Signal sequence contributes to the immunogenicity of *Pasteurella multocida* lipoprotein E

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ABSTRACT Recombinant *Pasterurella multocida* lipoprotein E (**PlpE**) has been shown to protect against fowl cholera. This study aimed to determine if the signal sequence may contribute to the antigenicity and protective efficacy of recombinant PlpE. A small antigenic domain of PlpE (termed truncated PlpE, **tPlpE**) was constructed with (**SP-tPlpE**) or without (tPlpE) the signal sequence and evaluated in vitro and in vivo. In vitro, the HEK-Bule hTLR2 Cells were used to evaluate the activation of NF-kB in the test associated with the stimulation of the SP-tPlpE and tPlpE proteins. When chickens were immunized, compared to the tPlpE vaccine group, the SP-tPlpE group showed higher antibody levels and enhanced $CD4^+$ T cell response. In a challenge test, the SP-tPlpE group showed a survival rate of 87.5% (n = 8), compared to 25% for the tPlpE group. It is confirmed that the inclusion of the native signal sequence enhanced protective efficacy against fowl cholera and may act as a vaccine adjuvant. The short SP-tPlpE construct is amenable to further vaccine engineering and has potential to be developed as a fowl cholera vaccine.

Key words: signal sequence, lipid moiety, lipoprotein E, fowl cholera, subunit vaccine

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INTRODUCTION

Bacterial lipoprotein with lipid modifications is a potent protein-based adjuvant to activate immune response through Toll-like receptor 2 (**TLR2**). Previous studies have shown that bacterial lipoproteins can enhance cellular and humoral immune responses (Huang et al., 2012; Huang et al., 2015; Hirschfeld et al., 1999). In actual application, a commercial vaccine against Lyme disease (licensed by the US Food and Drug Administration (**FDA**) in February 2002) was manufactured from 30 μg of recombinant lipoprotein *Borrelia burgdorfieri* (***B. burgdorferi***) outer surface protein A (**OspA**) expressed in *Escherichia coli* (***E. coli***). This study indicated that the full-length OspA gene expression using *E. coli* resulted in a lipidated OspA recombinant protein (attached lipid moiety, Pam3Cys). On the other hand, a constructed plasmid that lacked the OspA signal sequence was made to generate a nonlipidated OspA. When mice were immunized, the lipidated OspA induced a robust antibody and protected mice against *B. burgdorferi*. In contrast, immunized mice with nonlipidated OspA failed to protect mice against the challenge test (Erdile et al., 1993; Häupl et al., 1997). Based on this property, further study is needed to analyze and apply bacterial lipoproteins as a potent antigen for subunit vaccine development.

P. multocida PlpE is a potential protein-based antigen against fowl cholera. The disease is commonly caused by *P. multocida* serotype A, resulting in huge economic losses to poultry industries worldwide (Carpenter, 2003; Singh et al., 2014). Vaccination plays a central part in fowl cholera control, but limitations of current vaccines remain. Inactivated *P. multocida* vaccines are widely used and offer certain levels of protection against homologous strains, but provide poor protection against heterologous challenges. On the other hand, live-attenuated but are less frequently used because of considerable reversion to virulence (Bierer and Derieux, 1972; Adler et al., 1999; OIE, 2015). For subunit vaccine development, recombinant *P. multocida* PlpE was shown to

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provide certain levels of heterologous protection (60% survival) and 100% homologous protection (Wu et al., 2007), demonstrating that PlpE is a promising potential antigen for fowl cholera vaccine.

To further improve the cross-protection and protein production yield of recombinant PlpE, we seek to identify domains within PlpE critical for protection and to eliminate sequences not essential for immunity. Sequence analysis of PlpE (335 residues, from *P. multocida* serotype A3, GenBank: EF219455.1) revealed a segment (from 26-Gly to 86-Thr) of high hydrophilicity that may be surface-exposed and therefore immunogenic (Ayalew et al., 2004; Singh et al., 2010; Mostaan et al., 2020). This segment of PlpE is also highly conserved (≥93% similarity, using the Clustal W method in MegAlign of the DNASTAR software, Madison, WI, USA) among the prevalent *P. multocida* serotypes: A1(EF219452.1), A3 (EF219455.1), and A4 (EF219456.1), showing potential for cross-protection (Kumar et al., 2004; Shivachandra et al., 2006). By choosing this hydrophilic segment as a vaccine antigen, we are also eliminating other portions of PlpE that may hinder protein expression and folding; for example, the segment from 212-Thr to 330-Lys is predicted (using the Phyre2 server) to be a transmembrane β-barrel structure, which may be insoluble in solution.

In order to design a more compact recombinant PlpE, the inclusion of the signal sequence is necessary. Since the signal sequence of PlpE contains a consensus lipoprotein processing site (**Lipobox**): Leu-X-Ala-Cys (where X is a small, nonpolar amino acid) (using the SignalP 5.0 server) (von Heijne, 1989; Juncker et al., 2003). The lipobox contains a conserved cysteine residue at its C-terminus that functions as the attached lipid point. Moreover, the presence of the Lipobox of gram-negative *P. multocida* is expected to let the recombinant PlpE protein export to the surface of the outer membrane, which plays a major role in protein folding and stabilization (Tokuda, 2009; Kovacs-Simon et al., 2011; Singh et al., 2013). Because of these modifications (outer membrane localization and lipidation), the negative-bacterial lipoproteins are identified as foreign by the host, resulting in activation of innate immune response via TLR2 subfamily (TLR1, 2, and 6) (Hirschfeld et al., 1999; Kang et al., 2009; Halliday et al., 2016). After immunization, it is found that the humoral and cellular responses were enhanced with the recombinant bacterial lipoproteins (Kramer et al., 1996; Kiura et al., 2006; Huang et al., 2012; Leng et al., 2015). Therefore, we attempted to engineer the N-terminal lipoprotein, which contains the lipobox and the conserved PlpE as a protein-based fowl cholera vaccine.

