidase was subjected to SDS-PAGE, the molecular weight of the highest band was approximately 170,000 (data not shown). However, lower-molecular-weight fragments were seen on SDS-PAGE and probably represented fragments of the enzyme generated during the boiling of the preparation in 2-mercaptoethanol-SDS. The pH opti-

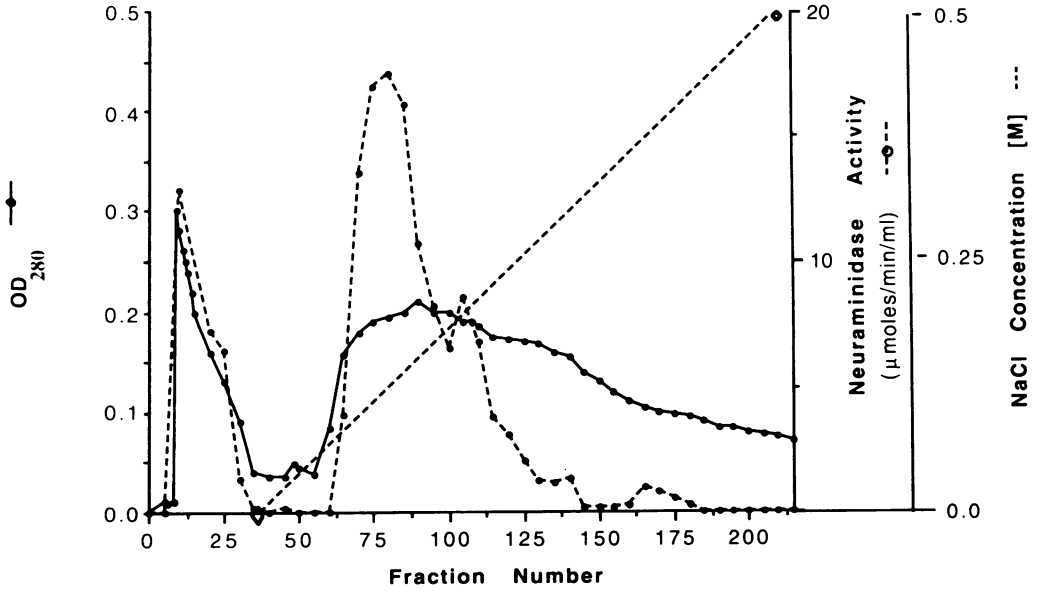

FIG. 2. Chromatography of stage II neuraminidase on DEAE-Sephacel. The peaks were monitored at 280 nm (OD_{280}) and eluted with an increasing NaCl gradient in 0.01 M sodium phosphate buffer (pH 7.5) at 4°C. Every fifth tube was assayed for neuraminidase activity by the method of Aminoff (1).

