OMPA FAMILY PROTEIN OF *PASTEURELLA HAEMOLYTICA*: PURIFICATION, PARTIAL CHARACTERIZATION AND POTENTIAL IMMUNOLOGIC SIGNIFICANCE

BY

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SIGNIFICANCE

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PREFACE

Pneumonic pasteurellosis (shipping fever) is the major cause of disease and death loss in feedlot cattle and a major source of economic loss for the beef cattle industry. Numerous studies were undertaken to develop an effective vaccine for the prevention of bovine pneumonic pasteurellosis. Current experimental studies revolve around the identification and incorporation of specific protective *Pasteurella* species antigen extracts into vaccines.

Recent studies have shown that outer membrane proteins (OMPs) of *Pasteurella haemolytica* are probably the major surface antigens in stimulation of protective antibody responses. This study was conducted to identify and characterize two OMPs (32 and 35 K, PomA and PomB), which may be important in stimulating protective antibodies against pasteurellosis.

The 32 and 35 K major outer membrane proteins were extracted from *P. haemolytica* by solubilization in n-Octyl polyoxyl ethylene (OPOE) and characterized. They were separated and partially purified by anion exchange chromatography, Their N-terminal amino acid sequence was determined, and one was shown to be related to OmpA, a major structural outer membrane protein of *Escherichia coli.* The importance of these proteins in immunity to pasteurellosis was also demonstrated by Western blotting with sera from vaccinated and subsequently experimentally challenged cattle.

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I thank all my family members for relieving me of responsibilities that made possible the continuation of my education. I am thankful to God for blessing me with good life.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Bovine pneumonic pasteurellosis, also known as shipping fever pneumonia, is the most common cause of cattle losses in North America (52). *Pasteurella haemolytica* biotype A serotype 1 is the principal etiologic agent of bovine pneumonic pasteurellosis (17, 18).

Much effort has been expended towards development of more efficacious vaccines for the prevention of bovine pneumonic pasteurellosis (30). To develop such vaccines, investigators attempted to better understand *P. haemolytica* virulence factors and the host immune response. Recently, Morton et al., (29) demonstrated that cattle vaccinated with *P. haemolytica* A1 outer membranes had enhanced resistance to experimental challenge with the homologus serotype. In a previous study, high antibody responses to *P. haemolytica* saline-extracted proteins with molecular masses of 16, 30, 40, 42, and 86 kDa consistently correlated with resistance to challenge with virulent *P. haemolytica* (31). It was proposed that a protein with a molecular weight of approximately 30 kDa is a major outer membrane protein (OMP), and that protein may be important in inducing immunity to *P. haemolytica* (13). The three tandomly arranged genes for *P. haemolytica* membrane lipoproteins, which are approximately 28-30 kDa and similar to an *Escherichia coli* 28 kDa lipoprotein, were recently cloned and

sequenced (13, 34). Serum antibodies to two of the lipoproteins correlated with resistance to experimental challange (13, 14).

Our preliminary studies suggested that another major OMP was similar to the *E. coli* OmpA protein. The outer membranes of numerous species of Gram-negative bacteria, both enteric and nonenteric, contain a major heat-modifiable protein structurally similar to OmpA (4). Those bacteria include *Actinobacillus actinomycetemcomitans* (50), *Haemophilus somnus* (46), *Haemophilus influenzae* (32, 48), *Haemophilus ducreyi* (44), *Salmonella typhymurium* (19), *Shigella dysenteriae* (5), *Neisseria gonorrhoeae* (22), *Pseudomonas aeruginosa* (23, 51) and *Serratia marcescens* (6). The OmpA protein is important in maintaining integrity of the *E. coli* outer membrane (43) and it elicits a strong antibody response (40). Also, OmpA may play an important role in virulence, because an OmpA-deficient mutant of *E. coli* K-1 has reduced virulence in an infant rat model of bacteremia (49). Antibodies against OmpA and several OmpA-family proteins are bactericidal, opsonic, or protective (24, 25, 28, 36, 41).

The aim of the present study was to purify and characterize two *P. haemolytica* OMPs that co-migrate with molecular weights of approximately 30 K on 12% SDS-PAGE gels, but separate as bands of 35 K and 32 K on 18% SDS-PAGE gels. These proteins were characterized by determination of their N-terminal amino acid sequence and evaluation of antigenicity by Western immunoblotting with sera from vaccinated and subsequently experimentally challenged cattle.

CHAPTER II

MATERIALS AND METHODS

Bacteria and culture conditions. *P. haemolytica* 89010807N (*lpp*) (35) was grown for 16-18 hours on brain-heart infusion (BHI) agar (Difco Lab; Detroit, Michigan) containing 10 μg/ml ampicillin and 20 μg/ml nalidixic acid. The culture was visually inspected for purity. Several colonies were inoculated into 100 ml of BHI broth containing 10 μg/ml ampicillin and 20 μg/ml nalidixic acid and incubated at 37°C, at 80 rpm on a rotary shaker overnight. Subsequently, 100 ml of the overnight culture was inoculated into 2 l of BHI broth containing 10 μg/ml ampicillin and 20 μg/ml nalidixic acid and incubated at 37°C for 16-18 hours on a rotary shaker at 80 rpm. Bacteria were harvested by centrifugation at 8000 x g for 10 min., resuspended and washed twice with sterile phosphate-buffered saline (PBS, pH 7.2) and centrifuged as above to obtain pellets. Cell pellets were stored frozen at -20°C for subsequent OMP extraction.

