

Isolation of immunogenic outer membrane proteins from *Mannheimia haemolytica* serotype 1 by use of selective extraction and immunoaffinity chromatography

Jerry K. McVicker, MS, and Louisa B. Tabatabai, PhD

Objective—To use antibodies produced by calves in response to infection with *Mannheimia haemolytica* in immunoaffinity chromatography for the identification and subsequent isolation of the dominant immunogenic antigens from bacteria grown in iron-deficient media.

Sample Population—Serum from 10 calves actively infected with *M haemolytica*.

Procedure—An outer membrane protein fraction was obtained from sonicated salt-extracted *M haemolytica* cells by extraction with N-lauroyl sarcosinate. The immunoglobulin fraction of serum from calves actively infected with *M haemolytica* was used to prepare an immunoaffinity column. The immunoaffinity column was used to isolate the dominant immunogenic proteins from the outer membrane protein fraction. The resultant immunogenic protein fraction was subjected to ELISA and immunoblot methods as well as carbohydrate quantification. Sequencing of the N-terminal was performed on the most prominent protein.

Results—5 immunogenic proteins with molecular weights of 42, 30, 24, 20, and 15 kd were isolated. The immunogenic protein fraction was found to contain 51% carbohydrate. The immunoaffinity column capacity was 1 µg of immunogenic protein/mL of gel. The N-terminal sequence of the 42-kd protein was Tyr-Gln-Thr-Tyr-Gln-Ser-X-Leu-Gln, where X could not be identified.

Conclusions and Clinical Relevance—Immunogenic proteins were isolated by use of immunoaffinity chromatography. A substantial amount of carbohydrates was co-purified in the process. Additional experiments are needed to determine whether the carbohydrates would hinder or enhance development of vaccine preparations. This method could potentially allow a more rapid production of antigens for use in vaccines. (*Am J Vet Res* 2002;63:1634–1640)

Mannheimia haemolytica, a gram-negative organism, is the major cause of pneumonic pasteurellosis or shipping fever in cattle as well as pneumonia and septicemia in sheep.^{1,2} Most disease outbreaks in cattle are attributable to infection with serovar-1 strains.^{3,4} Several virulence factors of *M haemolytica* serovar 1

have been identified, but the pathogenesis of this complex disease and the roles of these factors in producing disease have not been fully elucidated.⁵

A major problem in the control of pneumonic pasteurellosis in cattle is the lack of a vaccine that consistently induces protective immunity against *M haemolytica*.⁶⁻¹⁰ Although commercial vaccines (live and killed) are available, their efficacy in field trials has been variable. It has been reported that some of these attempts at vaccine production have resulted in poor protection or even an increase in disease severity.¹¹⁻¹⁶ Conversely, cattle that have recovered from natural infection appear to be resistant to subsequent infection, implying that an effective vaccine could be developed if the immunogens responsible for the natural protection could be identified.^{6,17}

A number of studies¹⁸⁻²³ have attempted to identify potentially important immunogenic proteins and membrane fractions of *M haemolytica* that could be used to develop safe, efficacious vaccines. Those studies have focused largely on cell-wall materials, capsular antigens, carbohydrate-protein fractions, leukotoxin, and lipopolysaccharide, whereas additional purification of specific proteins has been performed electrophoretically, chromatographically, or with recombinant techniques. It is only when the proteins or fractions are isolated that many of these investigations begin to evaluate the immunogenicity of the material. By performing investigations in this manner, a great deal of effort and time is expended searching for immunogenic components.

Another obstacle in the development of adequate vaccines has been the use of *M haemolytica* grown in vitro. A number of differences exist between the protein profiles of bacteria grown in vivo and those grown in vitro.^{6,24} It is clear that bacteria grown in vivo differ substantially from iron-sufficient bacteria grown in vitro in terms of the number and quantity of proteins expressed.²⁵ In fact, successful pathogens have evolved multiple iron-acquisition systems to overcome the limited availability of iron in vivo. *Neisseria meningitidis* can utilize iron from host transferrin, lactoferrin, haptoglobin, and hemoglobin.²⁶ Similar pathways have been described for *Haemophilus influenzae*²⁷ and *M haemolytica*.²⁸

The objective of the study reported here was to determine whether antibodies produced by calves in response to infection with *M haemolytica* could be used in an immunoaffinity chromatography system for the identification and subsequent isolation of dominant immunogenic antigens from bacteria grown in iron-

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From the Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Ames, IA 50011 (McVicker); and the USDA-Agricultural Research Service, National Animal Disease Center, 2300 Dayton Ave, Ames, IA 50010 (Tabatabai).

Address correspondence to Dr. Tabatabai.

deficient media. This approach would allow more rapid production of antigens and, subsequently, vaccines.