This study seeks to determine if the signal sequence may contribute to the antigenicity and protective efficacy of recombinant PlpE. To evaluate the effect of the signal sequence, the identified hydrophilic segment, tPlpE, was constructed with and without the signal sequence for comparison purposes. The designed tPlpE without the signal sequence may be helpful to infer a better solution to understand the mechanism of the antigenicity of the lipoprotein. In vitro, the HEK-Blue hTLR2 Cells were used to examine TLR2 activation. After immunization, the antibody and cell-mediated immunity elicited by the two constructs were analyzed. Finally, challenge tests in chickens were performed to evaluate protective efficacy.

MATERIALS AND METHODS

P. Multocida *A Strains*

A *P. multocida* strain, P-1059 (ATCC 15742, serotype A3), was obtained from the Bio-resource Collection and Research Center, Taiwan, for PlpE cloning. A virulent *P. multocida* A field strain was used for the challenge study, named **Chu01**. The Chu01 strain was confirmed with PCR using primers of the hyaD-hyaC gene (Townsend et al., 2001). The Brain Heart Infusion **(BHI)** broth medium was used to culture the *P. multocida* strains at 37°C for 24 h.

Recombinant PlpE Protein Production and Vaccine Formulation

Recombinant tPlpE was constructed with (SP-tPlpE) or without (tPlpE) signal peptide and formulated as vaccines. First, using Polymerase Chain Reaction (**PCR**), the full PlpE gene was cloned from the genomic DNA of P-1059 using primers listed in Table 1 and inserted into the plasmid vector pET32a (Novagen, Germany). Subsequently, primers were used for PCR to obtain SP-tPlpE and tPlpE gene fragments from the full PlpE template and gene products were inserted into pET32a. The resulting plasmid constructs were propagated in DH5α, and sequencing was performed to verify the accuracy of the inserts.

For recombinant protein expression, *Escherichia coli* BL21 (**DE3**) (Yeastern Biotech, Taiwan) harboring the constructed plasmid were cultured in Luria-Bertani

Table 1. Primers for gene cloning and recombinant protein construction.

Target gene		Sequence (5'-3')	RE site	Length (bp)	DNA templates
PlpE	F	*ggatcc*atgaaacaaatcgttttaaaaac	BamHI	1,009	ATCC 15742
	R	*gaattc*ttattgtgcttggtgacttttttc	EcoRI		
SP-tPlpE	F	*ggatcc*atgaaacaaatcgttttaaaaac	BamHI	258	PlpE
	R	*ctcgag*aaaggaggattgttgactattt	XhoI		
tPlpE	F	*ggatcc*ggtagcgctggaaatcg	BamHI	189	PlpE
	R	*ctcgag*aaaggaggattgttgactattt	XhoI		

Italics and underlines in the primers represent restriction enzyme (RE) sites.

(**LB**) medium at 37°C until the absorbance reached 0.6 to 0.8 at 610 nm. After that, the protein expression was induced by adding isopropyl-b-D-galactopyranoside (**IPTG**; Sigma, Germany) to a final concentration of 1 mM and the culture was grown for 4 h.

To quantify the expression levels of the recombinant proteins, the induced bacterial was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**) using Bovine Serum Albumin (**BSA**) protein standards. Western blot assay was performed using antibody His-tag as we reported previously (Doan et al., 2020). The recombinant proteins were screened as a soluble fraction and prepared for purification. The induced cells were harvested, lysed in native lysis buffer (300 mM KCl, 50 mM KH_2PO_4 and 5 mM Imidazole) and sonicated at 40% amplitude for 15 s. on/off. The soluble fraction was used for protein purification using Bioscale Mini Profinity (**IMAC**) cartridges 1mL (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The purified SP-tPlpE and tPlpE proteins were quantified independently using BSA standard.

Three vaccines were formulated with 50 μg/dose of the recombinant proteins: 1) SP-tPlpE +ISA71, 2) tPlpE +ISA71, and 3) PBS+ ISA71 as negative control. The water-in-oil adjuvant Montanide ISA71 (Seppic, France) was formulated with recombinant protein in a 4:6 (aqueous: oil) ratio for a final injection volume of 0.2 mL per chicken.

Evaluation of the SP-tPlpE and tPlpE to Activate TLR2

The HEK-Blue-hTLR2 Cells are genetically modified cell lines that can be stimulated through TLR2. In this study, the HEK-Blue-hTLR2 Cell lines (Catalog code. Lot C09-0801) were purchased from InvivoGen (San Diego, CA) and cultured following the manufacturer's instructions. Briefly, the reporter cells ($3\text{-}7 \times 10^6$) were recovered in a T75 flask at 37°C incubator with a humidified atmosphere of 5% CO_2. The cells were cultured in Dulbecco's modified Eagle's medium (**DMEM**, Sigma-Aldrich, MI, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Waltham, MA). The cells were gently washed twice with 5 mL PBS (37 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) when it reached 80 to 90% confluence and subcultured at a ratio of 1:3.