***P. haemolytica* envelope preparation.** Each bacterial pellet from 2 l of BHI broth was resuspended in 20 ml of solution containing 10 mM Na_2HPO_4, 5 mM $MgSO_4$ (pH 7.4) and 50 μg/ml DNase (Sigma Chemical Co., St. Louis, Mo.) and 50 μg/ml RNase (Sigma Chemical Co., St. Louis, Mo.) and sonicated on ice (Branson Sonifier Cell Disruptor, VWR Scientific., Danbury, Ct.) 5 times for 30 seconds each (continuously) with 30 second intervals using double step micro tip probe (50 watts) to

lyse the cells. The bacterial suspension was centrifuged at 950 x g for 10 min and the supernatant collected. The supernatant was then centrifuged at 205,800 x g for 1hr using a 55.2 Ti rotor (Beckman Ultracentrifuge). The envelope-rich pellet was resuspended in 10 ml distilled H_2O, and the protein concentration was determined (BCA protein assay, Pierce Biochemical).

OMP extraction. Two OMPs designated *Pasteurella* outer membrane (Pom) A (an upper band) and PomB (a lower band) identified by SDS-PAGE were extracted from the envelope by a modification of a previously described n-octyl polyoxyl ethylene (Octyl-POE, Bachem Bioscience Inc., Philadelphia, PA.) solubilization method (45). The envelope was resuspended by sonicating 3 times at a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 8.0), 0.5% OPOE. After centrifugation at 154,000 x g for 1 h at 20°C, the supernatant (designated supernatant #1) was collected. The pellet was resuspended by sonication as described above in 10 mM Tris-HCl, (pH 8.0), 3% OPOE. This step was repeated twice to obtain supernatants (#2 and #3). The resulting pellet was then resuspended in 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 3% OPOE by sonication and centrifuged again as described above. The supernatant (#4) was collected, and the steps were repeated for the remaining pellet to obtain supernatant (#5). The resulting pellet was resuspended in 50 mM EDTA, 0.4 M NaCl, 3% OPOE, sonicated and centrifuged as above. Supernatant (#6) was collected and pellet resuspended in deionized H_2O, sonicated and centrifuged as above. Both pellet and supernatant (#7) were collected. Outer membranes were also extracted separately in

0.5% sodium N-lauryl sarcosine, (Sarkosyl, Sigma Chemical Co., St. Louis, MO.) as previously described (29).

Anion exchange purification. Supernatant #4 (which contained PomA and PomB) was prepared for anion-exchange chromatography by equilibrating in 10 mM Tricine buffer (pH 8.9) (buffer A/binding buffer) by dialysis. An anion-exchange column (Econo-Pac High Q Cartridge, Bio-Rad, Calif.) was washed and equilibrated according to the manufacturer's instructions. The sample (1 ml) was applied to the column and washed with 10 ml of buffer A. The column was eluted by a linear gradient of 0.5 M NaCl to 2 M NaCl in 10 mM Tricine buffer (elution buffer/buffer B) at a flow rate of 0.5 ml/min. Fractions containing protein peaks were collected, concentrated by ultrafiltration with a PM 10 filter (Amicon Inc., Beverly, MA.). Fractions were dialysed against 10 mM Tricine buffer and proteins were identified in peak fractions by SDS-(18%)PAGE.

SDS-PAGE. Supernatants and pellets were subjected to SDS-PAGE as described (16, 27). Briefly, the samples were solubilized in sample buffer containing 5% ß-mercaptoethanol and heated to 100° C for either 5 or 10 min. Samples were loaded onto a 4.5% acrylamide stacking gel overlaying an 18% acrylamide resolving gel (except where stated when a 10% resolving gel was used), and separated by electrophoresis (Mini Protean II Electrophoresis Cell, Bio-Rad, Rockville Centre, N.Y.). Molecular weights of proteins were determined using known standards.

Western Immunoblots. Extracts were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose membranes as previously described (16,

31), and probed with a calf anti-*P. haemolytica* 35 kDa sera and rabbit anti-*E. coli* OmpA sera (the latter kindly provided by Dr. Ulf Henning, Tübingen, Germany) (20, 42). The antibody complexes were detected by incubating with either horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pel-Freez, Roger, Ark.) or HRP-conjugated rabbit anti-bovine IgG (Pel-Freez, Roger, Ark.). Serum samples described later from cattle were evaluated in Western immunoblots against PomA and PomB as previously described (13, 14, 31, 39). Briefly, the supernatant #6 containing mostly PomA from OPOE extraction and anion-exchange-purified PomB from supernatant #4 were subjected to SDS-PAGE, transferred to nitrocellulose membranes and probed with 1:25 dilution of cattle sera. Immune complexes were detected with affinity-purified, HRP-conjugated rabbit anti-bovine IgG (Pel-Freez, Roger, Ark.) (8). Color reactions were developed using 0.05% 4-chloro-1 naphthol dissolved in 20 ml ice cold methanol and 0.05% H_2O_2 in 100 ml Tris-buffer saline (TBS).