Materials and Methods

Serum from infected calves—Sera from 10 calves with active *M haemolytica* infection were obtained^a for use in the study. Calves had been inoculated with 2.8×10^9 colony forming units of *M haemolytica* serotype 1 in a 5-mL volume of RPMI administered via a needle inserted into the trachea. The inoculum was followed by 50 mL of PBS solution. The calves had clinical signs of acute disease, and blood samples were obtained 30 days after inoculation for harvest of serum. Titers (\log_2) determined by use of an indirect hemagglutination (IHA) assay ranged from 4 to 7 for these sera.⁹

Serum from calves inoculated with experimental vaccine—Sera from calves used in a study of an experimental *M haemolytica* vaccine were obtained^b for use in the study. The experimental vaccine was constructed from an isolate of *M haemolytica* from which the gene for the leukotoxin protein was deleted. Calves had been allocated into 3 groups (nonvaccinated control calves and calves inoculated orally or parenterally). An initial serum sample was collected on the day of inoculation (day 0) and on days 21, 28, and 33 after inoculation. The \log_2 titers determined by use of an IHA assay ranged from 2 to 4 for control calves, 2 to 8 for calves inoculated orally, and 2 to 6 for calves inoculated parenterally.⁹

Preparation of the immunoaffinity column—A polyphosphate method was used to isolate the immunoglobulin fraction of the pooled serum obtained from the *M haemolytica*-infected calves.²⁹ Ten grams of polyphosphate was added to 1,000 mL of pooled serum at 20°C. Hydrochloric acid (3.0M) was slowly added to the mixture until the pH reached 3.95. The solution was centrifuged ($7,500 \times g$ for 15 minutes), and immunoglobulins were recovered from the supernatant. Neutralization of the immunoglobulin fraction was achieved by slowly adding 10.0M sodium hydroxide until the solution reached pH 7.6. The immunoglobulin fraction was extensively dialyzed against a solution that consisted of 50mM Tris, 300mM NaCl, and 0.1% (wt:vol) sodium azide (pH, 7.4).

After dialysis, the immunoglobulin fraction was concentrated to a volume of 300 mL by use of polyethylene glycol (molecular weight, 40,000). Protein concentration of the fraction was 40 mg/mL, as determined by use of the bicinonic acid method. Concentration of bovine IgG was 20 mg/mL, as determined by use of an automated clinical chemistry analyzer.³⁰

The immunoglobulin fraction was dialyzed against a solution of 100mM potassium phosphate and 100mM sodium chloride (pH, 7.1) and subsequently was conjugated to 500 mL of aldehyde-activated agarose.^c Uncoupled aldehyde sites were blocked with a solution of 100mM potassium phosphate and 100mM ethanolamine (pH, 7.1). After coupling and blocking, the agarose was extensively washed with a solution of 100mM potassium phosphate and 500mM sodium chloride (pH, 7.1) and a final wash with a solution of 150mM Tris, 300mM sodium chloride, 10% (wt:vol) sucrose, and 0.1% (wt:vol) sodium azide (pH, 7.2). Binding efficiency was 97%.

Organism and culture—*Mannheimia haemolytica* serovar 1 (isolate L101) was isolated from infected bovine lung tissue as described elsewhere.⁹ Six 1-L bottles that contained 500 mL of RPMI-1640 buffered with 25mM HEPES (pH, 7.2) were inoculated (1% [vol:vol]) with a culture of *M haemolytica* grown for 4 to 5 hours in the same medium after inoculation from a blood agar plate. The culture was

incubated at 37°C for 20 to 24 hours with shaking until the absorbance at 600 nm reached a value of 0.5 to 1.0.

Isolation of outer membrane protein fraction—The *M haemolytica* culture was centrifuged at $15,000 \times g$. The cell pellet was washed once with 20mM Tris (pH, 7.5), suspended in 1.0M NaCl-0.1M sodium citrate, and extracted as described elsewhere.³¹ The salt-extracted pellet (10 g) was then resuspended in 10 mL of buffer solution (20mM HEPES and 0.5mM phenylmethylsulfonyl fluoride [PMSF]; pH, 7.4). Resuspended cells were sonicated as described elsewhere.²³ The sonicated cells were pelleted by use of centrifugation ($3,000 \times g$), resuspended with HEPES-PMSF buffer, and centrifuged at $50,000 \times g$ for 1 hour. The pellet was resuspended in 10 mL of HEPES-PMSF buffer and further extracted with 1% (wt:vol) N-lauroyl sarcosinate^d at 21°C for 20 minutes. After extraction with N-lauroyl sarcosinate, the material was centrifuged at $17,700 \times g$ for 1 hour and then separated into supernatant and the outer membrane protein pellet.

