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NOTE

Outer Membrane Protein H for Protective Immunity Against *Pasteurella multocida*

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***Pasteurella multocida*, a Gram-negative facultative anaerobic bacterium, is a causative animal pathogen in porcine atrophic rhinitis and avian fowl cholera. For the development of recombinant subunit vaccine against *P. multocida*, we cloned and analyzed the gene for outer membrane protein H (*ompH*) from a native strain of *Pasteurella multocida* in Korea. The OmpH had significant similarity in both primary and secondary structure with those of other serotypes. The full-length, and three short fragments of *ompH* were expressed in *E. coli* and the recombinant OmpH proteins were purified, respectively. The recombinant OmpH proteins were antigenic and detectable with antisera produced by either immunization of commercial vaccine for respiratory disease or formalin-killed cell. Antibodies raised against the full-length OmpH provided strong protection against *P. multocida*, however, three short fragments of recombinant OmpHs, respectively, showed slightly lower protection in mice challenge. The recombinant OmpH might be a useful vaccine candidate antigen for *P. multocida*.**

Keywords: outer membrane protein H, *Pasteurella multocida*, immunogenicity, subunit vaccine

Pasteurella multocida, a Gram-negative facultative bacterium, is one of the notorious animal pathogen causing widespread infections in various domestic animals; snuffles in rabbits, pneumonia and haemorrhagic septicaemia in cattles, sheep and goats, fowl cholera in chickens. In pigs, it causes severe respiratory diseases such as atrophic rhinitis (AR) and pneumonic pasteurellosis (Vasfi Marandi and Mittal, 1997). Significant global economic loss in swine production due to growth retardation is caused by AR, and therefore, vaccination has merged as the most attractive and effective approach in controlling AR (Foged *et al.*, 1989; Sakano *et al.*, 1997; Liao *et al.*, 2006).

Currently used vaccines, including inactivated and live vaccines, have their intrinsic problems as well as several advantages (Lubke *et al.*, 1994). A major advantage of live, attenuated vaccines is the fact that they are able to stimulate cross-protection against different *P. multocida* serotypes. However, because the basis for attenuation is not known, it is not surprising that vaccinated flocks with live, attenuated vaccines suffer outbreaks (Myint *et al.*, 1987; Avakian *et al.*, 1994). Moreover, when a killed bacterium was used for vaccination, immunity is generally serotype-specific (Carpenter *et al.*, 1991).

The outer membrane of Gram-negative bacteria contains lipopolysaccharides, several minor proteins and a limited number of major proteins present in very high copy numbers. One of the major outer membrane proteins, bacterial porins function as channels for the entrance and exit of hydrophilic low-molecular-weight substances. Porins have high immunogenicity as well as antigenicity, exposing epitopes on the bacterial surface. They are generally conserved in a bacterial species or even in bacterial families, and also share strong taxonomic associations in that they have high homology in both primary amino acid sequence and secondary structure (Jeanteur *et al.*, 1991; Bording *et al.*, 1994). Because of these properties, porins can be attractive vaccine candidates for heterologous immunity against Gram-negative bacterial infections (Tabaraie *et al.*, 1994; Rimler, 1996).

Porin H is the major outer memebrane protein in the envelope of *P. multocida* and conserved in several serotypes of *P. multocida* (Chevalier *et al.*, 1993). This protein exists as homotrimer in outer membrane, and the molecular weight of denatured monomer varies from 34 to 42 kDa (Chevalier *et al.*, 1993; Lubke *et al.*, 1994). The differences of the outer membrane protein H gene (*ompH*) by serogroups or serotypes in *P. multocida* are thought by the variety of the length and the sequence of the two variable regions in the *ompH* gene functioning as specific epitopes. The OmpH is a homologue of the P2 porin of *Haemophilus infuenzae* and a monoclonal antibody against OmpH could passively protect mice against infection (Vasfi Marandi and Mittal, 1997).