Heat modifiability. To determine heat modifiability of proteins, fractions were solubilized in sample buffer at 37°C or 100°C for 10 min. and subjected to SDS-PAGE.

N-terminal amino acid sequencing. Supernatant #4 was subjected to SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif.) and stained with Coomassie brilliant blue R-250 (Bio-Rad, Hercules, Calif.). The bands were identified by comparison with molecular weight markers. Strips of membrane containing PomA and PomB were excised and subjected to N-terminal amino acid sequencing (Molecular Biology Resource Facility, University of Oklahoma, Health Sciences Center).

Anti-*P. haemolytica* sera. Antibodies to PomA were produced by immunizing a 4 week old Holstein calf with gel-purified PomA as described previously (13). Briefly, supernatant #4 was subjected to SDS-PAGE. PomA was identified, excised with a sterile scalpel, and emulsified with an equal volume of Freund's incomplete adjuvant (FIA). The calf was immunized intramuscularly on days 0 and 15. Sera were collected on days 0, 15, 28, and 35. Antibody responses to PomA were determined by ELISA. Specificity was evaluated by Western immunoblots using a 1:200 dilution of sera.

Antibody responses in cattle. Antibody responses to PomA and PomB were quantified by Western immunoblots and densitometry as used previously in our laboratories (8, 13, 14, 31, 39). Serum samples were obtained from weanling cattle that had been previously experimentally vaccinated and challenged with *P. haemolytica* A1 (9, 10, 12, 38). Cattle had been previously vaccinated twice subcutaneously (days 0 and 7) with either PBS (n=5), formalin-killed *P. haemolytica* with aluminum hydroxide [Bacterin-AlOH (n=5)], formalin-killed *P. haemolytica* with FIA [Bacterin-FIA (n=5)], or 1 x 10^9 colony forming units (CFU) of live *P. haemolytica* (n=5). On day 21, cattle were challenged transthoracically into the caudal lung lobes with 5 x 10^9 CFU of *P. haemolytica*. Four days later, cattle were euthanatized and lung lesion scores ranging from 0 to 20 were assigned based on the criteria of intensity and spread of lesion (the higher the score the more intense the lesion) (38).

Reactivity of day 0 and day 21 sera to PomA and PomB on Western immunoblots was quantified by measuring the intensity of the bands using a 1-D gel scanner (Protein Database Incorporated, Huntington Station, N.Y.) The blots were

scanned, and the optical densities (Trace OD x mm) determined as indirect measurements of the antibody responses (8, 13, 14, 31, 39).

Serotype specificity of PomA. To determine the conservation of PomA in different *P. haemolytica* serotypes, whole cell lysates of serotypes 1, 2, 5, 6, 7, 8, 9, 10, 12, 13, 14, an untypable strain, strain 89010807N, and strain 89010807N (*lpp*) were solubilized in sample buffer at 100°C for 10 min. and subjected to SDS-PAGE. Supernatant #4 was solubilized at both 37° and 100°C, to provide a reference for heat-modifiable OmpA-like protein present in different serotypes. Proteins were transferred to nitrocellulose membranes and probed with a dilution of 1:200 calf anti-PomA serum to detect OmpA family protein bands.

Statistical analysis. Mean values for Trace OD x mm (mean OD values) for PomA and PomB were compared among the vaccine groups using 2-sample t tests (2). Within groups mean OD values were compared by paired t tests. Linear regression analysis was used to correlate experimental lesion scores with OD values for each band (31).

CHAPTER III

RESULTS

Fractionation of *P. haemolytica* 89010807N (*lpp*) by differential solubilization in OPOE detergent resulted in 7 supernatant fractions and an insoluble pellet. SDS-PAGE of all the supernatants and the pellet showed that supernatant fractions 4, 5, and 6 contained several membrane proteins with molecular weights ranging from 106 K to 18K (Fig.1). At approximately 35 K, a doublet of bands was seen in fractions 4, 5, and 6 with a progressive decrease in concentration of the lower band. The upper band (designated as PomA) was prominent and the lower band (designated as PomB) much less so. The apparent molecular weight of PomA and PomB varied with the % of the resolving gels used. We observed that PomA and PomB co-migrated at approximately 30 K on 12% gels. To electrophoretically separate these protein bands, we used SDS-(18%)PAGE. This resolved these proteins into 2 bands corresponding to a molecular weight of 35 K (PomA) and 32 K (PomB) (Fig. 1).