Isolation of immunogenic proteins—The outer membrane protein pellet that resulted from extraction with N-lauroyl sarcosinate was resuspended in 3.5 mL of a solution of 20mM HEPES, 150mM sodium chloride, and 4% (wt:vol) SDS (pH, 7.4) and incubated at 37°C for 1 hour. The extract mixture was then centrifuged at $50,000 \times g$ for 2 hours. The supernatant, representing the outer membrane proteins, was removed and dialyzed against a solution of 50mM Tris, 300mM sodium chloride, 5mM EDTA, and 0.1% (wt:vol) sodium azide (pH, 7.8).²¹ The sample was then heated to 60°C for 10 minutes and allowed to cool slowly. It was subsequently eluted by use of the immunoaffinity column (equilibrated with the same buffer). The column was eluted with 100mM glycine (pH, 2.8). Eluted proteins (second peak) were collected in a buffer solution (2.0M Tris and 3.0M sodium chloride [pH, 7.8]) at 5% (vol:vol) for neutralization. Additionally, SDS (2% [wt:vol]) was added to the eluted proteins of the second peak to maintain protein solubility. Eluent from the first peak was added to the column and eluted in the same manner 2 more times to ensure that the column was not overloaded and that all of the immunogenic proteins were collected.

Eluted immunogenic proteins from all 3 runs were pooled and applied to a desalting column.^e The final eluent was electrophoresed in a SDS-PAGE running gel (25mM Tris, 195mM glycine, 1% SDS [pH, 8.3]). Concentration was adjusted by use of polyethylene glycol (molecular weight, 40,000) to achieve a total protein concentration of 1.3 mg/mL.

SDS-PAGE and immunoblotting—The SDS-PAGE electrophoresis was performed by use of reducing conditions with 0.5-mm-thick gels^f (4–20% polyacrylamide gradient).³² Samples were adjusted to a total protein concentration of 1 mg/mL. A mini-gel system^g was used as described in the manufacturer's instructions. Following electrophoresis, gels were fixed with 20% (wt:vol) trichloroacetic acid and then stained with Coomassie blue stain^h or silver stain.ⁱ

For the immunoblot analysis, gels were soaked in blotting buffer (25mM Tris, 195mM glycine, and 20% [vol:vol] methanol) and electrophoretically blotted onto nitrocellulose at 30 V and 0.1 mA for 16 hours. Following blotting, membranes were blocked with 1% normal mouse serum in 100mM PBS solution (pH, 7.2) for 16 hours at 4°C. After 4 washes in PBS solution containing 0.1% Tween 20, incubation with the pooled serum from the actively infected calves (dilution of 1:25) was performed for 1 hour at 21°C, which again was followed by 4 washes. After addition of streptavidin-conjugated horseradish peroxidase (dilution of 1:250

in PBS solution containing 1% normal mouse serum), samples were incubated for 1 hour at 21°C, which was followed by 4 washes. Peroxidase activity was detected by use of a H₂O₂ and 4-chloro-1-naphthol substrate kit.¹ The reaction was stopped by rinsing membranes in distilled water.

N-terminal sequencing of an immunogenic protein—

Electrophoretic blotting of the immunogenic proteins was performed in accordance with the procedure described previously, except a polyvinylidene difluoride (PVDF) membrane was substituted for the nitrocellulose membrane, and the PVDF membrane was activated in methanol prior to soaking in blotting buffer. Following blotting, the PVDF membrane was stained with 0.125% Coomassie stainⁿ in a solution that consisted of 25% (vol:vol) 2-propanol and 10% (vol:vol) acetic acid for 5 minutes. The membrane was destained in a solution that consisted of 25% (vol:vol) 2-propanol and 10% (vol:vol) acetic acid; the solution was changed several times until the background cleared.

One of the immunogenic proteins (42 kd) was excised from the membrane, washed extensively with deionized water, and dried. The N-terminal sequence was determined with an automated protein sequencer.^k A computer-based search program^l was used to determine homology.³³

Carbohydrate determination—Carbohydrate content of the N-lauroyl sarcosinate fractions and the immunogenic proteins was determined.³⁴ Carbohydrate testing was performed to determine whether polysaccharides were isolated by use of the immunoaffinity column. To assess the portion of the immunogenic protein preparation that was polysaccharide rather than protein, we performed a method described elsewhere.³⁵ A solution of 80% phenol was added to samples and standards. Concentrated H₂SO₄ was then added, and the tubes were allowed to cool. Absorbance of the samples was determined at 485 nm. Sample concentrations were calculated from regression analysis of a plot of the standard concentrations of α -D-glucose versus absorbance. Amino sugars were not determined by this procedure.

