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Protection of chickens from fowl cholera by vaccination with recombinant adhesive protein of *Pasteurella multocida*

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Summary The recombinant adhesive protein (rCp39) of *Pasteurella multocida* strain P-1059 (serovar A:3) was prepared and purified with a hybrid condition of affinity chromatography. The rCp39 was highly protective for chickens from fowl cholera by challenge-exposure with parental strain P-1059 or heterologous strain X-73 (serovar A:1) compared to various kind of vaccines. Sixteen groups of ten chickens each were subcutaneously inoculated twice with 100, 200 or 400 μg proteins of rCp39, native Cp39, native outer membrane protein H (OmpH) or recombinant OmpH, or 100 μg proteins of crude capsular extract (CCE) of strains P-1059 or X-73 at 2 weeks interval. Five chickens of each group were challenge-exposed with each strain 2 weeks after the second inoculation. As the results, 60–100% protections were demonstrated in the chickens against both strains. Fisher's exact test indicated no significant differences ($P < 0.05$) in vaccine types and dosages. ELISA and Western blot analysis indicated that the chicken anti-rCp39 sera reacted to whole-cell lysate of parental or heterologous strains. In conclusion, rCp39 is a cross-protective recombinant adhesive antigen of *P. multocida* capsular serogroup A strains. Moreover, a hybrid condition of affinity chromatography was successfully demonstrated and protected the immunogenicity of recombinant protein.

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Introduction

Pasteurella multocida is a gram-negative bacterium and the causative agent of fowl cholera, bovine or buffalo hemorrhagic septicemia and swine atrophic rhinitis. The bacteria can be classified into five capsular serogroups A, B, D—F [1] and 16 somatic serotypes 1—16 [2]. Strains of capsular serogroup A and somatic serotypes 1, 3 and 4 are known as the causative agents of fowl cholera [1,3,4]. The virulence factors of *P. multocida* including capsular protein have been demonstrated [5—8]. Our previous studies demonstrated that the 39 kDa protein or native protein Cp39 in crude capsular extract (CCE) of strain P-1059 (serovar A:3) is an adhesive protein, a capsule-associated antigen and a cross-protective antigen among *P. multocida* capsular serogroup A strains [9—11]. The N-terminal sequence analysis demonstrated that the Cp39 was identical to the major outer membrane protein (OmpH) of strain X-73 (Borrathybay E., unpublished data). Recombinant Cp39 (rCp39) protein was prepared in *Escherichia coli* and the rabbit antisera against rCp39 indicated that the recombinant protein was a cross-reactive recombinant antigen among *P. multocida* capsular serogroup A strains.

Our preliminary experiments, the antibody induced by the recombinant proteins purified with the denatured condition of affinity chromatography could not react to the undenatured conformational epitopes in whole-cell lysate of strains P-1059 or X-73 because the immunogenicity of that recombinant proteins have changed and needs to be modified (Sthitmatee N., unpublished data). Therefore, the aims of this study were to demonstrate a hybrid condition of the affinity chromatography to purify the recombinant protein and to determine the protectivity in rCp39-immunized chickens from fowl cholera by challenge-exposure with parental strain P-1059 or heterologous strain X-73 (serovar A:1) in comparison with various kind of vaccines. Moreover, protectivity conferred by immunization with the major outer membrane protein and its recombinant protein (OmpH and rOmpH) of strain X-73 [12] were determined and compared to the rCp39. In addition, the antibody response was determined by indirect enzyme-linked immunosorbent assay (ELISA) and Western blotting.

Materials and methods

Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *P. multocida* strains were grown in tryptose broth (TB; Difco Laboratories, MD, USA) at 37 °C for 6 h and then subcultured on dextrose starch agar (DSA; Difco) at 37 °C for 18 h. *E. coli* strains PQE-cp39 [Borrathybay et al., submitted for publication] and PQE-ompH [12] were grown at 37 °C in Luria-Bertani (LB) broth or on agar supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin (Sigma—Aldrich, St. Louis, MO, USA).

Preparation of immunogen

Crude capsular extract

CCE of *P. multocida* strains was prepared using the saline extraction method as described previously [13]. Briefly, one single colony of strains P-1059 or X-73 on DSA plate was selected and cultivated in TB at 37 °C for 6 h. Then, 0.2 ml of 6-h culture were streaked onto DSA plates and incubated at 37 °C for 6 h. The bacteria grown on the plate were harvested in a 2.5% NaCl solution (5 ml/plate) and agitated at 56 °C in a water bath for 1 h. The bacterial suspension was centrifuged at 17,000 × *g* for 20 min. Supernatant was transferred to new centrifuge tube and centrifuged twice in the same manner. Supernatant was subjected to dialyze against 0.85% NaCl solution containing 0.01% thimerosal at 4 °C for 48 h. Supernatant was concentrated with carboxymethylcellulose sodium salt (Sigma). CCE was kept at −20 °C until use.