To test whether the two protein bands represented different proteins or were different forms of the same protein, we determined the N-terminal amino acid sequences. This analysis suggested that the two bands represented two different proteins. The N-terminal amino acid sequence of PomA is shown in Table 1. Comparison of this sequence with published sequences using the BLAST network

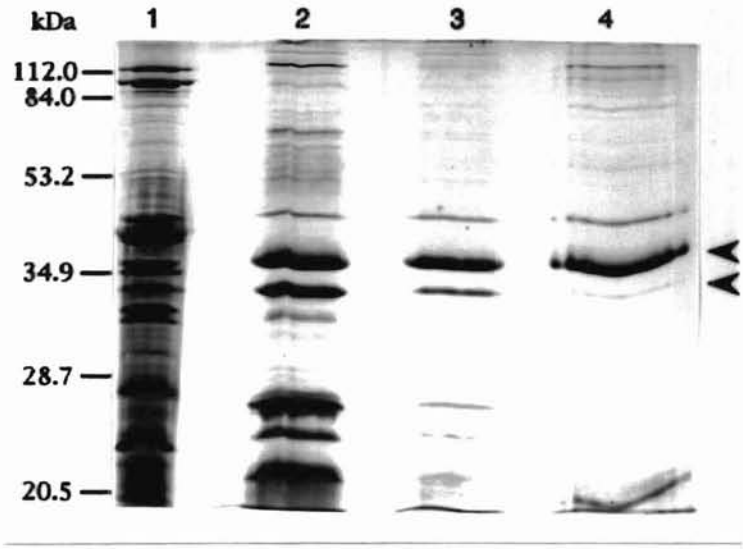

Figure 1. SDS-PAGE (18% acrylamide gel, silver stain) showing the outer membrane protein bands (including PomA and PomB) of *P. haemolytica* in various OPOE extracted supernatants and sarkosyl-extracted outer membrane proteins. Lane 1, outer membrane proteins of *P. haemolytica* 89010807N (*lpp*); Lane 2, Supernatant #4 (3% OPOE/50 mM EDTA soluble); Lane 3, Supernatant #5 (repeat of previous step); Lane 4, Supernatant #6 (3% OPOE/0.4 M NaCl/50 mM EDTA soluble). Upper arrowhead = PomA; lower arrowhead = PomB.

Table 1. Comparison of N-terminal amino acid sequence of PomA from *Pasteurella haemolytica* A1 with the sequences for *E. coli* OmpA and OmpA-related proteins.

Pasteurella haemolytica PomA	APQANTFYAGAKAGWASFHD	
Haemophilus influenzae P5 protein (1,33)[a]	APQENTFYAGYKAGQASFHD	(85.0%)[b]
Haemophilus influenzae Fimbrial protein (1)	APQENTFYAGVKAGQGSFHD	(80.0%)[b]
Enterobacter aerogenes OMP A (1)	APKDNTWYAGGKLGWSQFHD	(65.0%)[b]
Salmonella typhimurium OMP A (1,19)	APKDNTWYAGAKLGWSQYHD	(65.0%)[b]
Shigella dysenteriae OMP A (1,5)	APKDNTWYTGAKLGWSQYHD	(60.0%)[b]
Escherichia coli OMP A (1,3)	APKDNTWYTGAKLGWSQYHD	(60.0%)[b]
Serratia marcescens (1,6)	APKDNTWYTGAKLGWSQYHD	(60.0%)[b]
Actinobacillus actinomycetemcomitans 29 kDa protein (50)	APQANTFYAGAKA - - - - - - - -[c]	(100.0%)[e]
Haemophilus somnus OMP (46)	APQANTFYAGAK - - - - - - - - -[d]	(100.0%)[f]
Haemophilus ducreyi MOMP (44)	APQADTFYVGAKA - - - - - - - -[c]	(83.3%)[e]

[a]References
[b] identity of 20 amino acids with *P. haemolytica* PomA sequence.
[c] Sequence of the next 8 amino acids not available.
[d] Sequence of next 9 amino acids not available.
[e] % identity of 13 amino acids with *P. haemolytica* PomA sequence.
[f] % identity of 12 amino acids with *P. haemolytica* PomA sequence.

service (1) revealed that PomA had 60% identity with the N-terminal amino acid sequence of *E. coli* OmpA protein (4) and varied from 60%-100% identity with related proteins from several other Gram-negative bacteria (Table 1) (1, 5, 6, 19, 32, 33, 44, 51).

Further evidence that PomA was related to *E. coli* OmpA was obtained by Western immunoblot analysis (Fig. 2). Rabbit antiserum against the *E. coli* OmpA reacted strongly with PomA of *P. haemolytica*. Calf anti-*P. haemolytica* PomA sera also reacted with the same band.

The molecular weight of *P. haemolytica* PomB was 32 kDa. The N-termnal amino acid sequence was A D T I G F V D P S Y V L E N H P V L L D A S. A search of protein sequence databases using the BLAST network service (1) failed to find any substantial homology with other known bacterial proteins.