To test the involvement of TLR2 in SP-tPlpE and tPlpE-induced activity, the cell-based colorimetric assay was carried out using HEK-Blue Detection Kit. Briefly, the cells were counted to the final amount of 36,000 cells in 180 μL of resuspended HEK-Blue Detection (Catalog code: hb-det2) in each well of a flat-bottom 96-well plate. After that, 20 μL of each stimulator was added to corresponding wells. A 20 μL recombinant protein of SP-tPlpE and tPlpE (12.5 μg/mL each) were added to the plate. The inactivated JM109 *E. coli* component cells (activation at 56°C for 1.5 h, at a final concentration of 2,000 CFU) were used as a positive control. Negative controls included PBS mock stimulation and native elution buffer-only. The plate was incubated at 37°C and 5% CO_2 for 16 h. After incubation, the secreted alkaline phosphatase (**SEAP**) activity was determined using the HEK-Blue Detection Kit. The absorbance was measured at 625 nm using the Multiskan FC Microplate Photometer (ThermoFisher Scientific, Finland). Statistical significance between the SP-tPlpE and tPlpE proteins was determined using Student's t test, and only $P < 0.05$ was considered significant.

Animals, Immunization and Challenge Study

In this study, 24 five-wk-old brown Leghorn chickens were obtained from a local farm (Pingtung, Taiwan) and randomly assigned to 3 groups (n = 8). Before the experiments, all chickens had been screened and found healthy and free of *P. multocida*-specific antibodies.

The chickens were immunized twice subcutaneously with the formulated vaccines as follows 1) 50 μg SP-tPlpE + ISA71, 2) 50 μg tPlpE + ISA71, and 3) PBS + ISA71. All vaccine dosages have a final volume of 0.2 mL. At d 28 post-immunization, all the chickens were challenged with 3.2×10^5 CFU (**20 LD_{50}**) Chu01 via the intramuscular route with final volume of 0.1 mL per chicken. After the challenge test, the chickens were recorded for 14 d. Identified chickens with late-stage clinical signs of fowl cholera (depression, ruffled feathers, anorexia, diarrhea, increased respiratory rate, and head hanging) were euthanized using CO_2 asphyxiation. The survival rate was recorded. Additionally, the livers of chickens challenged with *P. multocida* at late-stage acute were dissected and observed for fowl cholera lesions. Positive *P. multocida*-infected liver samples were re-confirmed with PCR using primers for the hyaD-hyaC gene (Townsend et al., 2001). The experiment protocols were approved by the Animal Care and Use Committee, National Pingtung University of Science and Technology (**NPUST**-106-055).

Analysis of Antibody Response

To determine the antibody response elicited by the vaccines, indirect enzyme-linked immunosorbent assay (**ELISA**) was performed from immunized chicken's serum. The 96-well plates were coated with 50 ng/100 μL/well of purified full PlpE in the coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, Ph = 9.6) overnight at 4°C. The plates were washed with PBS containing 0.5% Tween-20 (**PBS-T**) four times and blocked by 5% skim milk in PBS at 37°C for 1 h. After blocking and washing, serial-diluted chicken serum was added into the wells (50 μL/well) and incubated at 37°C for 1.5 h as a primary antibody. After that, the plates were washed 4 times with PBS-T, and horseradish peroxidase (**HRP**)-conjugated anti-chicken IgG (Sigma-Aldrich, MI, USA) at 1:5,000 dilution was used as a secondary antibody at 100 μL/well. The plates were incubated at 37°C for 1 h, washed four times with PBS-T, and 100 μL 3,3′5,5′-Tetramethylbenzidine

(**TMB**) (KPL, Gaithersburg, MD) was used for color development. The reaction was stopped by adding 100 μL of TMB stop solution (KPL) after 15 min and optical density was read at 450 nm on the Multiskan FC Microplate Photometer (ThermoFisher Scientific, Vantaa, Finland).

Analysis of T Cell Population Expansion

To examine cellular immune response after vaccination, the percentages of $CD4^+$ and $CD8^+$ T cells in the peripheral blood mononuclear cells (**PBMCs**) of immunized chickens on d 1, 14, and 28 post-vaccination were analyzed by flow cytometry. For PBMC isolation, whole blood was collected in tubes containing disodium ethylene-diaminetetraacetic acid (**EDTA**). The blood cells were resuspended in equal volumes of PBS. The resuspended blood cells were then slowly layered on Ficoll-Paque (Amersham Biosciences, Buckinghamshire, UK) through centrifugation at $252 \times g$ for 40 min. The PBMC-containing fraction was collected to the new tubes. The cells were then washed twice and resuspended in PBS. Each chicken, freshly prepared PBMCs (at a density of 10^6 cells/100 μL) were resuspended in PBS containing 1 μL of anti-CD4-PE or anti-CD8-FITC antibodies (Arigo, Hsinchu, Taiwan) for 45 min at 4°C. The isolated PBMCs from the control group were used as the negative control. After incubation, the labeled and control cells were gently washed twice with PBS and resuspended in 700 μL PBS. For all samples, a number of 50,000 cells were analyzed using the BD Accuri C6 Flow Cytometer (BD Biosciences, San Diego, CA).

Analysis of Cytokine Response

To evaluate the type of immune response elicited by the vaccines, Th1 and Th2 type cytokines of PBMCs from immunized chickens (d 28) were analyzed. Freshly prepared PBMCs were added to 24-well plates with the final concentration of 2×10^6 cells/well. The cells were then stimulated with 10 μg/mL PlpE for a 3-h incubation at 37°C, 5% CO_2. Total RNA was then isolated from the stimulated cells using the Total RNA Extraction Miniprep System (Viogene, Taipei, Taiwan). The complementary DNA (**cDNA**) was synthesized from 1 μg of total RNA using the Reverse Transcriptase Kit (Applied Biosystems, Foster, CA) according to the manufacturer's instructions. Real-time PCR was then carried out using the SYBR Green Supermix (Bio-Rad, Hercules, CA) in the CFX96 Touch Real-Time PCR system (Bio-Rad Laboratories Inc., Hercules, CA). Primers for the cytokines are shown in Table 2. The housekeeping gene glyceraldehyde-3-phosphate dehydro-genase (**GAPDH**) was used to compare gene expression. For data analysis, the relative expression levels of these immune-related genes were normalized to that of the GAPDH gene and expressed as n-fold increase or decrease relative to the control (adjuvant-only).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software version 22 (SPSS Inc, Chicago, IL). One-way analysis of variance (**ANOVA**) and Tukey's post hoc test were used to compare differences among vaccine groups. The significance level was defined as $P < 0.05$, and all data are expressed as mean ± standard error of the mean (**SEM**).

