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Development of novel subunit vaccine based on truncated fiber protein of egg drop syndrome virus and its immunogenicity in chickens

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ABSTRACT

Egg-drop syndrome virus (EDSV) is an avian adenovirus that causes markedly decrease in egg production and in the quality of the eggs when it infects chickens. In this report, we engineered truncated fiber protein containing the entire knob domain and part of the shaft region as a vaccine candidate. The protein was obtained in the soluble fraction in *Escherichia coli* (*E. coli*), and expression level after nickel-affinity purification was 126 mg/L. By means of multiple characterization methods, it is demonstrated that the recombinant protein retains the native trimeric structure. A single inoculation with the structure-stabilized recombinant protein, even at the lowest dose of 2 μg, stimulated hemagglutination inhibition (HI) antibody responses in chickens, for at least 16 weeks. Neutralizing titers in sera from the protein immunized groups was similar to that of inactivated vaccine immunized group. The lymphocyte proliferation response and cytokine secretion were also induced in immunized SPF chickens. In addition, immunization with the fiber protein also significantly reduced the viral load in the liver. Taken together, these results suggest the truncated fiber protein as an effective single dose, long lasting and rapidly effective vaccine to protect against EDSV.

1. Introduction

The *Adenoviridae* family includes a large number of serotypes, which cause various diseases in humans and other species, resulting in considerable economic and social consequences. Whereas adenoviruses can be found infecting all 5 major vertebrate classes (Davison et al., 2003; Harrach, 2008), only *Aviadenovirus* that infects birds and *Mastadenovirus* that infects mammals have been well studied. As a member of the *Aviadenovirus*, the egg-drop syndrome virus (EDSV) appears to be widespread in serum of waterfowl and other aquatic birds, and becomes an economic problem for the poultry industry world-wide (Cha et al., 2013). The virus could cause a sudden significant drop in egg production in laying chickens accompanied by a reduction in egg quality, leading to substantial economic losses in the poultry industry.

EDSV is a non-enveloped double-stranded DNA virus with a diameter of 70–90 nm. The icosahedral virus consists of 11 proteins with molecular masses ranging from 14 to 97 kDa (Carmen San, 2012; Nazerian et al., 1991). The capsids are built with 240 hexons, 12 penton bases, and 12 fibers (Fabry et al., 2005). The hexon is a homo-trimeric protein, which forms the 20 facets of the icosahedron. The pentons form the 12 vertices (Fabry et al., 2005; Stewart et al., 1991). The fiber is a trimeric protein that attaches to a penton base and plays a crucial role in host cell attachment and entry (Nemerow et al., 2009). The fiber protein consists of three domains(Nguyen et al., 2016): a N-terminal tail that is noncovalently attached to the penton base, the long shaft with repetitive triple β-spiral motif (Raaij et al., 1999), and a C-terminal globular head (knob) domain which recognizes the primary cell receptor (Baker et al., 2019). Among the three major adenovirus capsid proteins, the hexon protein and the fiber protein contain important adenovirus-neutralizing epitopes (Bradley et al., 2012). Therefore, it was assumed that the adenovirus subunit vaccines could be developed based on the fiber protein (Harakuni et al., 2016).

In this study, we propose the use of the truncated fiber protein which contains the entire knob domain and a short repetitive sequence