Heat modifiability of PomA. SDS-PAGE analysis of PomA present in dialysed (against deionised water) supernatant #4 fraction demonstrated a molecular weight of 35 K following solubilization in sample buffer at 37°C (Fig. 2). In contrast, solubilization at 100°C resulted in a protein which migrated with a molecular weight of 40 K. On Western immunoblots, both 35 K and 40 K bands were identified with anti *E. Coli* OmpA and calf anti-*P. haemolytica* PomA sera indicating that they represent a single protein. N-terminal amino acid sequencing of the 35 K and 40 K bands demonstrated identical sequences. Differences in protein mobility were not seen in undialyzed fractions. PomB had the same molecular weight at both at 37°C and 100°C (data not shown).

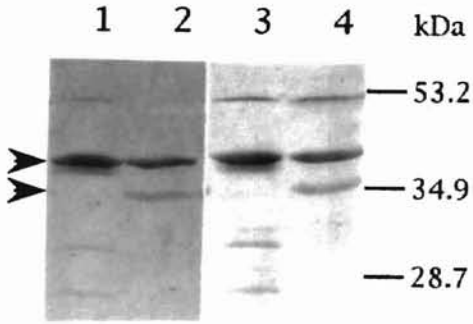

Figure 2. Western blot showing reactivity of rabbit anti-*E. coli* OmpA sera and calf anti-*P. haemolytica* (OmpA family protein) PomA sera with PomA of *P. haemolytica*. Lanes 1 and 2, loaded with supernatant #4 (dialyzed against deionised water) solubilized in sample buffer at 100°C or 37°C, respectively, and probed with anti-*E. coli* OmpA sera (1:200 dil). (In dialysed fracton the PomA is partially heat modified.) Lanes 3 and 4, same as in Lanes 1 and 2 except probed with calf anti-*P. hemolytica* OmpA family protein (PomA) serum (1:200 dil). Upper arrowhead = heat-modified PomA; lower arrowhead = unmodified PomA.

Serotype specificity of PomA. On Western immunoblots (Fig. 3) of whole cell lysates of different serotypes and an untypable strain, 2 bands reacted with bovine anti-*P. haemolytica* PomA sera. Their mobilities corresponded to heat-modified (40 K) and unmodified (35 K) forms of PomA present in supernatant fraction #4 (except serotype 9 in which the mobility of the bands are different this in consistence with unpublished data of Morton et al.,which suggests that the outer membrane profile of serotype 9 is different from other serotypes of A biotype). This suggested that all biotype A serotypes, including an untypable serotype, contain OmpA-family proteins.

Anion-exchange purification. Further OPOE extraction of supernatant #4 yielded partial purification of PomA. PomB was greately reduced in those extractions (supernatants #5 and #6). To determine if PomA and PomB could be separated and each partially purified from supernatant #4, we used anion-exchange chromatography. All proteins in supernatant #4 bound to the column (pH 8.9). Proteins were eluted with a linear gradient of elution buffer resulting in two protein peaks (Fig. 4). These proteins were identified in concentrated and dialysed peak fractions by SDS-PAGE (Fig. 5). PomB eluted with a salt concentration gradient of 0.62 - 0.66 M NaCl, and PomA eluted with a salt concentration gradient of 1.98 - 2.0 M NaCl. Thus it was possible to separate and partially purify PomA and PomB effectively with anion-exchange chromagraphy.

Antibody responses of cattle. To evaluate the antigenicity of PomA and PomB for cattle, antibody responses (OD values) to both proteins were determined in sera from cattle in 4 vaccinated groups (8, 13, 14, 31, 39). Mean antibody responses of cattle to both proteins and their corresponding lesion scores are shown in Table 2. There were significant differences between the mean lesion scores for PBS vs Live ($P < 0.01$), PBS

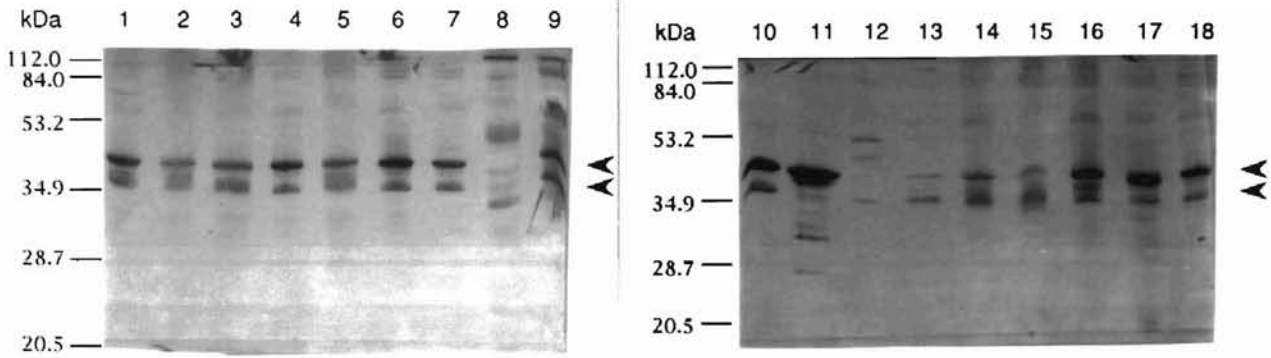

Figure 3. Western blot showing conservation of OmpA family PomA protein in different *P. haemolytica* A serotypes. The whole lysates of different serotypes and supernatant #4 were subjected to SDS-PAGE (18%), transferred to membrane, and probed with calf anti-*P. haemolytica* (OmpA family protein) PomA sera (1:250 dil). This sera detected the PomA equivalent bands in all serotypes.