In this study, we cloned and express the gene for outer membrane protein H (*ompH*) gene of pathogenic strain of *P. multocida* in Korea. Using the full-length and truncated form of recombinant OmpH we investigated the antigenic properties of the recombinant OmpH as a candidate for

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vaccine.

P. multocida used in this study was originally isolated from a case of swine atrophic rhinitis in Korea and grown on blood agar plate and grown for 12 h at 37°C. The isolate was grown overnight on Bacto™ brain-heart infusion (BHI) (Difco Laboratories, USA) agar at 37°C. Genomic DNA of *P. multocida* was isolated by the method of Sambrook and Russell (Sambrook and Russell, 2001). The *E. coli* DH5α and BL21 (DE3) were used for gene cloning and gene expression, respectively. The pGEM-T vector (Promega, USA) was used for cloning of PCR products, and the plasmid pET32a (Novagen, USA) and pSET B (Invitrogen, USA) were used for protein expression. Bacterial envelopes were prepared by sonication and centrifugation as described (Ryu and Kim, 2000). Outer membrane was extracted with 0.5% sodium *N*-lauroylsarcosine in 0.01 M Tris-HCl (pH 8.0) and insoluble fraction was collected by centrifugation (Dabo *et al.*, 1997).

Table 1. Oligonucleotide primers used in this work

Primer	Sequence	Amino acid position in OmpH
OmpH-F	5'-GTGAT[ACT]ATGAAAAAGACAATCG-3'	1
OmpH-R	5'-GATCCATTCCTTGCAACATATTGA-3'	3'-UTR
EOmpH-F1	5'-CAGCGGATCCA[GCA]ACAGTTTACAA-3'	21
EOmpH-R1	5'-CTAACAAAGCTTA[GAA]GTTACGCG-3'	341
EOmpH-R2	5'-AGTCACTTAAGCTTG[TGC]GTAGTC-3'	248
EOmpH-R3	5'-ACCTGAAGCTTC[GAA]ACCGCGACCG-3'	192
EOmpH-F2	5'-TTCAAGGATCCA[GAA]TTCAACGGT-3'	165

*Bam*HI sequences in forward primers and *Hin*dIII sequences reverse primers are underlined.
Sequences in boxes encode starting or ending amino acids of OmpH.