RESULTS

SP-tPlpE and tPlpE Expression Vectors Were Constructed

Recombinant tPlpE with (SP-tPlpE) or without (tPlpE) its native signal sequence was successfully cloned from a *P. multocida* A3 strain, P-1059, and expressed in *E. coli*. Hydrophilicity plot of PlpE is shown in Figure 1A. The SP-tPlpE gene was inserted into the expression vector pET32a (Figure 1B) and the native signal sequence containing the Lipobox is shown in Figure 1C.

Protein Analysis of Recombinant SP-tPlpE and tPlpE

After protein expression, SDS-PAGE analysis showed prominent bands at the predicted molecular weights of 31.5 and 28 kDa for SP-tPlpE and tPlpE, respectively (pET32a inserts a 20-kDa Trx-His-S-enterokinase tag at

Table 2. Primers for cytokine genes.

Target gene	Sequence (5'-3')	Length (bp)	Annealing temp. (°C)	GenBank
IFN-γ	F gacggtggacctattatt R ggctttgcgctggattc	255	50	HQ739082
IL-12	F ccaagacctggagcacaccgaag R gatccctggcctgcacagaga	163	61	AY262752.1
IL-4	F tgtgcccacgctgtgcttaca R cttgtggcagtgctggctctcc	193	61	AJ621249.1
IL-6	F caaggtgacggaggaggac R tggcgaggagggatttct	253	55	JQ897539
GAPDH	F tgctgcccagaacatcatcc R acggcaggtcaggtcaacaa	142	55	NM_204305

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; IFN-γ, interferon-gamma.

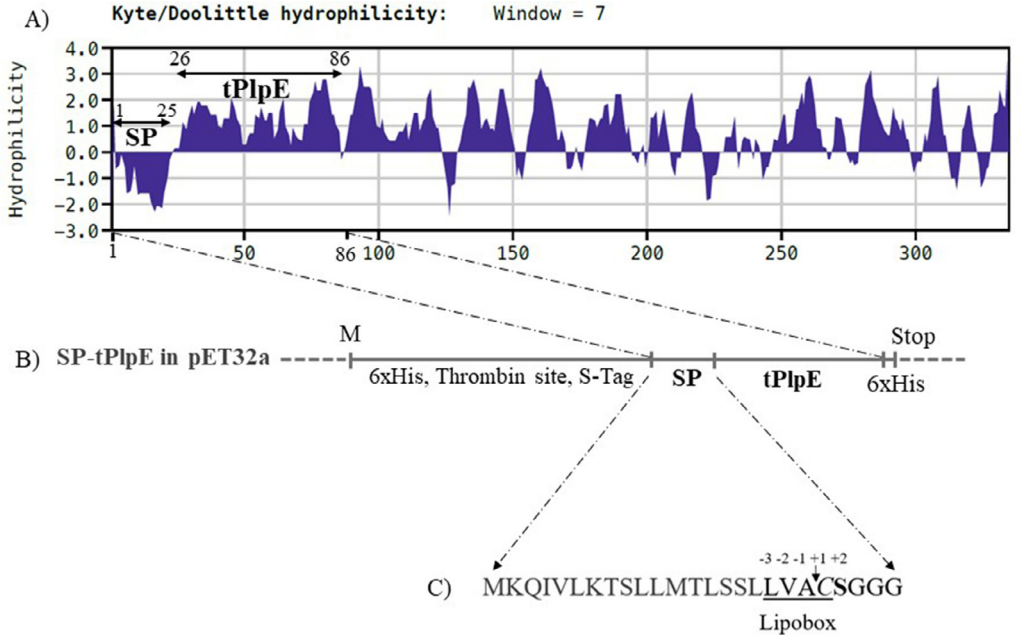


Figure 1. Hydrophilicity plot of PlpE and gene construction of SP-tPlpE. (A) Kyte-Doolittle plot of PlpE. (B) Schematics of the SP-tPlpE construct in plasmid vector pET32a. (C) Signal sequence of PlpE containing Lipobox.

the N-terminus, Figure 2A). Protein identity was confirmed with anti-His antibody using Western blotting (Figure 2B). When protein expression quantity was compared (Figure 2C), SP-tPlpE clearly showed a lower level compared to that of tPlpE, possibly as a result of the presence of the hydrophobic signal peptide. In terms of protein solubility, approximately 90% of both SP-tPlpE and tPlpE were expressed in a soluble form