Purification of Cp39 and OmpH proteins

Purified Cp39 (Cp39) of strain P-1059 and major outer membrane protein H (OmpH) of strain X-73 were prepared by the electroelution method as described previously [10,14,16]. Briefly, after separation of proteins by SDS-PAGE, the gel was dipped in distilled water for several seconds and then stained with 100 ml of 0.3 M $CuCl_2$ solution at room temperature for 5 min. The stained gel was washed with distilled water for 3 min to remove excess reagent, and then the target protein band was cut out. The cut strip of protein was destained twice by incubation in 0.25 M EDTA, 0.25 M Tris—HCl (pH 9.0) solution with gentle agitation at room temperature for 10 min. The target protein was purified by electroelution (electroelution electrophoresis apparatus, ATTO) in 20 mM Tris base, 150 mM glycine and 0.01% SDS buffer at 100 V for 1 h in icebox. The eluted protein was passed through the detergent removing minicolumn (Ampure DT, Amersham, Japan) in order to remove SDS from protein solution. Then, the eluted protein was kept at −20 °C until use.

Purification of recombinant proteins

E. coli strains PQE-cp39 and PQE-ompH (Borrathybay E., unpublished data, 12) that carried the *cp39* gene of strain P-1059 and the *omp*H gene of strain X-73, respectively, were used to produce the recombinant protein in this study as described in Qiagen Qiaexpressionist instructions. Briefly, 1 ml from overnight culture of *E. coli* strains in selective LB broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin was inoculated in pre-warm selective LB broth as above and incubated at 37 °C in shaking incubator until OD_{600} reached 0.5—0.7 (mid-log phase). Then, expression of 6-His-tagged fusion protein was induced by adding a final concentration of 1 mM of isopropyl-D-thiogalactopyranoside (IPTG; Takara, Shiga, Japan) and incubating continuously at 37 °C for 4—5 h. This fusion protein was easily purified by a hybrid condition of affinity chromatography with Ni-nitrilotriacetic acid resin (Ni-NTA; QIAGEN, Valencia, CA, USA). Briefly, bacterial cells were resuspended in lysis buffer B (100 mM NaH_2PO_4, 10 mM Tris—Cl, 8 M urea, pH 8.0) at 5 ml/g wet wt. Bacterial cells were stirred by a rotary shaker for 60 min at room temperature. Then, bacterial suspension

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or other relevant characteristics	Reference
P. multocida strains		
P-1059	A:3, Iridescent colony	ATCC15742
X-73	A:1, Iridescent colony	ATCC11039
E. coli strains		
PQE-cp39	6 × His-tagged fused *cp39* gene of strain P-1059 plasmid in *E. coli* strain M15 [pREP4]	Borrathybay E. (unpublished data)
PQE-ompH	6 × His-tagged fused *omp*H gene of strain X-73 plasmid in *E. coli* strain M15 [pREP4]	Luo et al. [12]

was centrifuged at 10,000 × *g* for 20 min at room temperature to pellet the cell debris. Four milliliters of supernatant was transferred and mixed gently by a rotary shaker with 1 ml of the 50% Ni-NTA slurry for 60 min at room temperature. The lysate—resin mixture was transferred carefully into an empty column and washed twice with 4 ml of washing buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0). The recombinant protein was eluted with 0.5 ml of elution buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 250 mM imidazole, pH 8.0) to a new tube and kept at −20 °C until use.

In addition, recombinant proteins were also purified with a denatured condition of the affinity chromatography as described in Qiagen Qiaexpressionist instructions and performed in immunoblotting.

Determination of protein concentration

Protein concentration was determined by Bradford's method [15] using bovine serum albumin standard (BSA; Sigma) and Bio-Rad Protein Assay (Bio-Rad, CA, USA).

Vaccine preparation

Immunogens were prepared as desired concentration and then completely emulsified with an equal volume of Freund's incomplete adjuvant (Wako, Japan) as desired concentrations and kept at 4 °C until used. The dosages of each immunogens are shown in Tables 2 and 3.

Chickens

Eight-week-old layers of *P. multocida*-antibody-free (RPM Farm & Feed Co. Ltd., Chiang Mai, Thailand) were used in this study. The animal welfare committee of Faculty of Veterinary Medicine, Chiang Mai University controlled use of laboratory animals with the laboratory animal ethics. Experiments were performed in closed system. Experiment rooms and instruments were cleaned with disinfectant for 2 weeks before and after experiment. Waste products were treated before released to environment.

Protection assay in chicken

Chickens were categorized into two experiment groups based on immunization strategies. The birds of each group were subcutaneously inoculated twice with 0.5 ml of each immunogen per dose at 2 weeks interval. At 2 weeks after the second inoculation chickens were challenge-exposed intramuscularly with 0.2 ml of bacterial suspension containing 10^3 to 10^4 cfu of strain X-73 or 10^5 to 10^6 cfu of strain P-1059 [16]. Non-vaccinated control chickens were also exposed with the bacteria in the same manner. The birds were observed for their mortality rates and clinical signs for 10 days. Necropsies and bacterial isolation were taken for dead chickens. Gross lesions were recorded and lungs, livers, spleens, kidneys and hock joints were collected for the bacterial isolation by direct culture using DSA plates.

Table 2 Protections in chickens immunized with rCp39

Group	Type of vaccine[a] (total protein/0.5 ml)	Survival rate of chickens challenge-exposed with	
		Strain X-73[b]	Strain P-1059[c]
1	Purified recombinant adhesive protein (rCp39: 100 μg)	3/5 (60)[d]	5/5 (100)
2	Purified recombinant adhesive protein (rCp39: 200 μg)	4/5 (80)	5/5 (100)
3	Purified recombinant adhesive protein (rCp39: 400 μg)	4/5 (80)	5/5 (100)
4	Purified native adhesive protein (Cp39: 100 μg)	4/5 (80)	5/5 (100)
5	Purified native adhesive protein (Cp39: 200 μg)	4/5 (80)	5/5 (100)
6	Purified native adhesive protein (Cp39: 400 μg)	4/5 (80)	5/5 (100)
7	CCE of strain P-1059 (100 μg)	4/5 (80)	5/5 (100)
8	No immunization	0/5 (0)	0/5 (0)

[a] Chickens were inoculated subcutaneously twice with various types of vaccine at 2 weeks interval.
[b] Chickens were exposed intramuscularly with 5.5×10^3 CFU/0.2 ml of strain X-73.
[c] Chickens were exposed intramuscularly with 7.1×10^5 CFU/0.2 ml of strain P-1059.
[d] No. of survived/no. exposed (%).

