Purification of *Haemophilus parasuis* Neuraminidase, Candidate Subunit Vaccine

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While new swine production technologies are making inroads into controlling many swine pathogens, *Haemophilus parasuis*-induced disease has unexpectedly emerged as an increasing problem (Nicolet, 1992; Boeckman, 1995; Radle, 1996; Zimmerman et al., 1997; Rapp-Gabrielson, 1999; Dee, 2000). Innovative new strategies of disease control are needed to meet the challenges associated with new production technologies for high health herds, including segregated early weaning and all-in all-out pig flow in production facilities.

*H. parasuis* normally colonizes the upper respiratory tract of pigs, the bacterium’s natural reservoir. Under undefined co-conditions, *H. parasuis* breaches the nasal mucosal barrier and causes pneumonia or systemic infection (polyserositis, polyarthritis, meningitis; Glasser's disease). The bacterium is readily transferred from sows to piglets prior to early weaning (transfer often occurs by 7-10 days of age). Morbidity and mortality can be devastatingly high in outbreaks in naive herds such as specific pathogen free (SPF) or other high health herds. *H. parasuis* can be a major cause of clinical disease in herds infected with PRRS virus (porcine reproductive and respiratory syndrome) and it can be a secondary invader in pigs with swine influenza virus or *Mycoplasma hyopneumoniae* infections. Available vaccines are less than optimal in preventing *H. parasuis* disease, in part because of the lack of cross protection between serovars (Rapp-Gabrielson, 1999).

An underinvestigated means to control bacteria infections is to investigate the pathogenesis (how the bacteria cause disease) and then develop directed vaccines or therapeutic interventions. A prime target of this analysis for *H. parasuis* is its enzyme neuraminidase (sialidase), an enzyme that hydrolyzes terminal sialic acid residues (group of unique nine carbon sugars) from sialoglycoconjugates. Whereas *H. parasuis* does not appear to synthesize any sialic acid containing substrates for the neuraminidase, sialoglycoconjugates are common in pigs and are potential substrates for the bacterial enzyme (Corfield, 1990; Troy, 1992; Pilatte et al., 1993; Lichtensteiger & Vimr, 1997). The role of neuraminidase in persistence or in virulence of *H. parasuis* is yet to be determined; possible roles include scavenging carbon or nitrogen for nutrition, unmasking receptors for invasion, or interfering with host defense systems (Corfield, 1990; Pilatte et al., 1993; Bagriacik & Miller, 1999; Vimr et al., 2000). Neuraminidase is a protective antigen in the related bacterium, *Pasteurella multocida* (Ifeanyi & Bailie, 1992; Lee et al., 1997) and also in the human influenza virus (Deroo et al., 1996; Martinet et al., 1997). Hence, neuraminidase is a good candidate for vaccine development regardless of its exact role in pathogenesis.

To facilitate further virulence investigations and vaccine development, we initially attempted to clone the *H. parasuis* neuraminidase gene by screening for neuraminidase expression, an approach that was successful in cloning the first bacterial neuraminidase gene (Vimr & Troy, 1985). We constructed genomic libraries of *H. parasuis* in lambda bacteriophage with 8 to 12 kb DNA fragments (partial Sau3A digestion) cloned into the BamHI site of the commercial system, Lambda-ZapR (Stragene, LaJolla, CA). Neuraminidase was not expressed in a functional form in screened plaques. We next screened over 4000 plaques (over ten fold the genome size) by hybridization with a degenerate probe based on sequence data from other bacterial neuraminidases. The probe did not hybridize any plaques in the *H. parasuis* library, adding evidence that the low overall conservation of the primary structure (20 to 30% conservation of amino acid sequence) among bacteria neuraminidases precludes this method of cloning the gene (Hoyer et al., 1992;
Roggentin et al., 1993; Vimr, 1994). The next approach was to determine and use the amino (N) terminus protein sequence data to identify the neuraminidase gene in genomic libraries for cloning. This protein sequencing requires adequate purification of the neuraminidase protein.