ELISA that used serum from calves inoculated with experimental vaccine—An ELISA was used to test the reactivity of serum from the calves vaccinated with an experimental *M haemolytica* vaccine. The immunogenic proteins were adsorbed overnight at 4°C to a 96-well immunoplate at a rate of 0.1 μ g/mL in carbonate-bicarbonate buffer (25mM sodium carbonate and 25mM sodium bicarbonate [pH, 9.6]). The plate was washed 4 times with PBS solution containing 0.1% Tween 20. After washing, the plate was blocked with 1% normal mouse serum in carbonate-bicarbonate buffer for 1 hour at 37°C. The plate was again washed with PBS solution containing 0.1% Tween 20. Serum samples were diluted 1:3,000 in PBS solution-Tween 20-1% normal mouse serum, added to the blocked plate, and incubated for 1 hour at 37°C. After the plate was washed again, horseradish peroxidase-labeled mouse anti-bovine IgG antibody (diluted 1:250 in PBS solution-Tween 20-1% normal mouse serum) was added. The conjugate was allowed to incubate at 37°C for 1 hour. After a final wash with PBS solution that contained 0.1% Tween 20, we added 3,3',5,5'-tetramethylbenzidine substrate^m to develop peroxidase reactivity. A plate reader was used to detect the absorbance of wells within the plate.

Results

Characterization of the outer membrane protein fraction—Total protein in the N-lauroyl sarcosinate pellet (ie, outer membrane protein) represented 28% of the initial material (sonication supernatant), whereas 66% was recovered in the N-lauroyl sarcosinate super-

natant. Total recovery for the pellet and supernatant fractions was 94%. The loss of 6% was considered negligible and could be attributed to inaccuracy in protein estimations or volume measurements.

Results of SDS-PAGE of the N-lauroyl sarcosinate extraction products for denaturing conditions revealed qualitative and quantitative differences in the protein bands (Fig 1). Examination of the supernatant fraction revealed results similar to those reported in another study.²¹ However, it appears that the 30-kd major protein identified in the supernatant in the study reported here corresponds to a major protein contained in the pellet of that other study. In addition, we identified a 42-kd protein band in the supernatant. If this is the same protein as the 42-kd protein found in the pellet, then it apparently was partitioned between the supernatant and pellet.

Isolation of immunogenic proteins—To solubilize the pellet that resulted from the N-lauroyl sarcosinate extraction, SDS (0.5% [wt:vol]) was added to the resuspension buffer. The SDS appeared to hamper binding of the immunoaffinity column, because elution peaks were not observed when SDS was included in the equilibration or sample buffer. Nonionic detergents did not resolubilize the pellet, so the pellet was heated at 60°C for 10 minutes prior to application to the immunoaffinity column.³³

The heat-solubilized pellet that resulted from N-lauroyl sarcosinate extraction was repeatedly applied to the immunoaffinity column constructed of immunoglobulins from actively infected calves. The pellet was repeatedly applied to ensure that all immunogenic proteins were removed from the sample. Chromatograms were evaluated (Fig 2), and when elution peaks were not detected, it was assumed that all the immunogenic proteins were removed. The capacity of the column was determined by dividing the total amount of protein isolated in all of the elution peaks by the total number of applications to the column. In this manner, the capacity was calculated to be 500 μ g/application (1 μ g/mL of gel). A total of 1,500 μ g was isolated.

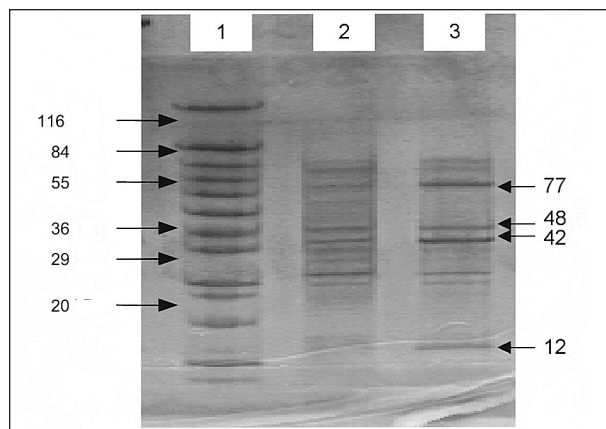


Figure 1—Gel resulting from SDS-PAGE of the pellet and supernatant fractions that were created by N-lauroyl sarcosinate extraction of sonicated *Mannheimia haemolytica*. Proteins were loaded onto a 8–25% gel and stained with Coomassie brilliant blue. Lanes were as follows: 1, molecular weight standards; 2, supernatant; and 3, pellet. Molecular weights (in kilodaltons) of the outer membrane proteins are indicated.