Lane 1 - Serotype 1
Lane 2 - Serotype A1
Lane 3 - Serotype 2
Lane 4 - Serotype 5
Lane 5 - Serotype 6
Lane 6 - Serotype 7
Lane 7 - Serotype 8
Lane 8 - Serotype 9
Lane 9 - Serotype 11
Lane 10 - Supernatant #4 at 37°C/10 minutes
Lane 11 - Supernatant #4 at 100°C/10 minutes
Lane 12 - *E. coli* whole cell lysate.
Lane 13 - *P. haemolytica* 89010807N (*lpp*)
Lane 14 - *P. haemolytica* 89010807N
Lane 15 - Untypable serotype
Lane 16 - Serotype 14
Lane 17 - Serotype 13
Lane 18 - Serotype 12

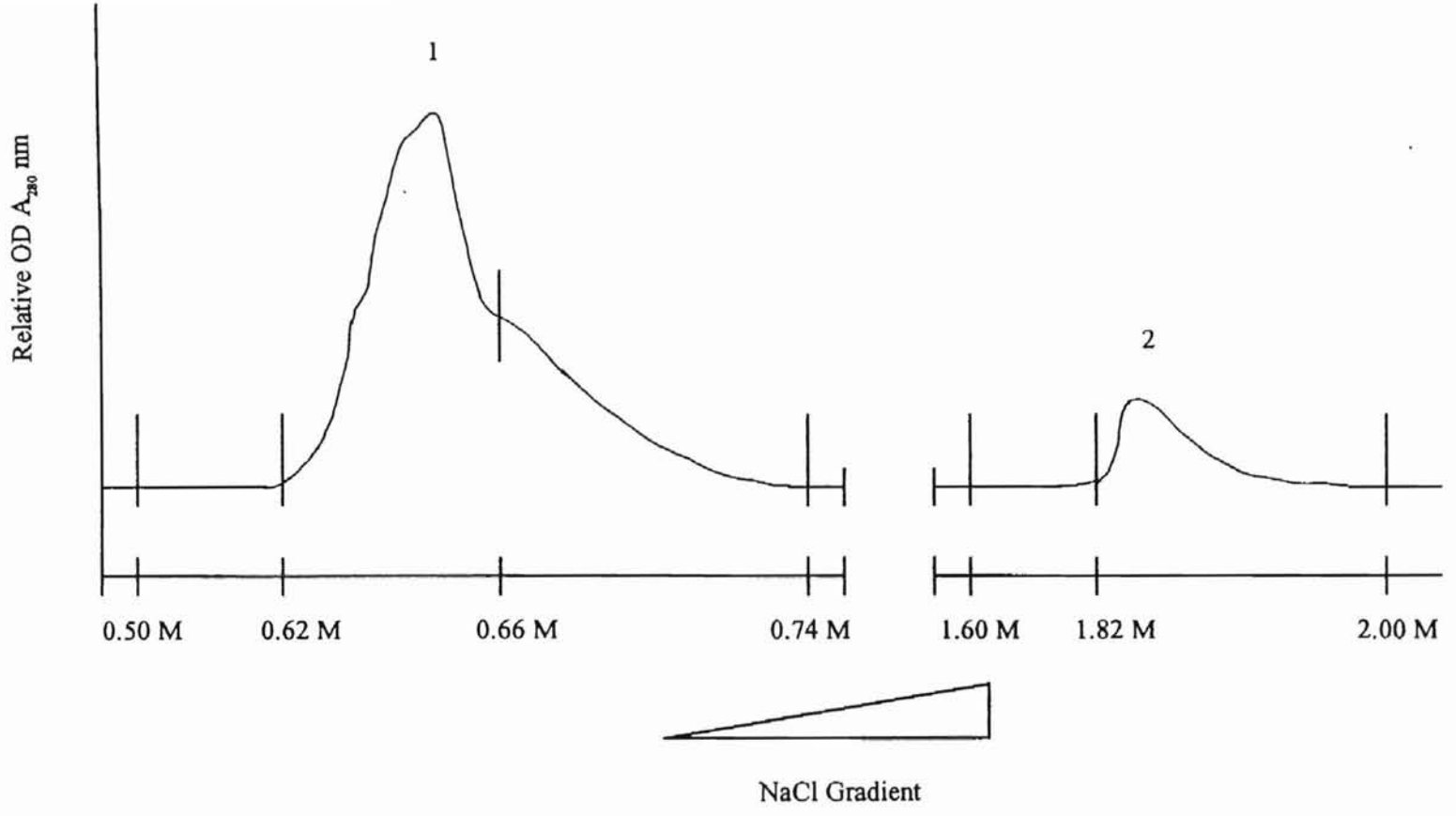

Figure 4. Anion exchange purification of PomA (1) and PomB (2) using binding buffer 10 mM Tricine (pH 8.9) and elution buffer 10 mM Tricine 2 M NaCl (pH 8.75).