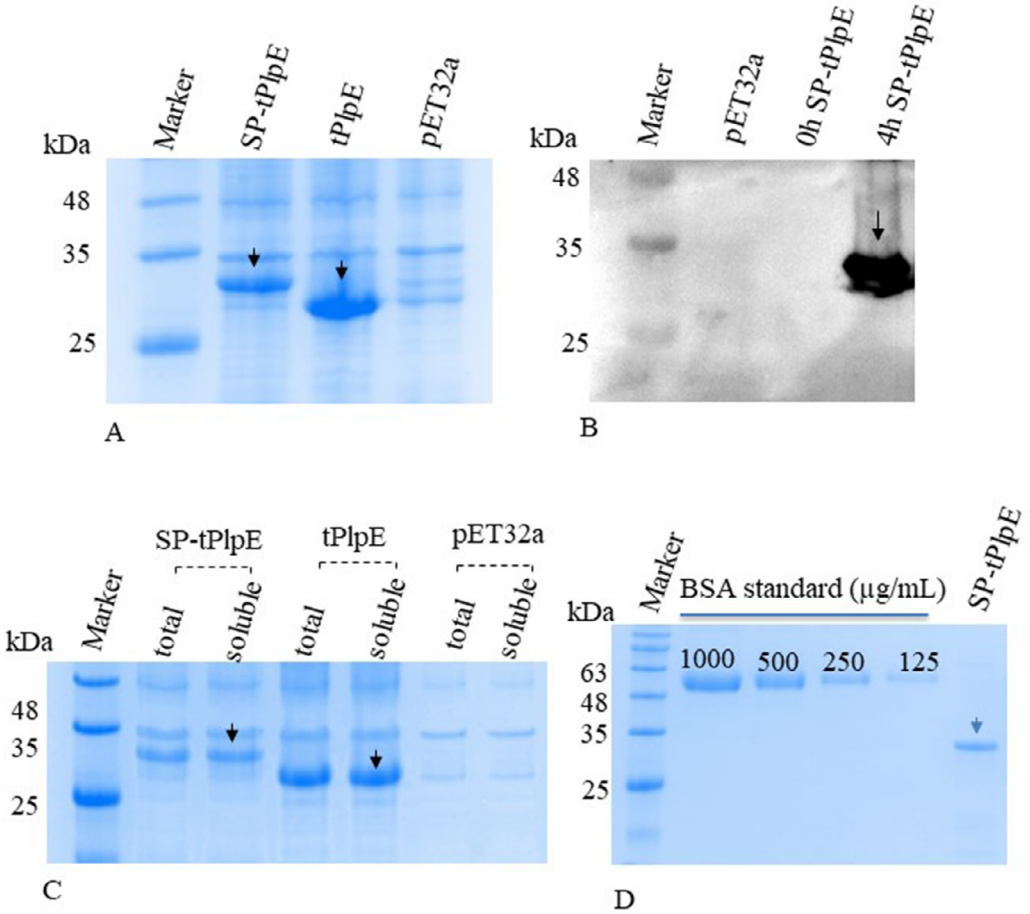


Figure 2. Protein analysis of recombinant SP-tPlpE and tPlpE. (A) SDS-PAGE analysis of the recombinant proteins. (B) Western blotting of SP-tPlpE 4h after induction. (C) Both SP-tPlpE and tPlpE recombinant proteins were expressed in a soluble form. (D) Protein quantitation using BSA protein standards, showing SP-tPlpE protein production at 289 mg/L after purification.

(Figure 2C). When the recombinant proteins were purified and quantitated (Figure 2D), the yield for SP-tPlpE was 289 mg/L (vs. 500 mg/L for tPlpE and 200 mg/mL for full-length PlpE, data not shown). Purified proteins were formulated as vaccines for immune response analysis and challenge study.

SP-tPlpE Activated TLR2 in HEK-Blue-hTLR2 Cells

The HEK-Blue hTLR2 Cells transfected with SEAP reporter genes were used to test whether the SP-tPlpE can activate the TLR2 signaling pathway. These cells were individually stimulated with SP-tPlpE and tPlpE for 16 h at 37°C, 5% CO_2. At the end of the incubation, the activation was measured using HEK-Blue Detection Kit. As shown in Figure 3, compared to the tPlpE construct, SP-tPlpE significantly increased SEAP secretion level. In contrast, a non-secreted SEAP level was observed in the control group. Therefore, the results suggested that the signal sequence of SP-tPlpE induced high immunogenicity by activating TLR2/NF-kB signaling in HEK-Blue-hTLR2 cells.

Signal Sequence Enhanced Antibody Production

The specific antibody titers in the serum of all experimental chicken were measured by Indirect Enzyme-linked Immunosorbent Assay (**ELISA**) using PlpE as the coating antigen. Upon high dilution of sera (1:10,000), the SP-tPlpE vaccine group still showed significantly higher antibody levels than the tPlpE group (d 21 and 28 after vaccination), indicating that the signal sequence in the construct resulted in a more immunogenic antigen (Figure 4).

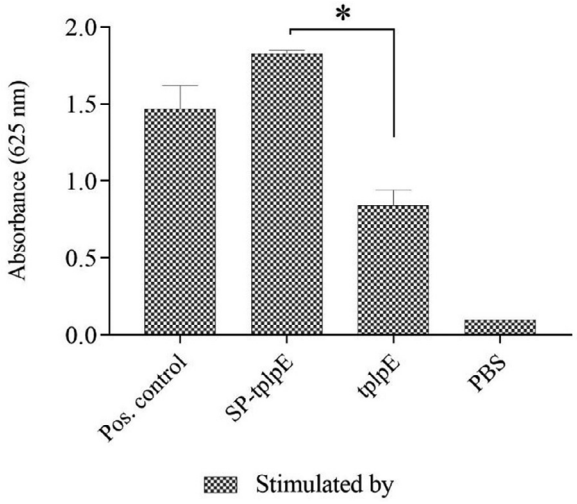


Figure 3. HEK-Blue-hTLR2 Cells were treated with SP-tPlpE and tPlpE at a final concentration of 12.5 μg/mL. The cells were treated with the inactivated *E. coli* (2,000 CFU/well) and SP-tPlpE, tPlpE at 37°C and 5% CO_2. Cell activation was determined after 16 h of incubation by measuring SEAP activity at OD_{625} using the HEK-Blue Detection assay. Results are expressed as means ± S.D. of triplicates and represented in three independent experiments. Statistical significance between SP-tPlpE and tPlpE-treated was determined using Student's *t* test ($P < 0.05$)

Signal Sequence Enhanced CD4+ T Cell Populations

To analyze cellular immune response after vaccination, the percentages of $CD4^+$ and $CD8^+$ T cells were determined by flow cytometry. One day after primary vaccination, the SP-tPlpE vaccine induced a significantly higher level of $CD4^+$ T cell expansion (16%) than the tPlpE vaccine (6%), indicating that the signal sequence enhanced cellular immune stimulation (Figure 5).