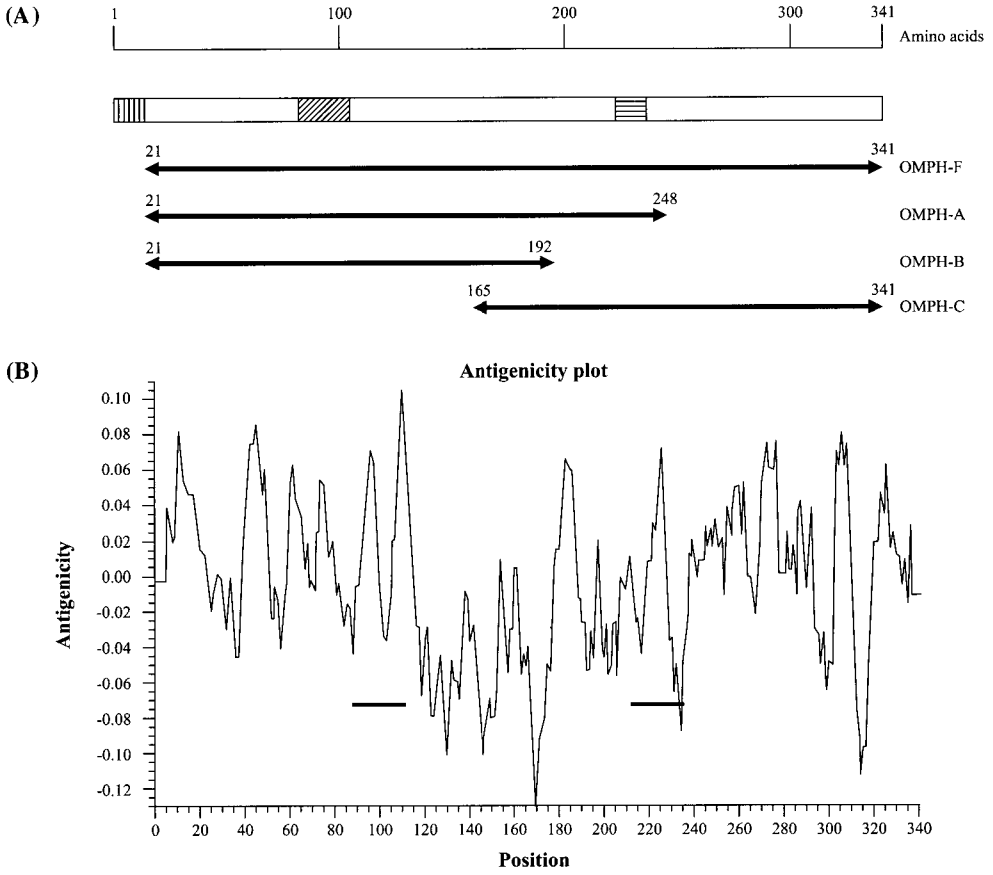

Fig. 1. Schematic illustration of the gene structure and antigenicity plot of OmpH protein. (A) The full-length and partial fragments of the *ompH* were cloned into pET32a for OMPH-F, and pRSET B for OMPH-A, OMPH-B, and OMPH-C, respectively. Numbers on the arrows present the start and end position of amino acid in each constructs. (B) Antigenicity plot of OmpH. Antigenicity was analyzed by the methods of Welling *et al.* (1985). The bars on the graph indicate sequence-variable regions of OmpH among serotype-specific strains.

The *ompH*-specific primer OmpH-F and OmpH-R for the gene amplification were designed by sequence analysis of several clones found in BLAST sequence database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA) (Table 1). The *ompH* gene fragment was amplified from the genomic DNAs of *P. multocida* isolate by PCR as described previously (Lee *et al.*, 2004). A predicted 1 kb DNA fragment was cloned into plasmid pGEM-T according to the manufacturer's instruction. The ORF of *ompH* is composed of 1,023 bp encoding 341 amino acid residues containing 20 amino acid long signal peptide (GenBank accession number AY603962). Hydrophilic domains within OmpH corresponded to predicted surface-exposed domains in secondary structure that are highly variable in their amino acid composition compared with similar OMPs. Furthermore, residues to 82-103 and 219-226 constituted variable regions which corresponded to hydrophilic domains of OmpH (Fig. 1). These two regions have various compositions in sequence and length. According to the analysis of antigenicity based on the amino acid sequence, these variable regions have relatively high antigenicity than other hydrophobic regions (Fig. 1).

The fusion expression vector for signal sequence-deleted OmpH to reduce the lethality of recombinant porin in *E. coli* was constructed. The DNA fragment encoding the truncated ORF (aa 21-341; deletion of signal sequence of 20 amino acids) was re-amplified using primer EOmpH-F1 and EOmpH-R1 to generate the OMPH-F clone using pET32a expression vector which has a thioredoxin (Trx) sequence in the upstream of multiple cloning sites. It was expected that N-terminal fused Trx could disturb and suppress the functional activity of recombinant OmpH. Recombinant expression plasmid for OMPH-F was transformed into *E. coli* BL21 (DE3). The gene expression was induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the recombinant protein was purified using the His-Bind® Kits (Novagen, Germany) according to the manufacturer's instruction. The