Table 3 Protections in chickens immunized with rOmpH

Group	Type of vaccine[a] (total protein/0.5 ml)	Survival rate of chickens challenge-exposed with	
		Strain X-73[b]	Strain P-1059[c]
1	Purified recombinant outer membrane protein (rOmpH: 100 μg)	5/5 (100)[d]	4/5 (80)
2	Purified recombinant outer membrane protein (rOmpH: 200 μg)	5/5 (100)	5/5 (100)
3	Purified recombinant outer membrane protein (rOmpH: 400 μg)	5/5 (100)	5/5 (100)
4	Purified native outer membrane protein of X-73 (OmpH: 100 μg)	5/5 (100)	5/5 (100)
5	Purified native outer membrane protein of X-73 (OmpH: 200 μg)	5/5 (100)	5/5 (100)
6	Purified native outer membrane protein of X-73 (OmpH: 400 μg)	5/5 (100)	5/5 (100)
7	CCE of strain X-73 (100 μg)	5/5 (100)	5/5 (100)
8	No immunization	0/5 (0)	0/5 (0)

[a] Chickens were inoculated subcutaneously twice with various types of vaccine at 2 weeks interval.
[b] Chickens were exposed intramuscularly with 5.5×10^3 CFU/0.2 ml of strain X-73.
[c] Chickens were exposed intramuscularly with 7.1×10^5 CFU/0.2 ml of strain P-1059.
[d] No. of survived/no. exposed (%).

Chicken sera

Blood was collected from all the chicken before each inoculation and exposure for ELISA and Western blot analyses. In addition, chicken antisera against P-1059 or X-73 bacterins and against denatured condition of the affinity chromatography-based rCp39 or rOmpH were also employed in this study for the determinations of antibody response (Sthitmatee, N. from personal control chicken sera).

Enzyme-linked immunosorbent assay

Antibody titers were evaluated by the indirect ELISA. Micro plates (Nunc-immuno™ plate, Denmark) were coated with antigens; rCp39, rOmpH or whole-cell of strains X-73 or P-1059 and an equal volume of coating buffer; 0.5 M carbonate buffer (pH 9.6), and then incubated at 4 °C overnight. Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T). Non-specific bindings were inhibited with blocking buffer; 1% skim milk (Difco), 1% bovine serum albumin (BSA; Difco), 0.1% sodium azide (Wako) and PBS (pH 7.2). A serial twofold dilution of each chicken serum was prepared in blocking buffer and added into each well. Then, the plate was incubated at room temperature for 1 h. After washed three times with PBS-T, each well was added with a 1:1000 dilution of horseradish peroxidase-conjugated anti-chicken IgY (IgG; Sigma) in blocking buffer and incubated continuously at room temperature for 1 h. Substrate solution containing of 30 mg *o*-phenylenediamine dihydrochloride (O-PDA; Wako) in 100 ml substrate buffer (0.2 M Na_2HPO_4—$12H_2O$; 0.1 M $C_6H_8O_7$; pH 4.8) was added to each well after a further washing and incubated without light at room temperature for 30 min. The reaction was stopped by adding 2N sulfuric acid (H_2SO_4; Wako) and absorbance values were recorded at wavelength 492 nm by ELISA reader (Immuno Mini NJ 2300, Intermed, Japan). End-point titers were expressed as the log of the highest dilution, which provided the optimal density more than 0.1 U above back ground value of the preimmune sera. The log mean titers and standard error of the mean of each group were calculated and were used to compare the statistical data.

SDS-PAGE and Western blotting

Samples for SDS-PAGE and Western blotting were prepared by two methods. Briefly, samples were resuspended in sample buffer and continuously incubated at 37 °C for 2 h or boiled for 5 min. Samples were analyzed on a 12.5% polyacrylamide slab gel [17] in a mini-slab apparatus (ATTO Corporation, Tokyo, Japan) and stained with Coomassie blue R-250 (Sigma). Then, proteins were transferred from SDS-PAGE slab gels to nitrocellulose membrane (Amersham Biosciences KK, Tokyo, Japan) and incubated with a dilution of 1:200 of chicken antisera and followed by incubation with a 1:1000 dilution of horseradish peroxidase-conjugated anti-chicken IgY (IgG; Sigma). Proteins were visualized by incubation with 3,3-diaminobenzidine (DAB; Sigma) in PBS (pH 7.2).

Statistical analysis

Comparisons of protection in immunized chickens were made by the Fisher's exact test. In addition, comparisons of protection in immunized chickens between rCp39 and rOmpH were also made. The log ELISA titers were calculated from the highest dilution of the number that more than the cut-off value as described above. Mean and standard deviation of the titers were calculated and are shown in Figs. 6 and 7. The statistical functions used in this study were calculated with the Microsoft® Office Excel 2003 (Microsoft® Office Professional Edition 2003, Microsoft Corporation).