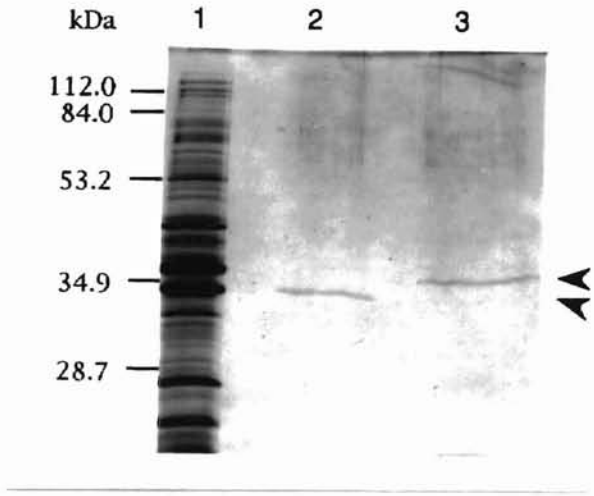

Figure 5. SDS-PAGE (18%) showing different eluted protein peak fractions on anion-exchange chromatography (silver stain). Lane 1, Supernatant #4; Lane 2, Peak 1 containing PomB; Lane 3, Peak 2 containing PomA. Upper arrowhead = PomA; lower arrowhead = PomB.

Table 2. Lesion scores and serum antibody responses to PomA and PomB of *Pasteurella haemolytica* for cattle vaccinated with various *P. haemolytica* vaccines.

Vaccine Group	No. of Cattle	Lesion Score	Antibody Responses[a] PomA Day 0	PomA Day 21	PomB Day 0	PomB Day 21
PBS	5	12.0 ± 4.1	0.066 ± 0.147	0.361 ± 0.113[e]	0.031 ± 0.049	0.019 ± 0.015
Bacterin-AlOH	5	6.5 ± 2.4[b]	0.000 ± 0.000	0.318 ± 0.074[e]	0.026 ± 0.029	0.090 ± 0.080
Bacterin-FIA	5	3.8 ± 4.3[c]	0.000 ± 0.000	0.458 ± 0.207[e]	0.009 ± 0.013	0.038 ± 0.037
Live *P. haemolytica*	5	3.6 ± 2.0[d]	0.040 ± 0.102	0.520 ± 0.166[e]	0.008 ± 0.015	0.277 ± 0.180[e,f]

[a]Expressed as mean trace OD x mm ± SD as determined by quantitative Western immunoblot analysis.
[b]Significant difference ($P < 0.02$) from PBS.
[c]Significant difference ($P < 0.01$) from PBS.
[d]Significant difference ($P < 0.01$) from PBS.
[e]Significant difference ($P < 0.05$) from Day 0.
[f]Significant difference ($P < 0.05$) from PBS at day 21.
AlOH = Aluminum hydroxide.
FIA = Freund's incomplete adjuvant.

vs Bacterin-FIA ($P < 0.01$), and PBS vs Bacterin-AlOH ($P < 0.05$). Antibody responses to PomA significantly increased between day 0 and day 21 for Bacterin-AlOH, Bacterin-FIA, and Live groups. The antibody response to PomA also significantly increased ($P < 0.05$) for PBS vaccinated group. The antibody response of live vaccinates to PomB was significantly ($P < 0.05$) greater on day 21 than on day 0.

There were no significant differences ($P \leq 0.05$) between mean antibody responses against PomA (day 21) among *P haemolytica*-vaccinated groups and PBS control groups. Significant differences ($P \leq 0.05$) were found, however, between antibody responses to PomB (day 21) for PBS and Live groups. There were no significant differences ($P \leq 0.05$) in antibody responses to PomB between PBS and Bacterin-AlOH or PBS and Bacterin-FIA. Regression analysis indicated significant correlations ($P < 0.05$) between high antibody responses to PomA and to PomB and low lesion scores ($r = -0.439$; $r = -0.458$), respectively.

CHAPTER IV

DISCUSSION

In this study, we partially purified and characterized two membrane proteins of *P. haemolytica* termed PomA and PomB (35 K and 32 K respectively), which eluted in fractions 4, 5, and 6 of an OPOE OMP extraction. Previous studies (34) identified 3 lipoproteins (28 - 30 kDa) that are in the Sarkosyl soluble and insoluble membranes of *P. haemolytica* A1. To prevent interference of these proteins in identification and characterization of PomA and PomB, we used a *P. haemolytica* lipoprotein mutant 89010807N (*lpp*) (35), which no longer produces these 3 lipoproteins. Even though supernatant #6 yielded partially purified PomA, it also had relatively small quantities of PomB. Hence for future studies of the OmpA family protein of *P. haemolytica*, anion-exchange chromatography, could be used to separate and purify PomA and PomB from supernatant #4.