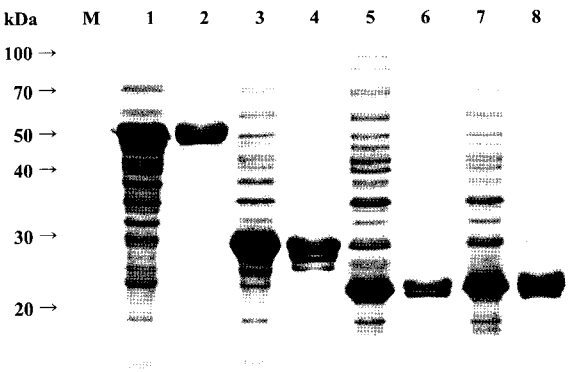

Fig. 2. Purification profile of recombinant OmpH protein. Lane M, protein molecular weight standard; lanes 1, 3, 5, 7, cell lysates of the *E. coli* host harboring the expression vector for OMPH-F, OMPH-A, OMPH-B, OMPH-C, respectively; lanes 2, 4, 6, 8, partially purified recombinant OMPH-F fused with TrxA and 6× His (*ca.* 53 kDa), recombinant OMPH-A fused with 6× His (*ca.* 29 kDa), recombinant OMPH-B fused with 6× His (*ca.* 22 kDa), recombinant OMPH-C fused with 6× His (*ca.* 23 kDa), respectively.

Trx-fused recombinant OmpH was successfully expressed and purified in *E. coli* (Fig. 2). The molecular mass of the fused OmpH (OMPH-F) was 53 kDa, which consist of 36 kDa-truncated OmpH and 17 kDa-Trx proteins provided by vector on the 12.5% SDS-PAGE.

According to the sequence and antigenicity analysis, we constructed expression vectors for three short fragments of OmpH, respectively. The DNA fragment encoding N-terminal to the middle region (aa 21-248) was re-amplified using primer EOmpH-F1 and EOmpH-R2 to generate the OMPH-A clone. The DNA fragment encoding N-terminal region of the protein (aa 21-192) was re-amplified using primer EOmpH-F1 and EOmpH-R3 to generate the OMPH-B clone. The DNA fragment encoding C-terminal region of the protein (aa 165-341) was re-amplified using primer EOmpH-F2 and EOmpH-R1 to generate the OMPH-C clone. All PCR products were digested with restriction enzyme *Bam*HI and *Hin*dIII, and then cloned into pRSET B for OMPH-A, OMPH- B, and OMPH-C, respectively (Fig. 1).

Recombinant expression plasmids for OMPH-A, OMPH-B, and OMPH-C were transformed into *E. coli* BL21 (DE3), respectively. The gene expressions were induced with 0.8 mM IPTG in transformed *E. coli* BL21 (DE3), respectively, and the recombinant proteins were purified busing Ni-NTA affinity chromatography, respectively (Lee *et al.*, 2004). The molecular weight of OMPH-A, OMPH-B, and OMPH-C including N-terminal tagging portion (4 kDa) provided by vector on 12.5% SDS-PAGE was about 29, 22, and 23 kDa, respectively. The expression efficiencies of these OmpH proteins ranged from 20-32% of the total cellular proteins. The purities of the recombinant OmpH proteins by Ni- NTA were up to 85% of the purified total proteins (Fig. 2).

To study the immunogenicity of recombinant OmpHs, female BALB/c mice were randomly divided among seven groups of 15 mice each: negative control vaccinated with PBS (group 1), commercial bacterin vaccine for porcine respiratory disease (consisted of *Bordetella bronchiseptica, P. multocida,* and *Haemophilus pleuropneumoniae*; group 2; Daesung microbiological lab. Co., Korea), formalin-killed *P. multocida* strain (group 3), OMPH-F (group 4), OMPH-A, (group 5), OMPH-B (group 6), and OMPH-C (group 7). Mice were subcutaneously immunized on days 0, and 14, respectively, with 50 μl of PBS with Freund's complete adjuvant (FCA) for group 1, 50 μg of formalin-killed *P. multocida* whole cells with FCA for group 3, 50 μg of OMPH-F with FCA for group 4, 50 μg of OMPH-A with FCA for group 5, 50 μg of OMPH-B with FCA for group 6, 50 μg of OMPH-C with FCA for group 7, respectively. As a known positive control, a commercial vaccine was administered intraperitoneally at 1/10 the stated swine dose as recommended by manufacturer (group 2). On day 7 and 21, five mice from each group were bled from the eyes, and the antisera were stored at -80°C.