Buffer exchange with the desalting column—All the pooled immunogenic proteins from the immunoaffinity chromatography were dialyzed against a solution of 50mM Tris, 300mM sodium chloride, 5mM EDTA, 0.1% (wt:vol) sodium azide, and 2% (wt:vol) SDS (pH, 7.8). Subsequent attempts at SDS-PAGE revealed smudging and clumping in the gels, so it was decided to exchange the proteins into the SDS-PAGE running buffer (25mM Tris, 195mM glycine, and 1% [wt:vol] SDS [pH, 8.3]).

SDS-PAGE of immunogenic proteins—After exchanging buffer with the desalting column and achieving a protein concentration of 1.3 mg/mL, SDS-PAGE was performed by use of denaturing conditions (Fig 3). Molecular weights of immunogenic proteins were determined to be 42, 30, 24, 20, and 15 kd.

The supernatant and pellet that resulted from N-lauroyl sarcosinate extraction were also examined by

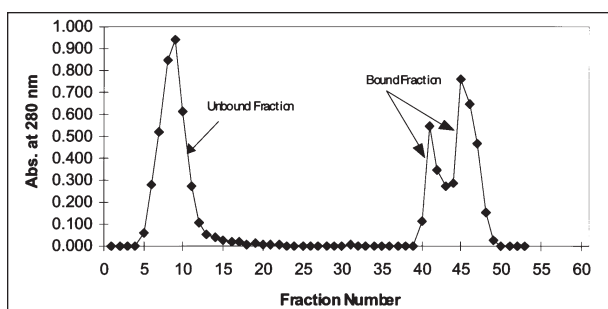


Figure 2—Representative results of immunoaffinity chromatography. The pellet that was created by N-lauroyl sarcosinate extraction of sonicated *M haemolytica* was loaded onto the immunoaffinity column, equilibrated in a solution of 50mM Tris, 300mM sodium chloride, 5mM EDTA, and 0.1% (wt:vol) sodium azide (pH, 7.8). Bound material was eluted with 100mM glycine (pH, 2.8). Absorbance of the effluent was monitored at 280 nm. Notice the 2 peaks of the bound fraction.

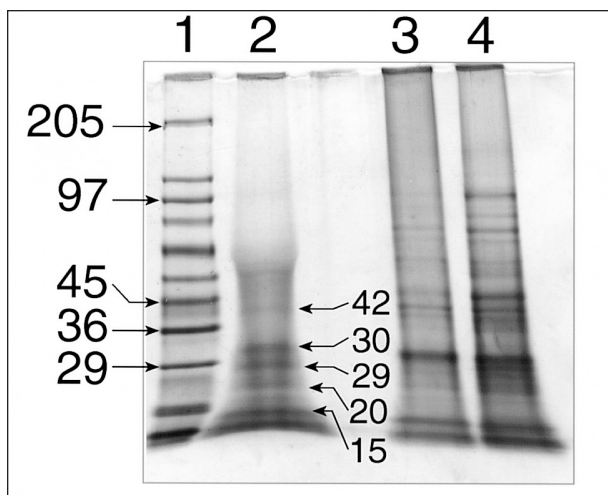


Figure 3—Gel resulting from SDS-PAGE of the eluted peak from the immunoaffinity column. Proteins were loaded onto a 4–20% gel and stained with silver stain. Lanes were as follows: 1, molecular weight standards; 2, immunogenic proteins (elution peak from the immunoaffinity column); 3, supernatant from the N-lauroyl sarcosinate extraction of sonicated *M haemolytica*; and 4, pellet from the N-lauroyl sarcosinate extraction of sonicated *M haemolytica*. Molecular weights (in kilodaltons) of the immunogenic proteins in lane 1 and the 5 major proteins in lane 2 are indicated.

use of SDS-PAGE. Molecular weights of the major bands in these samples were also determined. Interestingly, the 3 major bands in the pellet (42, 30, and 15 kd) were also evident in the supernatant. Because of this observation, it appears that the N-lauroyl sarcosinate extraction procedure more fully solubilizes the membrane proteins.

Detection of immunogenic proteins—Results of immunoblot analysis were obtained (Fig 4). Only 1 major band was observed for the immunogenic protein. Molecular weight of this band was 42 kd, which was the same as 1 of the 5 bands observed in the SDS gel of the immunogenic proteins.

Several protein bands were observed for the pellet resulting from the N-lauroyl sarcosinate extraction. The 2 major bands were determined to be 42 and 58 kd. The other bands appeared 1 after the other in an apparent smear between 20 and 42 kd. As expected, the 42-kd protein was evident in both lanes. It is possible that those proteins did not bind to the nitrocellulose or that conformation of the proteins was changed, thereby preventing binding of the antibody.