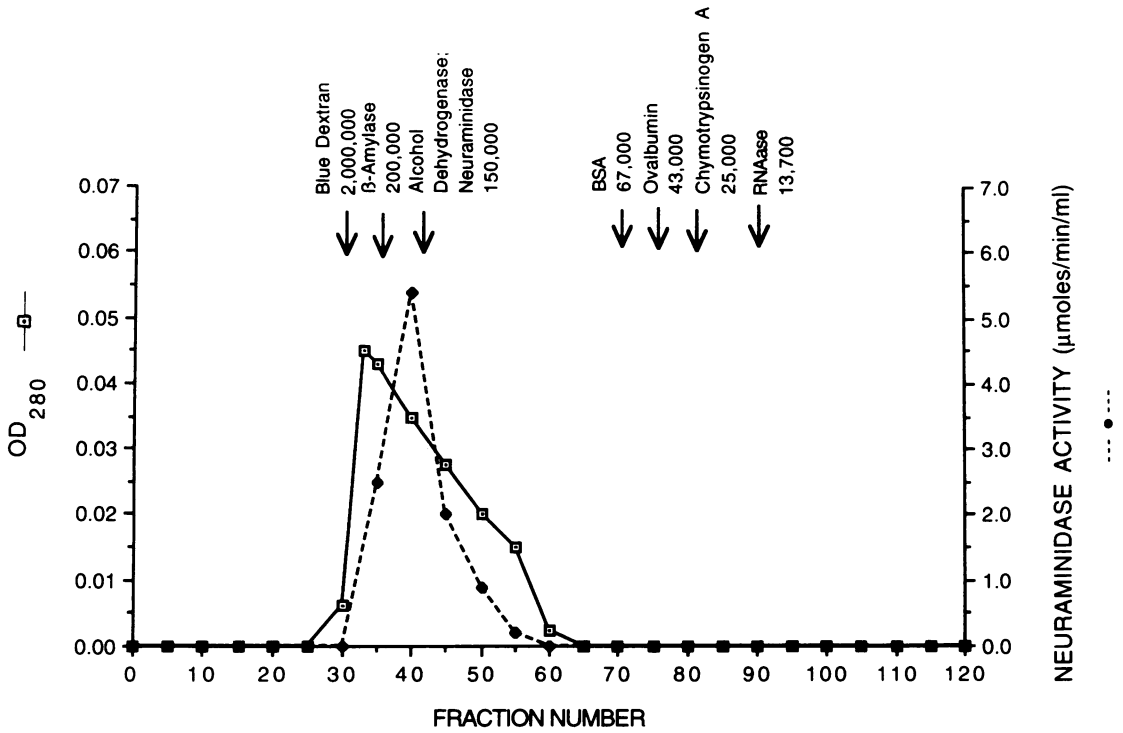

FIG. 3. Chromatography of stage III neuraminidase on a Sephadex G-200 column (2.5 by 90 cm). The peaks were monitored at 280 nm (OD_{280}) and eluted with 10 mM sodium acetate buffer (pH 6.5) at 4°C. Every fifth tube was assayed for neuraminidase activity by the procedure of Aminoff (1). Calibration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

mum was determined for the enzyme with human alpha 1-acid glycoprotein as the substrate. A peak of maximal activity occurred at pH 6.5 in 100 mM sodium acetate containing 10 mM $CaCl_2$ (Fig. 5).

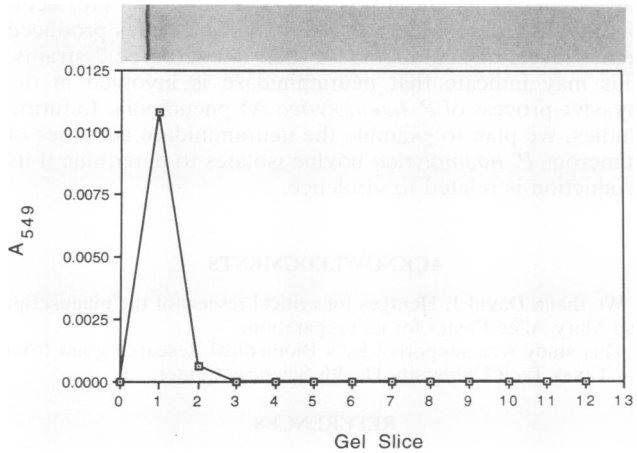

FIG. 4. PAGE of stage V *P. haemolytica* A1 neuraminidase in 7.5% polyacrylamide. Ten micrograms of protein was placed on the gel, which was run as described in Materials and Methods and stained with Coomassie brilliant blue. One lane was cut into 12 1-cm sections which were placed in dialysis bags and dialyzed at 4°C against 0.01 M sodium acetate, pH 6.5, for 72 h. After dialysis, the contents of each bag were analyzed for neuraminidase, employing human alpha 1-acid glycoprotein as the substrate, by the procedure of Aminoff (1).

DISCUSSION

Although there have been several studies documenting the production of neuraminidase by *P. haemolytica*, the production of the enzyme and its characteristics have not been described. Scharmann et al. (26) first examined *P. haemolytica* for neuraminidase production. They reported that the enzyme, if present (three of five strains examined), was in all instances associated with the bacterial cells. They made no further attempt at enzyme characterization. Only two other reports examining the neuraminidase of *P. haemolytica* can be found. Frank and Tabatabai (10) examined the neuramin-

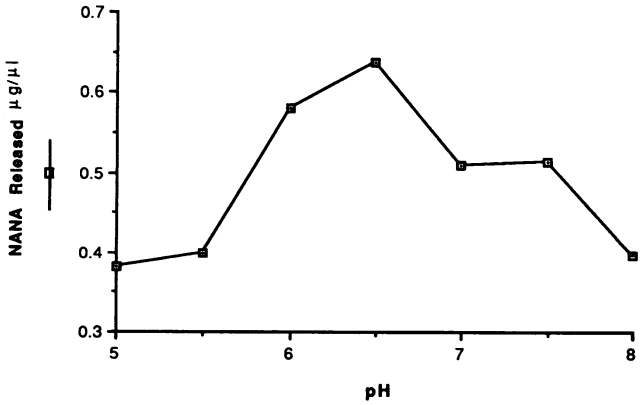

FIG. 5. pH optimum of *P. haemolytica* A1 neuraminidase in 0.01 M sodium acetate buffer. The substrate was human alpha 1-acid glycoprotein. NANA, *N*-acetylneuraminic acid.

idase activities of *P. haemolytica* cell suspensions. Their work demonstrated that neuraminidase activity levels of *P. haemolytica* isolates varied considerably and that the activity levels among the isolates they studied were serotype associated. All of the biotype T isolates they examined (serotypes 3, 4, and 10) and some of the biotype A isolates (serotype 11, untypeable isolates, and some bovine serotype 2 isolates) had no detectable enzyme activity. They also showed that there was considerably more neuraminidase released extracellularly by *P. haemolytica* than cell bound. Finally, the same investigators (29) attempted a partial characterization of the extracellular neuraminidase produced by *P. haemolytica* serotype 1. They showed that this enzyme was stimulated by the presence of Mn^{2+} and Ca^{2+}. They also showed that antibody prepared against whole *P. haemolytica* serotype 1 inhibited neuraminidase activity two- to threefold. This suggested to these investigators that the enzyme may be part of an antigenic complex.