Anti-OmpH antibody was determined by an enzyme-linked immunosorbent assay (ELISA). Preliminary assays were performed to determine appropriate serum dilution rate and antigen concentration to use in the assay. Wells of 96-well microtiter plates were coated with 0.1 μg of bacterial membrane fraction, and sealed and incubated at 37°C for 2 h. Plates were then washed three times with PBS containing

0.05% Tween 20 (PBST). Wells were coated with 200 μl of blocking solution containing 3% BSA in PBST, sealed, and then incubated at 37°C for 1 h. After washes three times with PBST, 100 μl of diluted mouse sera in antibody-diluting buffer (PBST with 1% BSA; 1:500) was added to each well. Control wells received antibody-diluting buffer only. The plates were sealed and incubated at 37°C for 2 h. Following incubation the plates were washed three times with PBST and 100 μl of a secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG; 1:10000) was placed in each well. The plates were sealed and incubated at 37°C for 1 h, and then washed three times with PBST. Fifty microliter of color development solution (3,3',5,5'-tetramethylbenzidine (TMB) dissolved in dimethyl sulfoxide) were used in colorimetric response. The incubation was carried out at room temperature in the dark place. After fully incubation, the absorbance values were measured with a 650 nm filter in a plate reader. All samples were subsequently tested in duplicate (Lee *et al.*, 2004). Mean antibody responses among the various vaccination groups were analyzed Kruskal-Wallis H Test and Dunnett's T3 post-hoc multiple comparisons. Antibody responses between days 0 and 21 were compared for each group by paired *t*-tests. Survival rates were compared among group by chi-square analysis (Armitage and Berry, 1994).

Vaccination with OMPH-F, OMPH-A, OMPH-B, and OMPH-C, respectively, resulted in a significantly ($P<0.05$) higher mean antibody responses against *P. multocida* outer membrane fraction between day 7 to 21 when compared to the responses of the other treatment groups (Fig. 3). Antibody responses for the OMPH-F, OMPH-A, OMPH-B, and OMPH-C vaccinated groups were not significantly different ($P>0.05$) from each other in both the first vaccinated sera (day 14) and

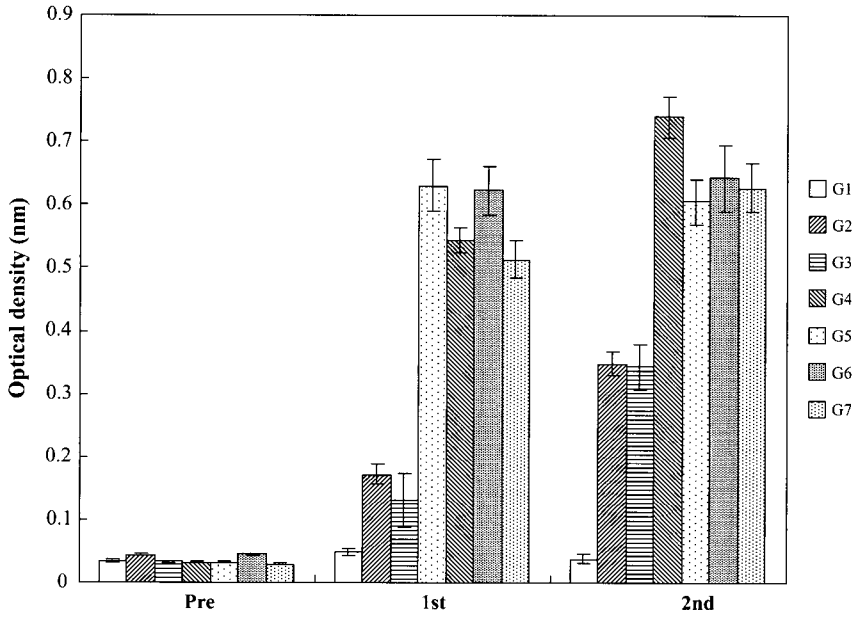

Fig. 3. Antibody titeration in antisera. Mice were immunized with PBS for negative control (G1), commercial vaccine (G2), formalin-killed whole cells of *P. multocida* strain (G3), OMPH-F (G4), OMPH- A (G5), OMPH-B (G6), and OMPH-C (G7). After vaccination, antisera were obtained from mice in each group, and the titers were measured by ELISA using outer membrane fraction as an antigen. Each value represents the Mean±Standard Deviation.