Cytokine Expression Profile

To validate the effect of the immune response elicited by the recombinant proteins, isolated PBMCs from vaccinated birds were stimulated with PlpE and cytokine-related mRNA expression was measured. Results showed that for both SP-tPlpE and tPlpE vaccine groups, expression of the TH2-type cytokine (**IL-4**) was more pronounced than that of TH1-type cytokines (IFN-γ and IL-12; Figure 6).

Signal Sequence Boosted Survival Rate to 87.5% in a Challenge Test

The protective efficacy of SP-tPlpE and tPlpE as vaccines were evaluated. When immunized chickens were challenged with a virulent *P. multocida* A, Chu01 strain, the tPlpE vaccine group showed a survival rate of 25% (2/8), whereas the SP-tPlpE group showed 87.5% (7/8) survival (Figure 7). The signal peptide sequence enhanced the protective efficacy of tPlpE.

Gross Lesion in Livers of Challenged Chickens at Late-Stage Acute of Fowl Cholera

Chickens were observed for clinical signs of fowl cholera post challenge with *P. multocida* Chu01. All the chickens in the PBS + ISA71 group (n = 8), 6 in the tPlpE + ISA71 group and only 1 bird in the SP-tPlpE + ISA71 group showed clinical signs of late-stage fowl cholera infection. These chickens were euthanized for examination of gross lesion of the liver. Livers from the PBS + ISA71 group showed severe symptoms with necrotic white foci (Figure 8A) while milder gross lesion was apparent in the slightly enlarged livers from the SP-tPlpE + ISA71 group (Figure 8B). Normal livers were found in the control group (Figure 8C). Presence of *P. multocida* was reconfirmed with PCR (Figures 8D and 8E).

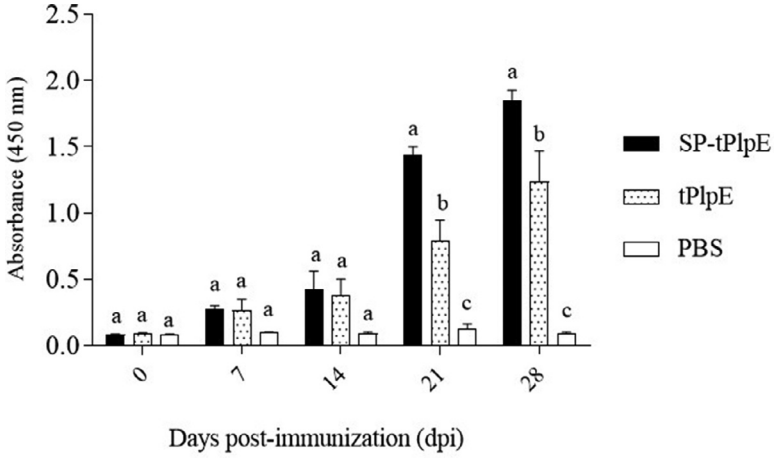


Figure 4. Antigen-specific antibodies of immunized chickens. Chickens (n = 3) were immunized twice with SP-tPlpE + ISA71, tPlpE + ISA71, or PBS + ISA71, and sera were analyzed by indirect ELISA using PlpE as the coating antigen. Data are presented as mean ± SEM. Different superscript letters indicate significant difference ($P < 0.05$) between vaccine groups at the same time point.

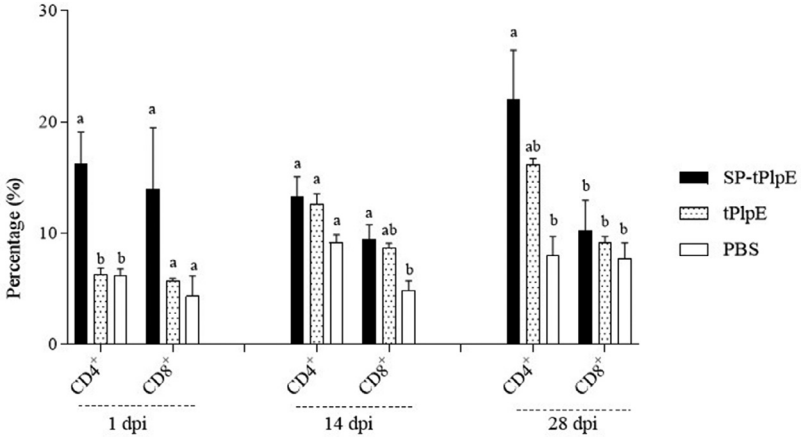


Figure 5. $CD4^+$ and $CD8^+$ T cell immune response of immunized chickens. Chickens (n = 3) were immunized twice with SP-tPlpE, tPlpE, or PBS and isolated PBMCs were stained with anti-CD4 or -CD8 antibodies for flow cytometric analysis. Data are presented as mean ± SEM. Different superscript letters indicate significant differences between vaccine groups at the same time point ($P < 0.05$).