We optimized neuraminidase purification to obtain a protein preparation adequate for sequencing. While optimizing, we developed a protocol to renature enzymatic activity after protein denaturation. After purification, over 90% of the activity was recovered with a marked enrichment in specific activity and purified preparations approached electrophoretic homogeneity. Protein sequencing identified ten amino acids at the amino terminus of a purified protein band with neuraminidase activity. The protein sequence did not match any sequence in GenBank database (http://www.ncbi.nlm.nih.gov); this may be due, in part, to the shortness of the sequence as well as the heterogeneity of bacterial neuraminidases.

Materials and Methods

Protein purification: The H. parasuis isolate, ATCC #19417, was grown in Haemophilus Test Medium broth or chocolate agar plates (Remel, Lenexa, KS). For purification, lawns of cells were grown for three to four days on chocolate agar at 37°C and 5% CO₂. During purification, the samples were processed at 4°C. The cells were collected (2 to 12, 100 millimeter diameter agar plates usually) by scrapping the cells in 50 millimolar (mM) Tris, 150 mM NaCl, 5 mM EDTA buffer at pH 7.2. The cells were collected and washed once in the buffer by centrifugation. The membrane fraction was extracted by sonication to lyse the cells (Branson Sonicator Cell Disrupter 185, Branson Ultrasonics, Danbury, CT; seven cycles of 30 seconds with intervening 45 second incubations on ice), low speed centrifugation (5 minutes, 1,500 x g) to remove large cell fragments in the pellet, and ultracentrifugation (30 minutes, 150,000 x g) to collect the membrane fraction (the ultracentrifugation pellet).

From the membrane fraction, the n-octyl-β-D-glucopyranoside (octyl glucoside; OcGl; Inalco Pharmaceutical, San Luis, CA) soluble fraction was extracted by resuspending the membrane fraction in 50 mM OcGl in the Tris, NaCl buffer. (OcGl is a non ionic detergent that is removable with dialysis.) After incubating 30 minutes on ice, the non soluble fraction was removed by ultracentrifugation (30 minutes, 150,000 x g), and the supernatant was collected. The sample was dialyzed (12 kilodalton [kDa] membrane exclusion) with 10 mM Tris, pH 7.2 and the protein collected by ultracentrifugation (30 minutes, 150,000 x g) and resuspended in the 50 mM OcGl buffer. Next, proteins were precipitated by fractionation with 35% to 65% NH₄SO₄ (Englard & Seifter, 1990). The enzymatically active protein fraction precipitated at 65% NH₄SO₄; proteins precipitated at 35% were discarded (centrifugations 10 minutes, 25,000 x g). The 65% precipitated fraction was resuspended in OcGl and further purified by ion exchange chromatography (Hi-Trap Q Sepharose HP column, Amersham-Pharmacia, Piscataway, NJ). The column was charged with the OcGl buffer and the sample loaded. After washing with 20 volumes of loading buffer, protein was eluted with 0.1 M NaCl. The eluant was dialyzed with water. The sample was concentrated about 20 fold by lyophylization and resuspension in water.

Enzyme analysis: Neuraminidase activity was detected with the fluorescent substrate 2′-(4′-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4MU-Neu5Ac). Neuraminidase activity was monitored in the different fractions during purification with a spot assay on impervious wax paper (Lichtensteiger & Vimr, 1997). For the spot assays, equal volumes (1 to 2 microliter [µl]) of sample and 4MU-Neu5Ac at 2 microgram (µg) µl⁻¹ were spotted together and the fluorescence viewed under long UV light. After purification, neuraminidase activity was determined by measuring relative fluorescent units from 4MU-Neu5Ac in aliquots held from each step of the purification (50 µl collected and held at 4°C). For the assays, 3 µl of sample in 222 mM sodium acetate buffer pH 4.5 to 5.5 (in the pH range of optimal activity of the enzyme) was mixed with 3 µl 4MU-Neu5Ac (6 µg) resuspended in 100 mM sodium acetate, 308 mM NaCl, 8 mM CaCl₂, pH 5.5 (Lichtensteiger &
At two and four minutes of incubation, 2 µl aliquots were taken and the reaction stopped by adding the aliquot to 3 ml of 100 mM glycine at pH 10. Fluorescence from hydrolyzed substrate was measured with UV light (Turner fluorometer; (Vimr et al., 1988)). A fluorescent unit was the relative amount of fluorescence released from 4MU-Neu5Ac per minute.