Results

Expression and purification of recombinant proteins

In transformation of the recombinant pQE-cp39 or pQE-ompH, which contained the *cp39* or *omp*H gene for the mature protein without the signal peptide, a large number of transformants on selective LB plate were grown. One colony of each was chosen and designated *E. coli* strains

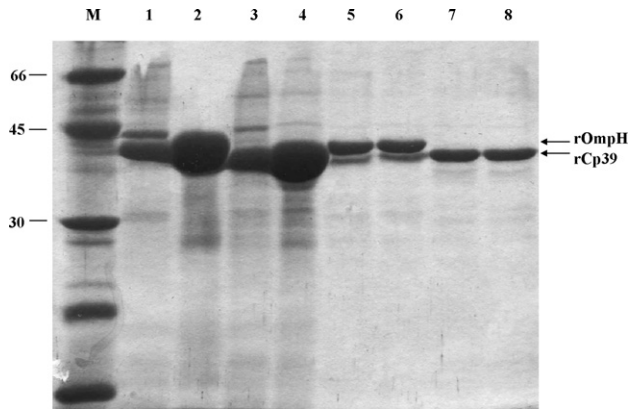

Figure 1 SDS-PAGE of rCp39 of strain P-1059 and rOmpH of strain X-73 on a 12.5% gel stained with Coomassie blue. Samples on lane numbers 1 and 3 were eluted by buffer D (pH 5.9), and numbers 2 and 4 were eluted by buffer E (pH 4.9) of the denatured condition. While, samples on lane numbers 5—8 were eluted by the elution buffer (pH 8.0) of the native condition under a modified hybrid condition. Lanes: M, molecular mass standards; lanes 1—2, 5—6, purified rOmpH; lanes 3—4, 7—8, purified rCp39. Arrow indicates the position of rCp39 or rOmpH. Numbers on the left indicate the positions of molecular mass standards (in kDa).

PQE-cp39 or PQE-ompH to express the recombinant protein. The recombinant proteins were 6 × His-tagged fusion protein which assist the recombinant protein bound the Ni-NTA resin. A hybrid condition of affinity chromatography was successfully demonstrated and the recombinant proteins rCp39 or rOmpH from *E. coli* strains PQE-cp39 or PQE-ompH, respectively, were purified by the chromatography. Molecular mass of recombinant proteins purified by a hybrid condition was identical to the denatured condition (Fig. 1).

Protectivities

Chickens of each group were immunized with 100, 200 or 400 μg proteins of native or recombinant proteins (Tables 2 and 3). Complete homologous protections of chickens from fowl cholera were obtained by vaccination with 100, 200 or 400 μg proteins of Cp39, or 100 μg proteins of CCE of strain P-1059 (Table 2). Similarly, complete homologous protections were also obtained in rCp39-immunized chickens. Fisher's exact test analysis indicated no significant difference in protection conferred by each rCp39 dosage ($P < 0.05$). Also, there was no significant difference in protection conferred between rCp39 and Cp39 ($P < 0.05$) whichever protein concentrations were used. Similarly, there was no significant difference in protection conferred by the CCE and rCp39 or Cp39 at any dosage ($P < 0.05$). Additionally, 60—80% protections from heterologous challenge-exposure were obtained. Fisher's exact test also indicated no significant difference ($P < 0.05$) in protection of chickens from homologous or heterologous challenge-exposure.

Almost complete protections conferred by rOmpH and its native immunogens of strain X-73 was also obtained (Table 3). Fisher's exact test indicated no significant difference in protection ($P < 0.05$) conferred by rOmpH, OmpH and CCE at any dosage against homologous and heterologous challenge-exposure. Additionally, protection of chickens challenge-exposed with strains X-73 or P-1059 resulted no significant difference in protection ($P < 0.05$) conferred by rCp39 or rOmpH at any dosage. No survivor was observed in the non-vaccinated control chickens exposed with both strains.

Clinical signs, gross lesions and bacterial isolation

Chickens of the non-immunized control groups started to show the peracute clinical signs, such as depression, inappetite, severe diarrhea at 6—8 h after exposure. Then, at 12 h after exposure chickens were found dead and all of the chickens in this group died within 24 h after exposure.

Chickens of immunized groups rarely showed the acute or peracute clinical sign. In the case of death, chickens died at 3rd day after challenge-exposure with strain X-73 while at the 5th day with strain P-1059. Clinical signs started as mild depression, anorexia and occasional diarrhea. Moreover, immunized-chickens challenge-exposed with strain X-73 showed severer clinical signs than with strain P-1059.

At the necropsies, the carcasses showed the typical gross lesions of fowl cholera, e.g. multiple necrotic foci at liver and/or spleen, lung congestion and edema, multiple petechiae in liver, hemorrhage in the small intestines and splenomegaly. *P. multocida* was recovered in pure culture from the specimens of all the dead chickens.

Determination of antibody response

The SDS-PAGEs of Cp39, rCp39 and whole-cell lysate of strain P-1059, and OmpH, rOmpH and whole-cell lysate of strain X-73 were analyzed before the Western blot analysis employing eight kinds of chicken antisera (Figs. 2—5). The results showed that protein or whole-cell lysate (boiled or incubated at 37 °C) were immunostained with chicken antisera against hybrid condition-based recombinant proteins (Fig. 2) and also with the control sera; chicken sera against P-1059 or X-73 bacterins (Fig. 4), chicken sera against Cp39 or OmpH (Fig. 5). In contrast, whole-cell lysate of strain P-1059 or X-73 incubated at 37 °C was not immunostained with the chicken antisera against denatured condition-based rCp39 or rOmpH (Fig. 3).