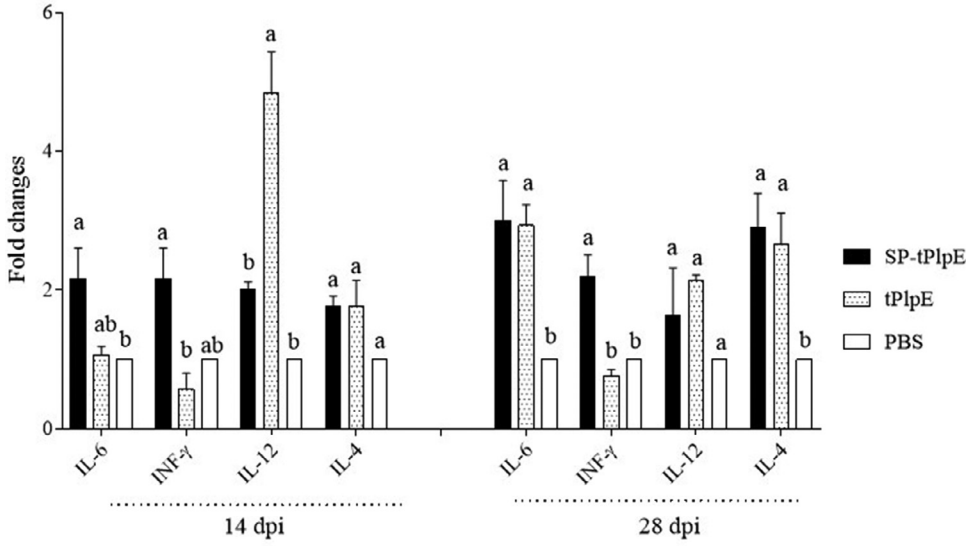


Figure 6. Cytokine expression of PBMCs from immunized chickens. Chickens (n = 3) were immunized twice with SP-tPlpE, tPlpE, or PBS and isolated PBMCs were stimulated with PlpE. Relative mRNA expression levels of IL-6, IFN-γ, IL-12, and IL-4 were determined. Data are presented as mean ± SEM. Different superscript letters significantly differ between vaccine groups ($P < 0.05$).

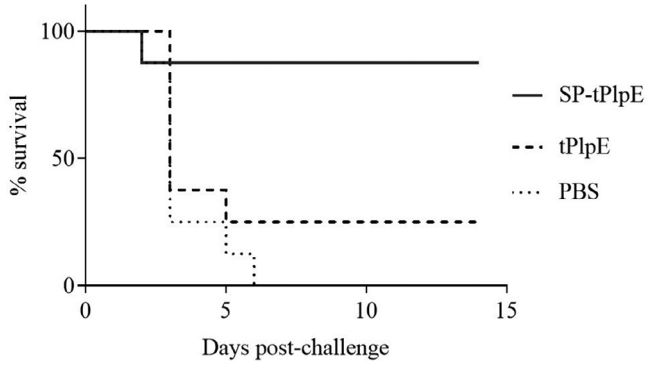
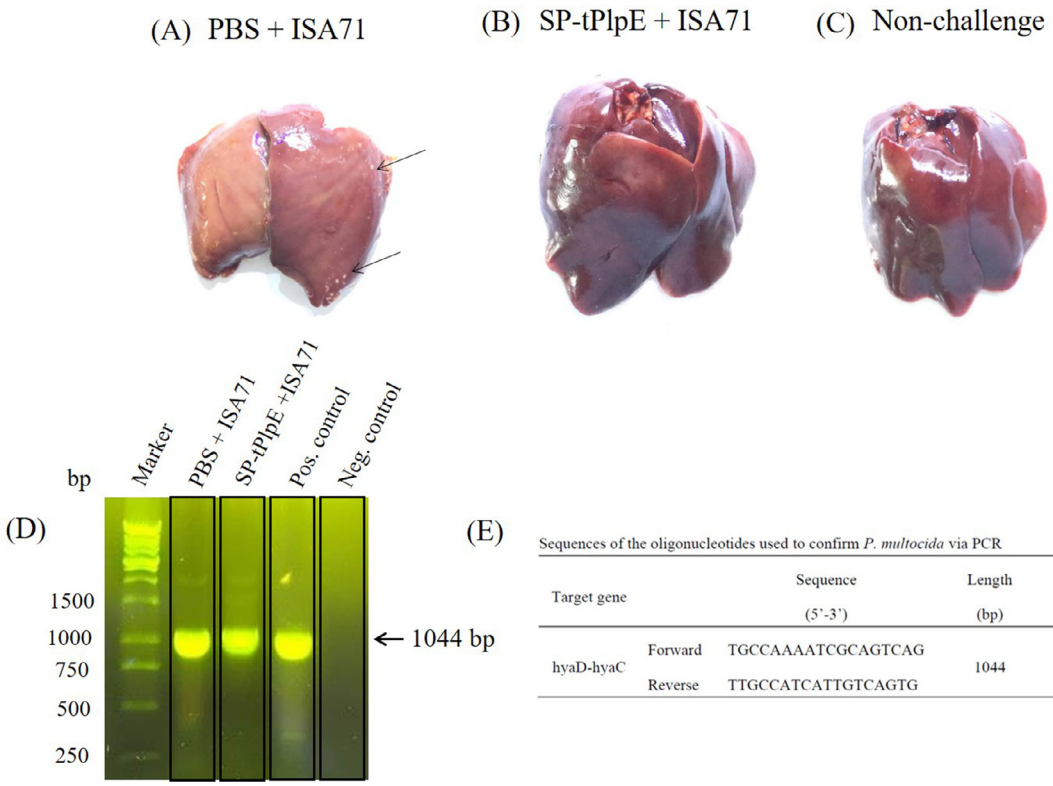


Figure 7. Survival rate of immunized chickens when challenged with *P. multocida* A. Chickens (n = 8) were immunized twice with SP-tPlpE, tPlpE, or PBS and challenged with 20 LD_{50} (3.2×10^5 CFU/dose) *P. multocida*.