N-terminal sequencing of an immunogenic protein—The N-terminal sequence of the 42-kd protein was Tyr-Gln-Thr-Tyr-Gln-Ser-X-Leu-Gln, where X could not be identified. Because the yield of the first residue (ie, tyrosine) was 1.0 pmol, only 9 residues could be determined. The bacterial protein database

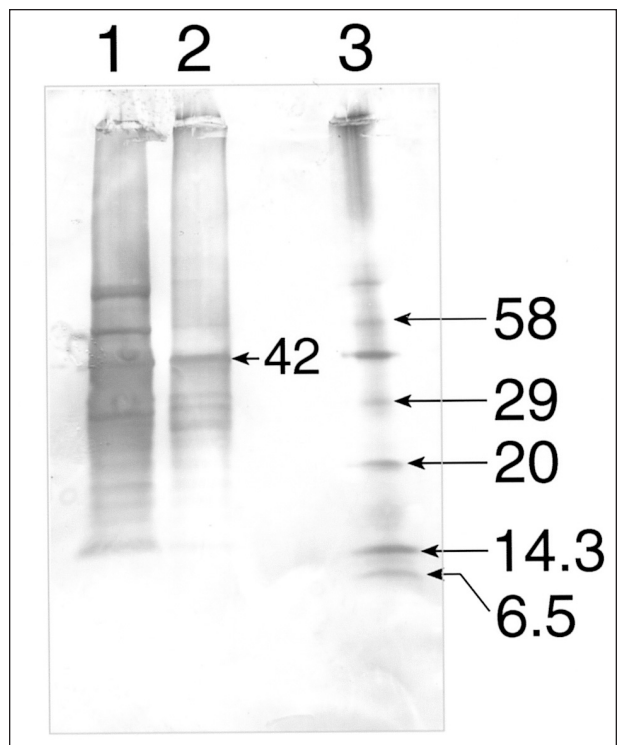


Figure 4—Immunoblot of immunogenic proteins obtained from the immunoaffinity column. Lanes were as follows: 1, pellet from the N-lauroyl sarcosinate extraction of sonicated *M haemolytica*; 2, immunogenic proteins (elution peak from the immunoaffinity column); and 3, biotinylated molecular weight standards (in kilodaltons). The primary antibody was the same serum that was used to prepare the immunoaffinity column. Notice the major immunogenic protein (molecular weight, 42 kd) in lane 2.

Table 1—Mean \pm SD absorbance values for an ELISA performed by use of antiserum from calves inoculated PO or IM with an experimental *Mannheimia haemolytica* vaccine

Day	Control group	PO inoculated group	IM inoculated group	P*	P†
0	0.766 \pm 0.117	0.848 \pm 0.284	0.787 \pm 0.258	0.57	0.87
21	0.765 \pm 0.129	0.932 \pm 0.261	0.743 \pm 0.309	0.24	0.89
28	0.793 \pm 0.211	0.937 \pm 0.272	0.845 \pm 0.244	0.36	0.72
33	0.850 \pm 0.363	1.071 \pm 0.316	0.833 \pm 0.237	0.37	0.93

Day 0 = Day of inoculation.
 *Results of 2-tailed paired *t* test that compared serum ELISA absorbance values of PO immunized group with those of the control group within a sample day. †Results of 2-tailed paired *t* test that compared serum ELISA absorbance values of IM immunized group with those of the control group within a sample day.

was searched.³⁴ A strong sequence identity was not detected. However, a number of sequence alignments were obtained, including 2 *Haemophilus influenzae* proteins; an aldose 1-epimerase (accession No. gi282091); a hemoglobin-haptoglobin binding protein (HhuA; accession No. gi1399345); a hypothetical *Neisseria meningitidis* protein (accession No. gi15794516); and an iron[III]-transport system permease protein (sfuB from *Fusobacterium nucleatum*, accession No. gi19703654).

Carbohydrate determination—The percentage of polysaccharide relative to total protein content was determined. Values were 6% for the supernatant resulting from the N-lauroyl sarcosinate extraction, 8% for the pellet resulting from the N-lauroyl sarcosinate extraction, and 51% for the immunogenic proteins.

ELISA that used serum from calves inoculated with experimental vaccine—The ELISA was performed to assess whether the experimental vaccine had induced an antibody response to the immunogenic proteins that were isolated by use of the immunoaffinity column. Mean absorbance of the serum from each of the 3 groups of calves was determined for each sample collection (Table 1).

Analysis of ELISA data revealed that the control group already had a high degree of antibody reactivity toward the immunogenic protein preparation on day 0. This suggests that the control group had prior exposure to *M haemolytica*. Because the immunogenic protein preparation contained 51% polysaccharide by weight, part of this reactivity undoubtedly was attributable to antibody to polysaccharide.