The ELISA titers are shown in Figs. 6 and 7. High titers of chicken sera were observed after immunization with rCp39 or rOmpH. There was no significant difference ($P < 0.05$) between the titers observed for two recombinant proteins at any dosages. No reactions were observed in the preimmune sera from all immunized groups with any coated antigens. In contrast, ELISA titers in the sera of chickens immunized with the denatured condition-based rCp39 or rOmpH showed very low titer against whole-cell of strains P-1059 or X-73.

Discussion

The Cp39, a cross-protective antigen of avian capsular serogroup A strains, is an interested protein and a can-

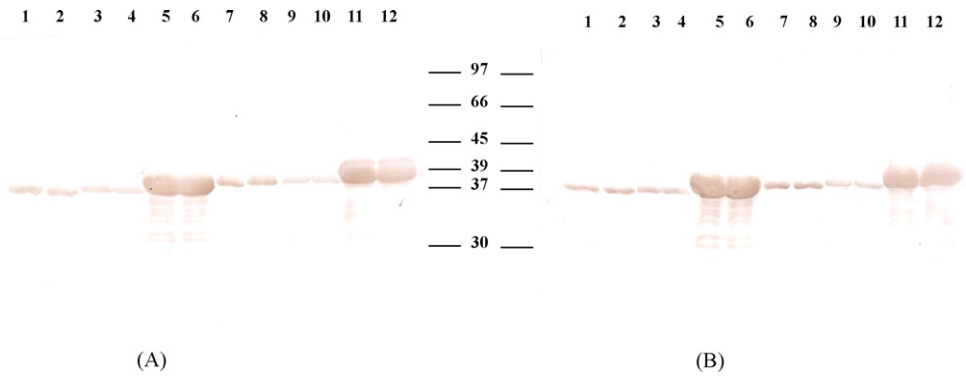

Figure 2 Western blot analysis of whole-cell, Cp39, rCp39 of strain P-1059 and whole-cell, OmpH, rOmpH of strain X-73 probed with (A) chicken antisera against hybrid condition-based rCp39 or (B) chicken antisera against hybrid condition-based rOmpH. Lane 1, boiled Cp39; 2, Cp39 incubated at 37°C; 3, whole-cell P-1059 incubated at 37°C; 4, boiled whole-cell P-1059; 5, boiled purified hybrid condition-based rCp39; 6, purified hybrid condition-based rCp39 incubated at 37°C; 7, boiled OmpH; 8, OmpH incubated at 37°C; 9, whole-cell X-73 incubated at 37°C; 10, boiled whole-cell X-73; 11, boiled purified hybrid condition-based rOmpH; 12, purified hybrid condition-based rOmpH incubated at 37°C. Numbers in the center indicate the positions of molecular mass standards (in kDa).

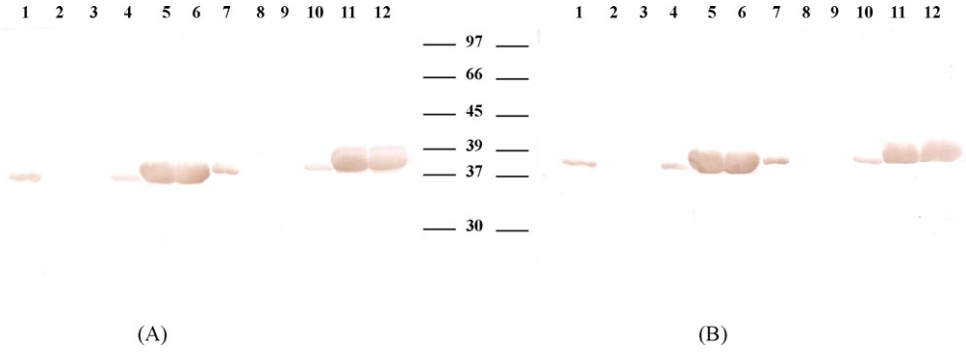

Figure 3 Western blot analysis of whole-cell, Cp39, rCp39 of strain P-1059 and whole-cell, OmpH, rOmpH of strain X-73 probed with (A) chicken antisera against denatured condition-based rCp39 or (B) chicken antisera against denatured condition-based rOmpH. Lane 1, boiled Cp39; 2, Cp39 incubated at 37°C; 3, whole-cell P-1059 incubated at 37°C; 4, boiled whole-cell P-1059; 5, boiled purified denatured condition-based rCp39; 6, purified denatured condition-based rCp39 incubated at 37°C; 7, boiled OmpH; 8, OmpH incubated at 37°C; 9, whole-cell X-73 incubated at 37°C; 10, boiled whole-cell X-73; 11, boiled purified denatured condition-based rOmpH; 12, purified denatured condition-based rOmpH incubated at 37°C. Numbers in the center indicate the positions of molecular mass standards (in kDa).