The N-terminal sequence of PomA and immunoblot analysis data indicate that this protein has substantial homology to and share epitopes with *E. coli* OmpA. Comparisons of N-terminal amino acid sequence of PomA with sequences from OmpA family proteins from 7 other bacteria indicated that PomA is probably a member of the OmpA family of proteins. These are major bacterial OMPs and are conserved among many Gram-negative bacteria. In addition, immunoreactive OmpA family proteins were

found in each of the *P. haemolytica* biotype A serotypes indicating conservation among *P. haemolytica* serotypes.

The N-terminal amino acid sequences for *P. haemolytica* PomA and PomB were different. We compared the sequence of PomB with other portions of the *E. coli* OmpA sequence and found no homology; therefore, it is unlikely that PomB is a cleaved portion of PomA or a member of OmpA family proteins.

The outer membranes of several strains of *E. coli*, other enteric bacteria, and a variety of nonenteric Gram-negative bacteria contain major heat-modifiable proteins similar to OmpA of *E. coli* K-12 (4). Several heat-modifiable proteins have been identified in *P. haemolytica.* (26). PomA was heat-modifiable like *E. coli* OmpA and OmpA family proteins of other Gram-negative bacteria such as the 37 kDa OMP of *H. somnus*, 29 kDa OMP of *A. actinomycetemcomitans*, and MOMP of *H. ducreyi*. PomA, however, was heat-modifiable only when the protein was dialysed against deionized water. This behaviour is most likely due to removal of components that stabilize the protein in its heat-unmodified form. Since many of the properties of PomA are similar to those of the *E. coli* OmpA, it is likely that PomA may also be surface exposed like other members of the OmpA family (7, 21, 37).

OmpA proteins confer stability to outer membranes (43), serve as receptors for certain bacteriophages (15, 47), and stabilize mating aggregates formed during F pilus-mediated conjugation (47). A recent study demonstrated that OmpA is a factor in determining resistance to complement-mediated serum killing in a virulent strain of *E. coli* K-1 by stabilising the outer membrane making it more resistant to the effects of

complement (49). To determine the potential role of *P. haemolytica* PomA and PomB in immunity, antibody responses to those proteins were determined in sera from cattle that had been experimentally vaccinated with live or killed *P. haemolytica*. The Western immunoblotting / densitometry procedure has often been used to help determine potentially important immunogenic proteins (8, 13, 14, 31, 39). Increases in the antibody responses between day 0 and day 21 in Bacterin-AlOH, Bacterin-FIA, and live vaccinates were observed suggesting that PomA and PomB were present in these vaccines and were immunogenic. The antibody response to PomA, however, also increased in the PBS-vaccinated controls. The reason for this response is not known with surety. During the course of cattle experiments, one frequently finds a small increase in antibodies to *P. haemolytica* due to inhalation of nasal bacteria. Other Gram-negative bacteria are also part of the normal nasal flora and inhalation of them could have stimulated cross-reactive antibodies. The possibility, however, that this represented antibodies to naturally acquired active *P. haemolytica* infection seems unlikely because antibody responses to PomB actually declined in the PBS controls, and antibodies to whole *P. haemolytica* also did not increase in proportion to that seen for PomA (data not shown).

Regression analysis indicated significant correlations ($P < 0.05$) between high antibody responses to PomA and PomB and resistance to experimental challange. This suggests that serum antibodies against those bands may aid in increasing the resistance to pasteurellosis. Morton et al. (29) found that vaccination of calves with outer membranes of *P. haemolytica* protected them against experimental challange. Mosier et al. (31)

demonstrated that antibody responses to *P. haemolytica* surface proteins of 86, 66, 51, 49, 34, 31, and 16 kDa correlated both resistance to experimental pneumonic pasturellosis. Whether the 34 kDa protein identified in those studies is PomA of *P. haemolytica* and the 31 kDa protein is PomB is not known.

In previously published reports, serum antibodies to saline-extractable antigens from *P. haemolytica*, including 15 kDa and 30 kDa proteins, were associated with resistance to experimental bovine pneumonic pasteurellosis (11, 31). Recently, Craven et al. (13) and Dabo et al. (14) observed high antibody responses to two different 30 kDa lipoproteins also correlated with resistance to experimental challange. Therefore it appears that antibody responses to several *P. haemolytica* proteins in the range of 30-35 kDa may be important immunogens. It is possible that these proteins could be incorporated into a subunit vaccine to enhance protection of cattle against pneumonic pasteurellosis.

In summary, this study indicates that PomA of *P. haemolytica* is related to *E. coli* OmpA and a member of the OmpA family of proteins. It shares many properties of OmpA family proteins such as heat modifiability, N-terminal amino acid sequence. The N-terminal amino acid sequence of PomB does not have homology with other known bacterial protein sequences. Antibodies against these two proteins correlated with resistance to experimental pneumonic pasteurellosis. The role of these proteins in pathogenesis and immunity in bovine pasteurellosis can now be further studied.

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