Titers determined by the use of the IHA test, which reflect antibody to capsular polysaccharide, were plotted against ELISA absorbance values on the basis of the immunogenic proteins. There was not a relationship between the titers and ELISA absorbance values to the immunogenic protein fraction, suggesting differ specific antibodies were measured by the 2 tests.

Discussion

Because of all the unsuccessful attempts to prepare an effective *M haemolytica* vaccine, it would appear questionable whether a useful vaccine may ever become available.¹¹⁻¹⁶ However, the observation that cattle that have recovered from natural infection are resistant to subsequent infection implies that an effective vaccine could be developed.⁶ The emphasis, it appears, needs to be focused on isolating the immuno-

genic proteins responsible for the natural protection to develop a vaccine capable of controlling pneumonic pasteurellosis. Potter et al²³ reported that an experimental vaccine containing **transferrin binding protein A (TbpA)** and **transferrin binding protein B (TbpB)** protected animals against challenge exposure and that the protection correlated with anti-TbpB antibody concentrations. Additionally, those authors suggested that TbpA contributed to protection through the induction of a non-antibody-mediated immune response.

A number of differences exist between the protein profiles of *M haemolytica* grown in vivo and in vitro.^{6,24} Investigators have simulated in vivo growth conditions by limiting the iron content in the growth media.^{8,26,27} In fact, 2 proteins produced in media that lack free iron can bind bovine transferrin.²³ Therefore, iron-restricted growth conditions were used in the study reported here so that the organisms would express most of the same proteins in vitro that are normally expressed in vivo.

Perhaps the best initial characterization of cell surface proteins of *M haemolytica* was provided by Squire et al.²¹ Because cell surface proteins are believed to be the most important antigens in protective immunity, the results of that study have been of particular interest to vaccine researchers. In that study, investigators were able to separate and characterize inner and outer membrane proteins with a simple N-lauroyl sarcosinate extraction.

The N-lauroyl sarcosinate extraction was used in the study reported here because of its ability to separate inner and outer membrane proteins.²¹ Our intention was to obtain only the proteins from the outer membrane to select for those proteins responsible for natural protection. This was accomplished by initially extracting the periplasmic proteins with a hypertonic salt solution, then disrupting the cells by sonication and collecting the crude membranes. This was followed by the N-lauroyl sarcosinate extraction to yield N-lauroyl sarcosinate-soluble (inner membrane) and N-lauroyl sarcosinate-insoluble (outer membrane) fractions.

The pellet created by the N-lauroyl sarcosinate extraction in the study by Squire et al²¹ and the pellet obtained after the N-lauroyl sarcosinate extraction in the study reported here were similar. Squire et al²¹ reported that the pellet fraction contained 2 major proteins (30 and 42 kd), as determined on the basis of Coomassie blue staining intensity. The pellet fraction in the study reported here also contained these 2 major proteins in addition to a 15-kd protein. However, supernatant fractions resulting from the N-lauroyl sar-

cosinate extraction were not as consistent between the 2 studies. The supernatant extracted in the study reported here had the same 3 proteins that were identified in the pellet fraction. In the study of Squire et al,²¹ the 2 supernatant and pellet fractions did not contain the same major proteins. It appears as though the N-lauroyl sarcosinate extraction solubilized more of the membrane proteins in our study, leading to a partitioning of the major proteins. This was somewhat surprising, because the percentages of N-lauroyl sarcosinate-solubilized proteins were comparable between the 2 studies (68 and 64%). Although it appears that some quantity of proteins were lost during the N-lauroyl sarcosinate extraction, sufficient amounts remained for the immunoaffinity chromatography procedures. It is also possible that the proteins were not the same proteins, but merely had the same molecular weight.

Most research on *M haemolytica* vaccines is based on isolation and characterization of each of the outer membrane proteins. Characterization of each protein would include a large investment of time and resources, hopefully resulting in an appropriate vaccine. Instead, we chose to identify a group of immunogenic (protective) outer membrane proteins by making use of naturally produced immunoglobulins directed against the outer membrane proteins. This was accomplished by extracting immunoglobulins from the serum of calves that were actively infected with *M haemolytica*. Total immunoglobulins were used, as opposed to only IgG, in case the calves had not reached the IgG phase of the immune response. These immunoglobulins were then used to prepare an immunoaffinity chromatography column. Because the calves were actively infected with *M haemolytica*, a portion of the immunoglobulins on the immunoaffinity column should have been directed against those immunogens that are used for acquiring immunity. Use of this immunoaffinity column in combination with the outer membrane proteins, which contains the majority of the immunogenic proteins, should have allowed the isolation of all of the proteins that the calves' immune systems recognized as being important, rather than a single naturally immunogenic protein. Isolation of all of the immunogenic proteins was intentional. If an individual protein was chosen on the basis of its apparent concentration as the result of staining intensity, researchers would simply be isolating the immunogenic protein that existed in the highest concentration but that may not necessarily have been the most immunogenic protein. In other words, an important protein that is not contained in high quantity might appear as a weak band, whereas a poorly immunogenic protein contained in high quantity could appear as a strong band. Therefore, a more complete vaccine preparation could be obtained by isolating all of the proteins from the immunoaffinity column, rather than isolating a specific single protein.