The protein in purification fractions was also analyzed by electrophoresis in sodium dodecyl sulfate (SDS) 10% polyacrylamide gels (Garfin, 1990). For electrophoresis, the samples were boiled in SDS detergent and 2-mercaptoethanol which, as expected, denatured the enzyme and caused complete loss of neuraminidase activity. The neuraminidase was renatured by washing the gel in 0.5% Tween-40 (polyoxyethylene sorbitan monopalmitate) in 100 mM sodium acetate, 50 mM NaCl buffer at pH 8.0: two washes (from one hour to overnight) followed by two rinses with pH 8.0 buffer and one rinse with buffer at pH 5.0 (rinses one hour each). For all renaturation steps the gels and buffers were at 4°C. To detect neuraminidase activity, the gel was equilibrated with room temperature and overlain with 4MU-Neu5Ac, 2 to 10 µg in 3 ml 0.5% low melt agarose. Fluorescence was detected and imaged with a computer operated camera system (Alpha Imager™ 2000; Alpha Innotech Corp., San Leandro, CA). After fluorescent imaging, protein bands were detected in the gel by Coomassie blue R250 stain and imaged with the same camera system under white light. In the gels, two standards were resolved in parallel with the samples: colored rainbow protein size standards (Amersham, Arlington Heights, IL; now Amersham Pharmacia, Piscataway, NJ) and purified recombinant *Vibrio cholerae* neuraminidase (Vimr et al., 1988).

Protein concentration was measured in aliquots taken during purification steps by chromophore binding standardized to bovine serum albumin (BCA kit; Pierce, Rockford, IL).

**Protein sequencing:** To sequence the amino (N) terminus, the final purified protein preparation was resolved in 10% polyacrylamide gel by electrophoresis and then transblotted onto polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA). The transblotting was done in a 10 mM CAPS at pH 11, 5% methanol, 0.075% SDS buffer with 350 milliamp of current overnight followed by 40 minutes at 500 milliamp. (CAPS is 3-[cycolhexylamino]-1-propanesulfonic acid.) The blotting buffer was continually cooled by circulating tap water in adjacent coils. The PVDF membrane was washed in water three times (15 minute each) and the proteins were stained with Coomassie blue (0.1% dye in 1% acetic acid and 50% methanol (5 minutes). The membrane was then destained with 1% acidic acid and 50% methanol, washed in 10% methanol, and air dried overnight. The dried membrane was submitted to the University of Illinois Keck Center for sequencing of the amino terminus by Edman chemistry (Procise 494 HT; Perkin-Elmer, Wellesley, MA).

**Results**

The spot assays of neuraminidase activity (fluorescent substrate, 4MU-Neu5Ac) in the purification fractions at each step indicated that very little enzyme was lost. Neuraminidase activity was essentially found only in the expected fractions with the exception of a little activity in the pelleted large cell fragments after sonication in some preparative runs. When the large fragments were resonicated, most of the neuraminidase activity was recovered. The NH₄SO₄ protein precipitation markedly decreased the total protein in the preparation. For example in the extraction in Table 1, the total protein decreased from 446 mg in OcGl extracted sample to 16.8 mg in the NH₄SO₄ precipitate fraction.