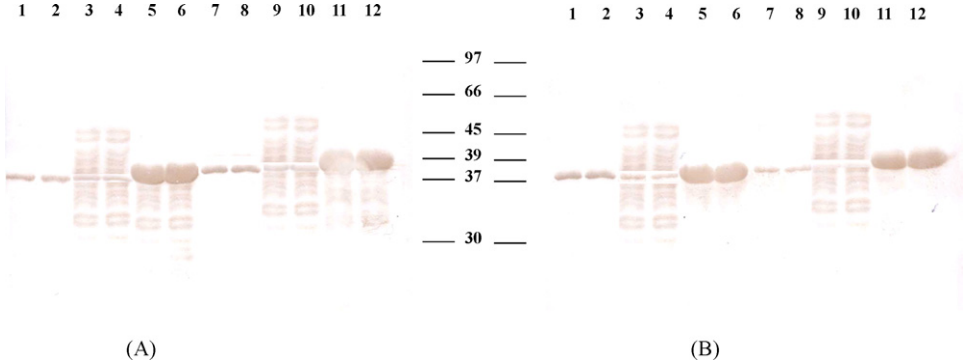

Figure 4 Western blot analysis of whole-cell, Cp39, rCp39 of strain P-1059 and whole-cell, OmpH, rOmpH of strain X-73 probed with (A) chicken antisera against P-1059 bacterin or (B) chicken antisera against X-73 bacterin. Lane 1, boiled Cp39; 2, Cp39 incubated at 37°C; 3, whole-cell P-1059 incubated at 37°C; 4, boiled whole-cell P-1059; 5, boiled purified hybrid condition-based rCp39; 6, purified hybrid condition-based rCp39 incubated at 37°C; 7, boiled OmpH; 8, OmpH incubated at 37°C; 9, whole-cell X-73 incubated at 37°C; 10, boiled whole-cell X-73; 11, boiled purified hybrid condition-based rOmpH; 12, purified hybrid condition-based rOmpH incubated at 37°C. Numbers in the center indicate the positions of molecular mass standards (in kDa).

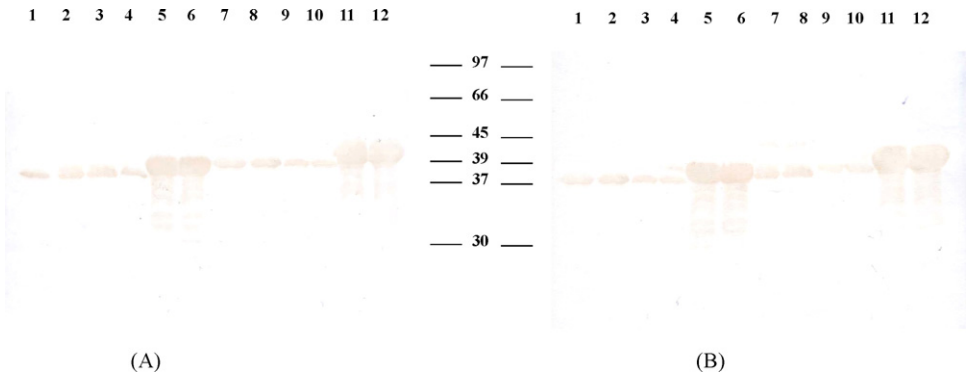

Figure 5 Western blot analysis of whole-cell, Cp39, rCp39 of strain P-1059 and whole-cell, OmpH, rOmpH of strain X-73 probed with (A) chicken antisera against Cp39 or (B) chicken antisera against OmpH. Lane 1, boiled Cp39; 2, Cp39 incubated at 37 °C; 3, whole-cell P-1059 incubated at 37 °C; 4, boiled whole-cell P-1059; 5, boiled purified hybrid condition-based rCp39; 6, purified hybrid condition-based rCp39 incubated at 37 °C; 7, boiled OmpH; 8, OmpH incubated at 37 °C; 9, whole-cell X-73 incubated at 37 °C; 10, boiled whole-cell X-73; 11, boiled purified hybrid condition-based rOmpH; 12, purified hybrid condition-based rOmpH incubated at 37 °C. Numbers in the center indicate the positions of molecular mass standards (in kDa).

didate of future fowl cholera vaccine [9]. Gene encoding the Cp39 is identical to the major outer membrane protein (MOMP) of *P. multocida* strain P-1059 and also suggested that OmpH may be origin of Cp39 and may filtrate into capsule from outer membrane, and work as an adherence factor inducing higher pathogenicity of the encapsulated *P. multocida* (Borrathybay E., unpublished data). Ali et al. [9] suggested that the Cp39 is a capsule-associated protein by the immunoelectron microscopy employing monoclonal antibody recognized 39 kDa protein (Cp39 in this paper). In addition, the MOMP of *P. multocida* is also a cross-protective antigen [12,19,21,24,25]. The immunogenicity of MOMPs among *P. multocida* have been clarified and recombinant MOMPs were also prepared and performed as an immunogenic antigen in various hosts including natural hosts [7,18—23]. Recently, an outer membrane-associated protein with a similar molecular mass of approximately 39 kDa was demonstrated and supposed to be one of the cross-protective antigens [24,25]. The 39 kDa cross-protective factor protein of *P. multocida* membrane proteome was also identified and suggested that this antigen was *Pasteurella* lipoprotein B or PlpB [25].