We have confirmed the findings of Frank and Tabatabai (10, 29) that *P. haemolytica* produces an extracellular neuraminidase. Our data appear to indicate that the best time to harvest *P. haemolytica* A1 cells for maximal enzyme yield is during the stationary phase (Fig. 1). This is because this strain of *P. haemolytica* A1 does not appear to produce a protease that destroys the neuraminidase in the stationary phase, as has been reported for three different streptococcal strains (5, 19, 28). Indeed, when the stage I neuraminidase preparation was examined for protease activity, none was detected. Extracellular neuraminidase production paralleled the growth of *P. haemolytica* A1, as reported for group A streptococci (5) and for *Streptococcus sanguis* (28).

Purification of the *P. haemolytica* A1 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 enzyme preparation eluted from Sephadex G-200 gave one band on PAGE. This band was shown to possess neuraminidase activity (Fig. 4). The purified enzyme possessed a specific activity of 100.62 μmol of sialic acid released per min per mg of protein when assayed with alpha 1-acid glycoprotein as the substrate. The specific activity of this enzyme is considerably greater than that of the neuraminidase produced by *Streptococcus pneumoniae* (11), which had a specific activity of 45 μmol/min/mg of protein when alpha 1-acid glycoprotein was used as the substrate. With the same substrate, the neuraminidase of *S. sanguis* had a specific activity of 174.4 μmol/min/mg of protein (28). There is only one other report (29) of a specific activity of a neuraminidase produced by *P. haemolytica*. These investigators demonstrated a specific activity of 0.53 μmol of sialic acid released per min per mg of protein for *P. haemolytica* neuraminidase when fetuin was used as the substrate. The specific activity of our *P. haemolytica* A1 neuraminidase for this substrate is much higher, undoubtedly because of its extensive purification.

The molecular weight estimates for the *P. haemolytica* A1 neuraminidase based on gel filtration (Fig. 3) and SDS-PAGE were 150,000 and 170,000, respectively. These estimates indicate a decidedly larger molecular weight for the *P. haemolytica* A1 neuraminidase than for any other neuraminidase examined to date. For example, the neuraminidase produced by *Arthrobacter sialophilus* was reported to have a molecular weight of 87,000 as determined by gel filtration (8), and the neuraminidase produced by group A streptococci was shown by gel filtration to have a molecular weight of approximately 90,000 (5). Another bacterial neuraminidase with a molecular weight comparable to those of the *A. sialophilus* and *Streptococcus pyogenes* neuraminidases is produced by *S. pneumoniae*; it was reported to have a molecular weight of 88,000 (6). The neuraminidase produced by *S. sanguis* was estimated to have a molecular weight of 85,000 (28). There are several bacterial neuraminidases with molecular weights of less than 87,000. These include neuraminidases produced by *Vibrio cholerae* (molecular weight, 68,000 [6]), *Clostridium perfringens* (molecular weight, 56,000 [2, 3]), and *Corynebacterium diphtheriae* (molecular weight, 65,000 [22]). The single bacterial neuraminidase with a molecular weight similar to that of the *P. haemolytica* A1 neuraminidase is that of type III group B streptococci. This neuraminidase has been shown to have a molecular weight of 125,000 by gel filtration chromatography (21).

The extracellular neuraminidase of *P. haemolytica* A1 had a K_m value of 1.1 mg/ml when human alpha 1-acid glycoprotein was used as the substrate. A K_m value of 3.84 mg/ml has been reported for the A1 neuraminidase, but a different *P. haemolytica* strain was used and fetuin was the substrate, so it is impossible to compare the two values (29). The same investigators reported a V_{max} of 12.15 nmol/min for the *P. haemolytica* A1 neuraminidase with fetuin as the substrate. We found the V_{max} for the *P. haemolytica* A1 neuraminidase with human alpha 1-acid glycoprotein as the substrate to be 1.81 μmol/min.

The role of neuraminidase in the pathophysiology of pneumonia caused by *P. haemolytica* A1 remains speculative. Neuraminidase is produced by many species of bacteria and has been shown to be associated with virulence in some situations (20, 24, 27). Certain *P. haemolytica* serotypes have been more frequently isolated from cattle with pneumonia than have others (9). It is not known whether there is a relationship between the ability of *P. haemolytica* to induce pneumonia in cattle and to elaborate neuraminidase. Serotypes 1 and 2 are the primary serotypes isolated from the nares of cattle (9). *P. haemolytica* A1 is most frequently isolated from calves with respiratory distress and is most frequently isolated from cases of acute fibrinohemorrhagic pneumonias that develop in market-stressed feeder/stocker calves after shipment (15). Frank and Tabatabai (10) demonstrated that serotype 1 *P. haemolytica* isolates produced higher levels of neuraminidase than did serotype 2 strains. This may indicate that neuraminidase is involved in the invasive process of *P. haemolytica* A1 pneumonia. In future studies, we plan to examine the neuraminidase activities of numerous *P. haemolytica* bovine isolates to determine if its production is related to virulence.

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