Table 2. Evaluation of protection conferred to immunized mice against live *P. multocida* challenge

Experimental group[a]	Immunization	No. of dead/No. challenged[b]	Survival rate (%)
G1	PBS	10/10	0
G2	Commercial vaccine	3/10	70
G3	Formalin-killed whole cells	2/10	80
G4	OMPH-F	3/10	70
G5	OMPH-A	5/10	50
G6	OMPH-B	7/10	30
G7	OMPH-C	5/10	50

[a] Mice were immunized with PBS for negative control (G1), commercial vaccine (G2), formalin-killed whole cells of *P. multocida* (G3), OMPH-F (G4), OMPH-A (G5), OMPH-B (G6), and OMPH-C (G7). After vaccination, the mice were intraperitoneally challenged with live virulent *P. multocida* (4.3×10^3 CFU) and observed for 96 h.

[b] The protection index was calculated as the ratio of dead mice to total mice used in a group.

second vaccinated sera (day 21). The levels of antibody response in commercial vaccine and formalin-killed whole cell vaccinates were relatively lower than those of the recombinant OmpHs.

After vaccination, the mice were challenged with 100 μl intraperitoneal injection of live virulent *P. multocida* (4.3×10^3 CFU) and monitored for 96 h (Tabatabaei *et al.*, 2002). The protection index was calculated as the ratio of dead mice to total mice used in a group. The results of protection studies are shown in Table 2. Vaccination with commercial vaccine, formalin-killed whole cells, OMPH-F, OMPH-A, OMPH-B, and OMPH-C resulted in protection from challenge; however, the levels of the protection were different. The commercial vaccine and formalin-killed whole cells induced similar level of lower antibody titers against outer membrane fraction of *P. multocida* than other OmpH proteins, and showed relatively efficient protections against *P. multocida* challenge (70% and 80% survival rate, respectively). The OMPH-F vaccination showed high antibody titer against outer membrane fraction of *P. multocida*, and had high protection against *P. multocida* challenge (70%). The protection level was slightly lower than that of formalin-killed whole cell vaccination; however, difference was not significant ($P>0.5$). The OMPH-A and OMPH-C induced high antibody titers against outer membrane fraction of *P. multocida*, respectively. But they had significantly lower protective property (50%) than OMPH-F and Formalin-killed whole cell. In case of OMPH-B, it induced similar level of antibody against outer membrane fraction of *P. multocida*, compared to OMPH-A, and OMPH-C; however, the protection was lower (30%) than those of other OmpH proteins.