With this in mind, outer membrane proteins from the N-lauroyl sarcosinate extraction were applied to the immunoaffinity column. Five proteins were isolated and termed the immunogenic proteins. Two of the proteins corresponded in molecular weight to major proteins in the outer membrane protein extract (42

and 30 kd). The other 3 immunogenic proteins were considered minor proteins in the outer membrane protein extract as determined on the basis of Coomassie blue staining intensity.

None of the 5 proteins isolated here appeared to be transferrin binding proteins described in another study.²³ This was surprising, because the organism in the study reported here was also grown in iron-restricted conditions. Furthermore, in that other report, transferrin binding proteins were immunogenic and protective.

Results for the ELISA and immunoblotting assay that used antiserum from experimentally vaccinated calves revealed that there is reactivity to the immunogenic proteins and that the expression of antibodies increases with exposure time. Although a large quantity (51%) of the immunogenic protein fraction contained carbohydrate, these results provided evidence of reactivity to the proteins. Collectively, these results indicate that infection with a challenge strain of *M haemolytica* results in antibody production against the immunogenic proteins and that the polysaccharide content of the immunogenic protein fraction indicates prior exposure to *M haemolytica*.

The N-terminal sequence analysis was performed on the 42-kd protein. This protein was chosen for sequencing simply because it had the most intense staining on the PVDF membrane. The sequence was determined to be Tyr-Gln-Thr-Tyr-Gln-Ser-X-Leu-Gln, where X could not be identified. A computerized search for homologous sequences revealed a number of sequence alignments including 2 *H influenzae* proteins, an aldose-1-epimerase (accession No. gi282091), and a hemoglobin-haptoglobin binding protein (HhuA; accession No. gi1399345). Sequence alignment was also found with a hypothetical *N meningitidis* protein (accession No. gi15794516) and an iron[III]-transport system permease protein (sfuB from *F nucleatum*; accession No. gi19703654).

The capacity of the immunoaffinity column was determined to be 1 µg of immunogenic protein/mL of gel. Although this may be an adequate yield for preparing an experimental vaccine, it is uncertain whether this would be practical from a manufacturing standpoint for a commercial vaccine, because it is impossible to estimate the concentration at which the proteins would need to be administered to provide immunity. Once adequate quantities of the proteins are isolated (approx 25 mg), a second immunoaffinity column could be prepared from the proteins, which could then be used to purify immunoglobulin from infected calves. This affinity-purified immunoglobulin could then be used to prepare a third column that would have a theoretically higher capacity.

One disadvantage to the use of total antibody to purify immunogenic proteins was that the total antibody pool contained antibodies to polysaccharides. As a result, the affinity-purified protein fraction also contained polysaccharides. This became evident during our evaluation of the ELISA in which we used sera from experimental vaccinated calves, which resulted in high initial absorbances.

²³ Provided by Dr. Glynn H. Frank, USDA-Agricultural Research Service, National Animal Disease Center, Ames, Iowa.

^bProvided by Dr. Robert E. Briggs, USDA-Agricultural Research Service, National Animal Disease Center, Ames, Iowa.
^cActigel-ALD, Sterogene, Carlsbad, Calif.
^dSarkosyl, Sigma Chemical Co, St Louis, Mo.
^eTrisacryl GF05M, BioSeptra, Rockville, Md.
^fGradient gels, BioRad, Hercules, Calif.
^gMighty Small, Pharmacia Biotech, Piscataway, NJ.
^hCoomassie brilliant blue R-250, Kirkegaard & Perry Laboratories, Gaithersburg, Md.
ⁱSilver stain kit, Kirkegaard & Perry Laboratories, Gaithersburg, Md.
^jCN substrate kit, Kirkegaard & Perry Laboratories, Gaithersburg, Md.
^kProcise model 494, Applied BioSystems, Foster City, Calif.
^lBlastp, available online at www.ncbi.nlm.nih.gov, National Center for Biotechnology Information, Bethesda, MD.
^mTMB-substrate solution, Kirkegaard & Perry Laboratories, Gaithersburg, Md.

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