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from the shaft region as the vaccine antigen for several reasons. Firstly, the truncation contains most adenovirus-neutralizing epitopes (Gahery-Segard et al., 1998; Harakuni et al., 2016). Fibers are the major surface-exposed structural proteins of adenoviruses. It is the key mediators of virus antigenicity (Bradley et al., 2012; Wang et al., 2018). Besides, the trimeric knob domain of the fiber, which serves as the receptor binding domain, provides most of the neutralizing epitopes (Henry et al., 1994). Consequently, the truncated fiber protein contains sufficient adenovirus-neutralizing epitopes. Secondly, the truncated fiber protein can restore the native conformation of the fiber, which would benefit the protein immunogenicity. It has been shown that trimerization of the EDSV fiber requires at least two elements, including the entire knob head and at least the adjacent 15 amino acids of the shaft (Green et al., 1983; Hong and Engler, 1996; Hong et al., 1997). Indeed, each 15-residue segment of the shaft contains two short β-strands and two β-bends, forming a long, narrow amphipathic β-sheet. Thus, the short sequence of the fiber shaft is essential for the truncated fiber protein to form stable trimer, which is required for virion assembly (Louis et al., 1994; Mitraki et al., 1999). Finally, we also expect the recombinant protein containing the short shaft region to be highly soluble when it was expressed in *E. coli*. While the knob domain was readily expressed in the soluble fraction in *E. coli*, a previous study demonstrated that the fiber knob plus 34 shaft amino acids was predominantly expressed in inclusion bodies in *E. coli* (Fingerut et al., 2003). Thus, the study indicated that the 34 amino acids of the shaft region might reduce the protein solubility. In this study, to improve the protein solubility, the shaft sequence was extended to 60 amino acids based on the analysis of hydrophilic property of the peptide. In addition, the codon usage bias was adjusted to fit the highest expression profile of *E. coli*. And the expression conditions were also optimized. Taken together, all the optimization measures could lead to the high solubility of the recombinant protein when expressed in *E. coli*.

Herein, the recombinant fiber protein was expressed using *E. coli* expression system. To estimate the aggregation state of the protein, we analyzed the sample using size exclusion chromatography (SEC), dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS). Further, we evaluated the immunogenicity of the recombinant fiber protein by measuring HI and the level of neutralizing antibodies. Moreover, the protection against EDSV challenge was tested in SPF chickens. The aim of the present research was to develop a subunit vaccine against EDS using the truncated fiber protein expressed in *E. coli*.

2. Materials and methods

2.1. Virus

EDSV strain 127 was propagated in duck embryo fibroblasts (DEFs) prepared from 10-day-old duck embryos. All animals were treated according to the regulations of Chinese law and local Ethical Committee. All animal experiments were performed following the management guidelines of the Institutional Animal Care and Use Committee (IACUC) under the approval of Henan Academy of Agricultural Sciences (Approval number SYXK 2014-0007).

2.2. Plasmid construction

To express the fiber protein of EDSV strain 127 (GenBank accession number Y09598) in *E. coli*, the coding sequence for the knob and the adjacent 60 amino acids of the shaft (GenBank accession number MK386577) was cloned into the prokaryotic expression vector pET-28a after codon optimization, based on the most commonly occurring codons in *E. coli*, yielding the recombinant expression plasmids pET-Fiber. The resultant recombinant clone contains a 6×His tag at the N-terminus, with the molecular mass of approximately 30 kDa.

2.3. Protein expression and purification

The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells. Protein expression was induced with 0.1 mM sopropyl β-D-thiogalactopyranoside (IPTG) overnight at 20 °C. The cell pellets were re-suspended in lysis buffer (20 mM Tris, pH 8, 250 mM NaCl), lysed by sonication and then centrifuged to remove the insoluble pellet. The expressed protein was purified by Ni-NTA His Bind Resins (Merck, Darmstadt, Germany), and further purified by a gel filtration column Superdex200 10/300 G L (GE Healthcare, Sweden). Then the purified fiber protein was subjected to 12% SDS-PAGE and western blotting analysis with mouse anti-His mAb. The protein was quantified with a Micro BCA™ protein assay kit 120 (Pierce Biotechnology, USA).

2.4. Dynamic light scattering (DLS) analysis

The shape, size, size distribution and zeta potential of the obtained fiber protein were characterized by Dynamic Light Scattering (DLS) (Malvern, Worcestershire, UK). Three measurements were acquired for 1 ml of each sample and averaged at 25 °C. The sample (5 mg/ml) was diluted in a buffer of 20 mM Tris − HCl, 150 mM NaCl, pH 8. All agents were filtered through a 0.22 μm Millipore filter membrane to remove any dust particles prior to DLS measurement. The cuvette was then inserted into the unit and left to equilibrate for 2 min at 25 °C before the measurement.