However, too many proteins were still in the preparation (Figure 1A, lanes 2 and 3). Because of the additional proteins still present, we expanded the purification to include ion exchange chromatography with another round of dialysis and lyophylization. Results from one of several runs with the expanded purification protocol are listed in Table 1. Elution efficiency was not increased
with increased ionic strength as 0.2 M NaCl essentially did not recover any more neuraminidase activity after elution with 0.1 M NaCl (Table 1). The specific neuraminidase activity was increased 136 fold and essentially all of the neuraminidase activity recovered. In other runs with aliquot testing, about 90% was recovered (data not shown).

Aliquots were collected during purification and the samples analyzed with gel electrophoresis. In addition to (and prior to) staining proteins in the gels, neuraminidase activity was determined with an agarose overlay of 4MU-Neu5Ac (Figure 1B). To do so, we devised a method to renature the activity of the neuraminidase. We routinely renatured the neuraminidase in the gel by washing with 0.5% Tween-40 to remove the SDS and renature the enzyme to its active form. We originally renatured the neuraminidase with 0.25% poloxoxyethylene 7 lauryl ether that was known to renature viral influenza neuraminidase. We determined that both Tween-40 and NP-40 (nonylphenoxy polyethoxy ethanol) renatured the protein to its active form. The Tween-40 results were similar to lauryl ether and Tween-40 was a much less expensive detergent. NP-40 worked adequately, but subjectively the activity (fluorescence) was slightly less.

Figure 1B indicates a single band of neuraminidase activity in the cell pellet and two NH₄SO₄ precipitation preparations of *H. parasuis* (Lanes 2, 3, and 4). The activity comigrates with the neuraminidase activity of *Vibrio cholerae* recombinant protein (Lane 6) (Vimr et al., 1988). In the preparation photographed in Figure 1A, the Coomassie blue stained protein band corresponding to the *H. parasuis* neuraminidase activity was too pale to photograph well in contrast to the *V. cholerae* recombinant neuraminidase. The moderately prominent band in the Figure 1A near the level of the *V. cholerae* is just above the neuraminidase activity. When the purification scale was increased by starting with a larger *H. parasuis* cell pellet, the neuraminidase protein was readily visible just below the more prominent, non neuraminidase band in Figure 1A. This non neuraminidase protein was the major limitation to electrophoretic homogeneity. Another change in the larger and more extensive preparations was the addition of slightly smaller bands of activity in the final preparations (interpreted as fragmentation caused by proteolysis during handling).

The initial sequencing attempts failed to generate amino acid sequence with much assurance of correct interpretation. When we scaled to larger preparations; these preparations had three bands of neuraminidase activity in the transblot apparently due to fragmentation of the neuraminidase band (two bands of activity slightly below the single expected one). However, one preparation yielded a 10 amino acid sequence from one of the lower, apparently fragmented, protein bands that also corresponded to neuraminidase activity: threoanyl-valyl-phenylalanyl-leucyl-lysyl-cystyl-aspartyl-leucyl-tyrosyl-glutamic acid. The sequence had no homology in GenBank. Using

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**Figure 1.** Renaturization of *H. parasuis* neuraminidase activity and apparent molecular size of the enzyme. **A.** Coomassie blue staining of protein resolved in a SDS polyacrylamide gel. **B.** Agarose and 4MU-Neu5Ac overlay of gel in Fig. 1A to detect neuraminidase activity; done prior to protein staining. Gel lanes: Lane 1, colored protein standards with size listed to the left in kilodaltons (kDa); Lane 2, *H. parasuis* cell pellet; Lanes 3 and 4, two NH₄SO₄ precipitated preparations of *H. parasuis* OcGl extracted, membrane protein; Lane 5, blank; and Lane 6, purified *V. cholerae* recombinant neuraminidase, which resolves at 82 kDa (arrow).
the sequence data, we designed an 18 nucleotide degenerate probe: 5'-GT(I) TT(T/C) (C/T)T(I) AA(G/A) TG(T/C) GA(C/T)-3'. The nucleotide probe was synthesized (IDT Inc, Coralville, IA) and end radiolabeled. The probe failed to bind *H. parasuis* DNA in a Southern blot analysis; therefore, it was not a valid probe to screen the lamada phage *H. parasuis* gene library for neuraminidase activity.