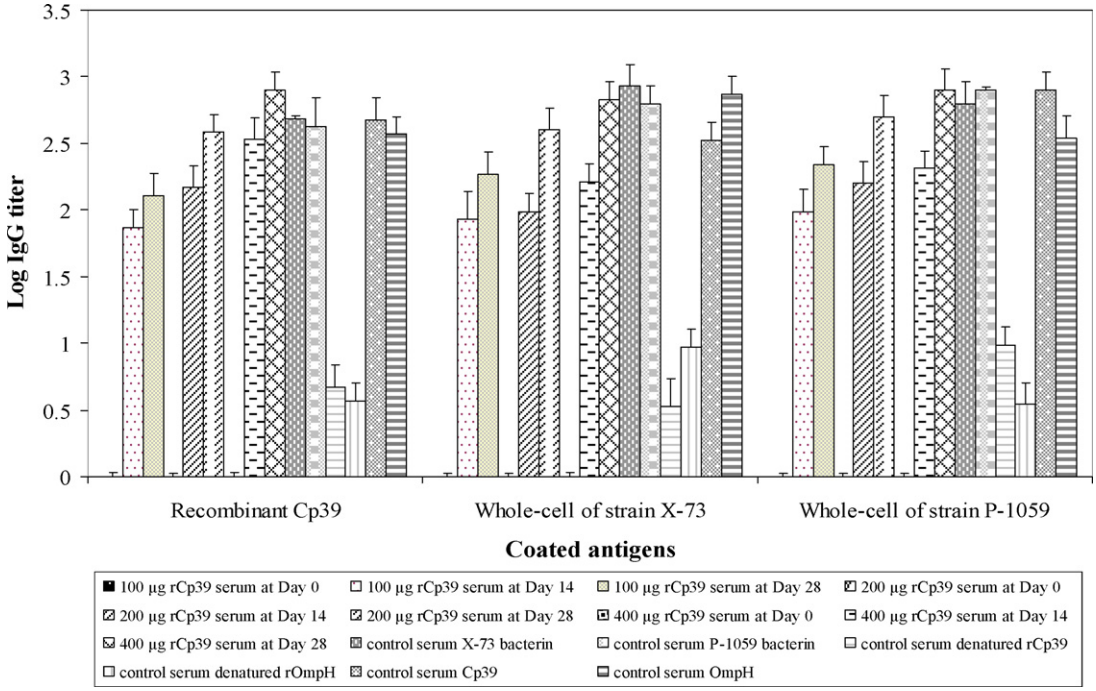

Figure 6 Antibody responses in the sera of chicken immunized with rCp39. ELISA plates were coated with 10 µg protein of rCp39 or whole-cell of *P. multocida* strain P-1059 or X-73. The chicken sera against *P. multocida* strain P-1059 or X-73 bacterins or chicken sera against denatured condition-based rCp39 or rOmpH were used as the control sera. The data are presented as the log mean endpoint titers and the bars indicate standard error of the means.

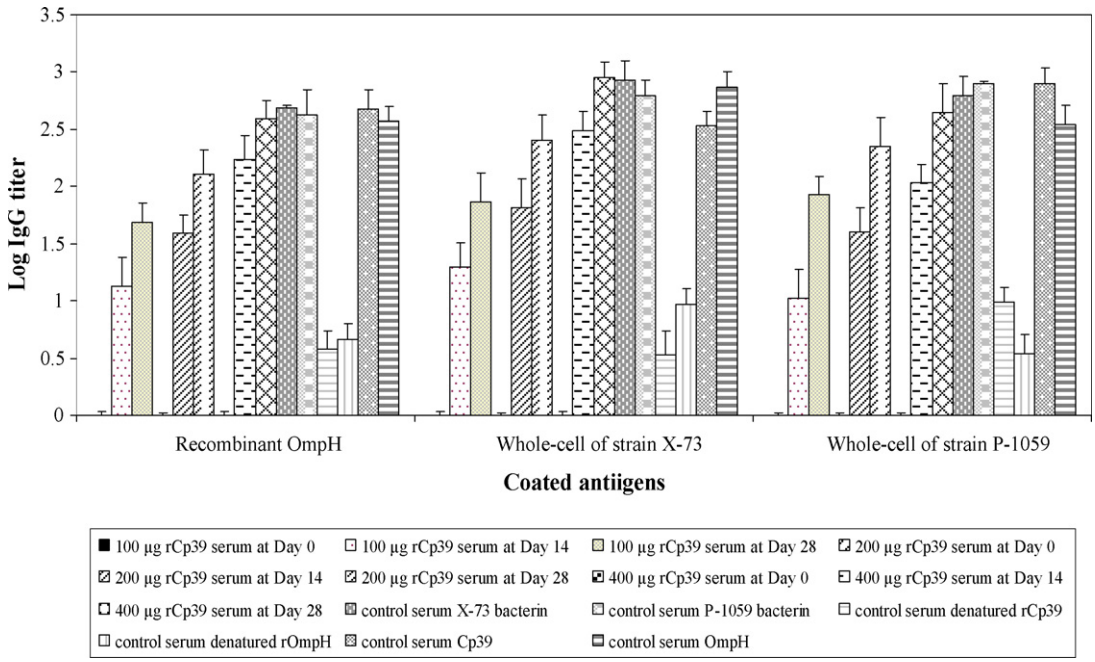

Figure 7 Antibody responses in the sera of chicken immunized with rOmpH. ELISA plates were coated with 10 μg protein of rOmpH or whole-cell of *P. multocida* strain P-1059 or X-73. The chicken sera against *P. multocida* strain P-1059 or X-73 bacterins or chicken sera against denatured condition-based rCp39 or rOmpH were used as the control sera. The data are presented as the log mean endpoint titers and the bars indicate standard error of the means.