DISCUSSION

Our study found that the inclusion of its native signal sequence in the recombinant tPlpE enhanced immunogenicity and protective efficacy. For a potential explanation of this observation, the literature review offers a hypothesis: the signal sequence resulted in lipidation of tPlpE and the lipid moiety activated TLR2 for a robust immune response. In Gram-negative bacteria, lipoproteins are firstly synthesized in the cytoplasm as precursors containing the signal peptide. The precursor is subsequently translocated across the inner membrane through the Sec translocon. Diacyglycerol is then transferred onto the sulfur of the cysteine residue in the Lipobox (lipidation). After that, the amino acid side of the cysteine residue is cleaved by signal peptidase II and third fatty acid is added to the new amino terminus, resulting in a mature lipoprotein. It is triacylated and localized to the inner or outer membrane (Bos and Tommassen, 2004; Tokuda, 2009; Braun and Hantke, 2019). The lipid moiety has been demonstrated to greatly enhance the immune response and protective efficacy of *B. burgdorfieri* (Lyme disease) outer surface protein A (Erdile et al., 1993; Kramer et al., 1996; Häupl et al., 1997). Later studies found that acylated lipopeptides initiates immune response through TLR2 and the ligand-TLR crystal structure has been solved (Chua et al., 2008; Hirschfeld et a., 1999; Khan et al., 2009; Zaman and Toth, 2013).

In attempts to re-design PlpE as an antigen, this study demonstrated that the short protein fragment SP-tPlpE contains protective epitopes and this signal sequence-TLR2 activation hypothesis was proved. In the current investigation, this signal sequence-TLR2 activation was tested using HEK-Blue-hTLR2 Cells. The result indicated that the SP-tPlpE significantly enhanced the production of SEAP. However, a small amount of SEAP production was also detected in the reported cells of tPlpE stimulation, it is possible the purified tPlpE contains negligible endotoxin contamination from purification progress. Although our experiments demonstrated that the signal sequence of SP-tPlpE signaled through TLR2 independent of both TLR1 and TLR6. Further studies would be necessary to



Sequences of the oligonucleotides used to confirm *P. multocida* via PCR

Target gene		Sequence (5'-3')	Length (bp)	Annealing temp. (°C)	Reference
hyaD-hyaC	Forward	TGCCAAAATCGCAGTCAG	1044	51	Townsend et al., 2001
	Reverse	TTGCCATCATTGTCAGTG			

Figure 8. Gross lesions in the livers of chickens challenged with *P. multocida* Chu01. (A) Liver of group PBS + ISA71, necrotic white foci on the surface; (B) liver of immunized SP-tPlpE + ISA71 group, swollen; (C) liver of non-challenge chicken, no lesion was observed. (D) Confirmation of *P. multocida* from livers of inoculated chickens using PCR. (E) The primers were used to amplify the hyaD-hyaC gene.

elucidate the interaction of the signal sequence-TLR2 through heterodimers with TLR1 or TLR6 independent manner. Moreover, further experiments using LC-MS/MS method should be performed to confirm that the expressed SP-tPlpE is indeed a lipoprotein.

To design a more compact recombinant PlpE, the inclusion of the signal sequence becomes an interesting question. Since signal peptides are hydrophobic, they are usually excluded from recombinant antigen designs for better solubility. Indeed, our result showed that SP-tPlpE was slightly insoluble, and only 80% of the recombinant protein was in the soluble form. The lipid moiety is, however, very hydrophobic and may decrease the solubility of the antigen, creating a trade-off between immunogenicity and protein yield (the yield for SP-tPlpE was 289 mg/L vs. 500 mg/L for tPlpE, Figure 2C). This trade-off may be acceptable considering the fact that expressed full-length PlpE recombinant protein commonly form inclusion bodies (Wu et al., 2007, Hatfaludi et al., 2012, Okay et al., 2012), and after nickel-affinity purification, protein yield can be as low as 200 mg/L (data not shown). Thus, the overexpressed SP-tPlpE can be considered an improved antigen over the full-length PlpE.

SP-tPlpE may also be an ideal antigen for multivalent subunit vaccines because of its reduced size. The full-length PlpE has been approved as an antigen against *P. multocida* (Wu et al., 2007, Hatfaludi et al., 2012, Okay et al., 2012). In this study, the amino acids $_{26}$Gly to Thr$_{86}$ of this protein are predicted as an immunogenic domain using a bioinformatics tool (https://web.expasy.org/protparam/). The expressed protein of this domain as tPlpE only provided 25% protection when challenged with *P. multocida*. However, by simply including the short signal sequence in the SP-tPlpE construct, protective efficacy can be greatly enhanced to 87%. In all experimental groups, the chickens survived and revealed no differences in behavioral between vaccinated and control chickens during the 28-d immunization period. After challenge, survived chickens showed no clinical symptoms of fowl cholera except depression in the first 2 d. The results of the study suggest that vaccination with SP-tPlpE offers significantly high protection compared to tPlpE group. There is a high possibility that SP-tPlpE was lipidated while tPlpE cannot be lipidated since it lacks the signal sequence. Our results support the continued development of signal sequence as a vaccine adjuvant. Moreover, future designs of SP-tPlpE may include the addition of other peptides that have adjuvant properties, such as flagellin that can activate TLR5 (Doan et al., 2020), for the stimulation of multiple TLRs. If the combined adjuvant effect is sufficient, the use of additional chemical adjuvants may no longer be necessary.

CONCLUSIONS

The current study showed that inclusion of the signal sequence enhanced the protective efficacy of tPlpE. As a vaccine candidate, when compared to the full-length PlpE, the SP-tPlpE construct results in higher protein yield, and its smaller molecular weight may make it more amenable to further vaccine engineering.

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DISCLOSURES

All authors have no financial or personal relationships with other people or organizations that could inappropriately influence or bias their work.

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