Since the first event tried by Louis Pasteur at immunization against infection with the Gram-negative facultative bacterium, like *P. multocida*, it has been known that many of the bacterial outer membrane proteins are strong antigens. Several outer membrane proteins have been identified in various strains of *P. multocida* and have been used in protective experiments against agents of severe pasteurellosis (Lubke *et al.*, 1994; Pati *et al.*, 1996; Dabo *et al.*, 1997). It has been suggested that certain serological types could be the etiologic agents of severe pasteurellosis, and that a cross-protective immune response might be achievable by immunization with a mixture of serotype-specific antigens that are recognized by convalescent sera (Pati *et al.*, 1996; Luo *et al.*, 1999; Haesebrouck *et al.*, 2004). Recently, characterization and diversity of pathogenic *P. multocida* has been investigated on the basis of not only capsular type but also OmpH type by PCR analysis (Davies *et al.*, 2003). In this study, a gene for outer membrane protein, *ompH*, of *P. multocida* from pigs suffered from atrophic rhinitis was isolated and analyzed. The protective immunity of OmpH was assessed using recombinant OmpH expressed in *E. coli*. The amino acid sequences showed high level of homology to other bacterial porins and had the highest similarity to OmpH of other serotypes of *P. multocida*. The signal peptide region of the OmpH had the common characteristic composition such as a stretch of hydrophobic amino acids and an Ala-X-Ala cleavage site (Jeanteur *et al.*, 1991). The compositions of amino acid sequences of the OmpH had typical characteristic of Gram-negative bacterial porins: highly negative hydropathy index, lack of stretches of hydrophobic residues, high glycine content, low proline content and lack of cysteine (Luo *et al.*, 1999). Hydrophilic portions of OmpH, which were predicted as surface-exposed domains in secondary structure, had highly variable composition of amino acids compared with OmpH in other serotypes. The overall structure of OmpH is conserved in serotypes; however, there are small variable regions in the proteins of each serotype. This indicates that the variable regions may work as strain-specific epitopes that take an important role in serotype-specific immune response.

Bacterial porin genes are sometimes difficult to clone in *E. coli* because foreign porins are usually lethal for *E. coli*. Initial attempts to clone the entire *ompH* gene into expression vector were unsuccessful. This failure could be explained due to the leaking expression of the primary protein without IPTG induction, and lethality of recombinant porin protein in *E. coli*. Although we also tried to use ectopic expression of truncated form of OmpH which has deleted signal peptide, the *E. coli* harboring the expression vector was not grown well, and also the expression level of the recombinant gene was relatively low (data not shown). Therefore, the fusion expression vector system for signal sequence-deleted OmpH to reduce the lethality of recombinant porin in *E. coli* was used in this study.

Outer membrane proteins of *P. multocida* have been recognised as immunodominant antigens (Confer, 1993) and are thought to be responsible for cross protective immunity, since LPS alone induces only partial protection against pasteurellosis in mice (Ryu and Kim, 2000). Several studies have successfully used antibodies against *P. multocida*, in ELISA, as a predictor of enhanced resistance against challenge. Those studies included cattle (Confer *et al.*, 1996), buffalo (Pati *et al.*, 1996), rabbit (Klaassen *et al.*, 1985), chickens (Hofacre *et al.*, 1987), and mice (Wijewardana and Sutherland, 1990). In case of outer membrane protein, Oma87, An 87 kDa outer membrane antigen, had been cloned, sequenced, and expressed in *E. coli* (Ruffolo and Adler, 1996). Antisera raised against recombinant Oma87 protected mice against homologous challenge and it was therefore suggested that Oma87 was a protective outer membrane protein. In this study full-length and short fragments of recombinant OmpH could produce high titration of antibodies specific to outer membrane fraction of *P. multocida*. The level of antibody response to outer membrane portion in these full-length and short fragments of recombinant OmpH were not significantly different ($P>0.05$) (Fig. 3); however, the resistance against challenge was different. Only full-length OmpH showed high protection like as formalin-killed whole cells, other short fragments had relatively low protection. The levels of antibody response in commercial vaccine and formalin-killed whole cell vaccinates were relatively lower than those of the recombinant OmpHs; however, they showed higher the protections against challenge than short fragments of recombinant OmpH. These results can be explained; outer membrane fraction was used in ELISA as an antigen, and the vaccinated antigen source in case of commercial vaccine and formalin-killed whole cell vaccination were complex, not unique. Also it means that strong antibody response alone is not always a good predictor of protection. In fact, several studies have

identified that antibodies to some bacterial antigens may be detrimental to the host defenses (Harper *et al.*, 2006).

In conclusion, OmpH is a major antigenic outer membrane protein from *P. multocida* and has high immunogenicity in antibody production. Although the short fragment of recombinant OmpH has lower protective immunity, antibodies against full-length of recombinant OmpH appear to be protective in mice. Therefore, recombinant OmpH is a desirable immunogen for stimulating protection against *P. multocida* infection.

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