In our previous study, the rabbit antisera against Cp39, rCp39 and OmpH or rOmpH of strain X-73 [12] had been prepared and used for the Western blot analysis and adhesion assay (Borrathybay E., unpublished data). Western blot analysis demonstrated that the OmpH or rOmpH were strongly immunostained with the rabbit antiserum against Cp39 or rCp39. Additionally, Borrathybay E. (unpublished data) suggested that the rCp39 was confirmed as a recombinant adhesive protein and a cross-reactive recombinant antigen among *P. multocida* capsular serogroup A strains. Our preliminary protection assays in mice by vaccination with Cp39 or OmpH have been performed and the results showed that the efficient protections against challenge-exposure with *P. multocida* strains (data not shown). In contrast, low or no protection was observed in mice immunized with any dosage of the denatured condition of the affinity chromatography-based rCp39 or rOmpH ($P < 0.05$). The Western blot and ELISA analyses of mice sera in that preliminary experiments indicated that mice antibody induced by the denatured form of recombinant protein could not react with the whole-cell of both strains. Moreover, whole-cell of both strains incubated at 37 °C were not immunostained with the control chicken sera against denatured condition-based rCp39 or rOmpH and the ELISA also showed the low antibody response to the denatured condition-based recombinant protein (Figs. 6 and 7). Therefore, the results of our preliminary experiments coupled with the present results suggested that the immunogenicity of recombinant protein have been changed during purification of the protein. In the present investigation, the purifying condition to protect immunogenicity of recombinant protein was demonstrated as suggested from the previous protection assay in mice. Purification of recombinant protein by the affinity chromatography was modified into a hybrid condition. The expressed recombinant protein in *E. coli* may aggregate and form insoluble inclusion bodies within bacterial cell. The inclusion bodies were resolved by the strong detergent (urea) under the denatured condition and imidazole was used to elute recombinant protein from Ni-NTA under the native condition. Among the entire eluents, imidazole is a mildest eluent for elution than EDTA or pH-based eluent (The QIAexpressionist, QIAGEN). The instruction manual suggested that protein would be damaged by the reduction in pH or when the presence of metal ions in the eluate and may have an adverse effect on the purified protein. This suggestion may explain the low or no protection in mice in our previous protection assay and also in the previous study [12].

The present investigation tried to clarify the protection conferred by immunization with rCp39 in the natural host chickens. The protection against challenge-exposure with parental strain P-1059 and heterologous strain X-73 was obtained by two inoculations of 100, 200 or 400 μg of rCp39 proteins in chickens. Likewise, high ELISA titers were induced by rCp39 or rOmpH and a cross-reaction was shown between strains P-1059 and X-73 (Figs. 6 and 7). Fisher's exact test indicated that there was no significant difference ($P < 0.05$) in efficacy of the rCp39 and the native immunogens (Cp39, CCE) at any dosages. These results supported that vaccines prepared from the bacterial OMP were able to induce the sufficient protection against parental strain [18,21,23,26,27]. Moreover, the present rCp39 was also able to induce the cross-protection against heterologous challenge-exposure. However, low mortalities were observed in chickens immunized with recombinant proteins against heterologous challenge-exposures. This might be

due to higher virulence of strain X-73 and difference in somatic antigenicity of the two strains.

Protection induced by immunization with recombinant protein in the present study is interesting. Luo et al. [12] demonstrated that immunization of purified native OmpH was able to confer the protection of chickens from fowl cholera by challenge-exposure with parental strain X-73 but the recombinant OmpH of strain X-73 induced little protection against challenge-exposure of parental strain. Western blotting and ELISA indicated that the undenatured OmpH of strain X-73 could not induce the antibody against native OmpH of strain X-73 [12]. While, in the present investigation, the protection conferred by native or recombinant OmpH in chickens was observed even though challenge-exposure with high bacterial numbers of homologous or heterologous strain after purification condition was modified. Western blotting showed that the hybrid condition-based recombinant proteins were undenatured protein and the induced antibodies were able to react to the whole-cell lysate of both strains (Fig. 2) while the denatured condition-based recombinant proteins were not (Fig. 3). ELISA have been performed and showed the high antibody titers against native conformational epitopes of Cp39 or OmpH in the whole-cell lysate of both strain. These indicated that antisera against rCp39 or rOmpH were expressed *in vivo* and a hybrid condition was able to protect the immunogenicity of protein. Interestingly, these observations correlated to the previous investigation that protective immunity was obtained by the immunization with the undenatured form of other porin protein [21]. However, the efficacy of this modified method to protect the immunogenicity and the solubility of the recombinant protein need to be determined.

The protein modification in host and the host response mechanism are interesting and these may affect upon the protection. Recently, Boyce et al. [28] described how the expression of OMP of *P. multocida* changed during infection in the hosts. A total of 35 proteins of OMP were identified from host samples. Analysis of the proteins concluded that the OMP will modify itself during infection in host in order to function. Then, host response and modification of the OMP during infection affect upon protection *in vivo* [28].

In conclusions, the homologous and heterologous protections were conferred by the recombinant adhesive protein (rCp39) of *P. multocida* strain P-1059 in chickens. A modified hybrid condition of affinity chromatography successfully protected immunogenicity of the recombinant protein. The Fisher's exact test indicated no significant differences ($P < 0.05$) in protections among vaccine types and dosages. Therefore, the rCp39, an emerging candidate recombinant vaccine, was able to induce a sufficient immunity for fowl cholera in the natural host chickens.

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