<table>
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<th>Preparation</th>
<th>Quantity (ml)</th>
<th>Protein (mg)</th>
<th>Per unit (fu/ul)</th>
<th>Total (fu)</th>
<th>Specific (fu/mg protein)</th>
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<td>3680</td>
<td>2208</td>
<td>1283</td>
<td>136</td>
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</table>

¹ fu: fluorescent unit, the relative amount of 2'- (4'-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4MU-Neu5Acsubstrate) hydrolyzed per minute.  
² Purification factor: the relative increase in specific activity compared to the starting preparation (fold increase in fu/mg protein). The factor equals the specific activity of the preparation divided by the specific activity of the initial cell pellet.  
³ Membrane fraction: fraction derived by sonication of the cell pellet followed by ultracentrifugation (150,000 x g) to collect of the membrane fraction after low speed centrifugation (1,500 x g) to discard large cell debris and unbroken cells.  
⁴ Column: ion exchange chromatography with the preparation in 50 mM OcGl detergent.  
⁵ Neuraminidase preparation: column elute was dialyzed, lyophylized, and resuspended in a small volume of water.

**Discussion**

We identified a segment of ten amino acids in *H. parasuis* neuraminidase. The GenBank protein sequence data base had no homologous structure to the sequence of these residues. This may be due to the limitation of a short sequence but is also compatible with the sequence structure variability among bacterial neuraminidases. A larger initial preparation and resolving the final preparation in a longer gel before transblotting may increase (double) the length of sequence that can be identified. In addition, other cloning vector systems have been derived that may allow direct cloning for expression screening. For example, we have recently cloned genes, after other methods failed, with a new fosmid system with increased stability (CopyControl™ Fosmid Library; Epicenter
Technologies, Inc, Chicago, IL). With genes that are refractory to cloning, numerous methods need to be tried as there is no predictor of which will be successful. If direct cloning is successful, our protein purification method will facilitate purification of the recombinant neuraminidase.

The neuraminidase activity was localized to the membrane fraction of the cell pellet. This puts the enzyme close to host sialoglycoconjugate substrates. Contact with host substrates may be increased if the enzyme is secreted (not apparent in our earlier in vitro studies) or released in membrane blebs (appears to occur at a low level in vitro) (Lichtensteiger & Vimr, 1997).

We determined the size of the H. parasuis via our protocol that renatures the neuraminidase activity after resolving the enzyme preparation in a denaturing polyacrylamide gel. The H. parasuis neuraminidase co-resolves with that of V. cholerae neuraminidase (82 kDa), indicating H. parasuis neuraminidase belongs to the “large” (> 80 kDa) versus “small” (about 40 kDa) neuraminidases (Vimr, 1994). Our results suggests H. parasuis synthesizes a single neuraminidase (the two additional slightly smaller bands in some preparations are likely fragmentation during extensive purification). The single protein contrasts with the related P. multocida (both bacterial species in the HAP group; Haemophilus, Actinobacillus, Pasteurella) in which two neuraminidase genes (proteins of 80 and 120 kDa) were recently cloned after years of controversy of the size of its neuraminidase (claims of 36 to 250kDa) (Mizan et al., 2000).

Interestingly our renaturation protocol failed to renature the neuraminidases from P. multocida (data not shown) although it readily renatured neuraminidases of V. cholerae (82 kDa; Fig 1A) and Salmonella typhimurium LT2 (42 kDa; data not shown (Hoyer et al., 1992)) as well as the H. parasuis enzyme (Fig 1A). Hence critical differences can occur in neuraminidases in related bacterial species and more distant species may have similar neuraminidases properties. This is compatible with the lateral horizontal transfer of neuraminidase genes (Roggentin et al., 1993).

References