2.5. Small angle X-ray scattering (SAXS)

To further check the oligomeric state and determine the molecular envelop of the protein, the recombinant fiber protein (5 mg/ml) was analyzed using SAXS technique. The qualities of the scattering curves were analyzed using the program PRIMUS (Konarev et al., 2003) to ensure that there was no obvious aggregation and radiation damage before further analysis. The initial *R*g values were calculated from the Guinier plot; only data from low q values were used for the calculation. The pair distance distribution functions, P(r), was calculated with the program GNOM (Petoukhov et al., 2012). The molecular weight was estimated directly from the SAXS MoW server (http://www.if.sc.usp.br/~saxs/saxsmow.html) using the P(r) distribution function.

The low-resolution global shape of the protein in solution was modeled by the program DAMMIF (Franke and Svergun, 2009) in the asymmetric unit and P3 symmetry using both the original scattering curve and the calculated P(r) distribution curve. The structural models of the fiber head and part of the shaft region were modeled by the SWISS-MODEL server (http://www.swissmodel.expasy.org/) (Arnold et al., 2006). The trimeric fiber head and fiber shaft models from the SWISS-MODEL server were superimposed onto the low-resolution model of the trimeric fiber in solution using the program Chimera (Pettersen et al., 2004).

2.6. Vaccination and challenge in SPF chickens

All the animal experiments presented in this study were performed using SPF White Leghorn chickens housed in filtered-air, negative-pressure isolation units. At 6 weeks of age, a total of 54 chickens were randomly divided into six groups with nine chickens in each group. Group 1 were inoculated with PBS as a negative control. Group 2 were vaccinated with a commercial inactivated whole-virus vaccine and served as a positive control. The other four groups were inoculated with 2 μg, 10 μg, 30 μg and 60 μg of fiber protein emulsified with the adjuvant Montanide ISA 71 at a ratio of 1:3 respectively. All groups were injected intramuscularly in the pectoral muscle, and given in a single injection. The serum samples were collected weekly after vaccination for analyzing the antibody responses and cytokine expression in chickens. To assess the protective efficacy of recombinant EDSV fiber protein, chickens were challenged with EDSV 127 strain at 4 weeks

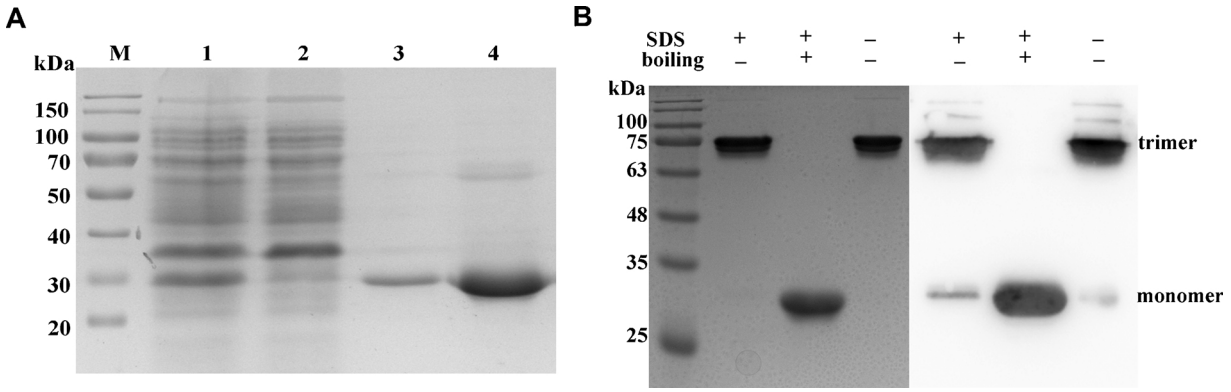

Fig. 1. The fiber protein was expressed and analyzed by Western blotting. The empty vector pET-28a was used as a control. A: a, SDS-PAGE analysis of the expression of the fiber protein. Lane 1, pET-Fiber cell lysate after induction; Lane 2, pET28a vector control; Lane 3, 4, purified fiber protein. B: SDS-PAGE (left panel) and Western blot (right panel) of fiber constructs under different conditions. The fiber protein has a molecular mass of 90 kDa; when boiled and denatured with sodium dodecyl sulfate (SDS), these fibers dissociate into monomeric proteins which migrate at an estimated molecular mass of 30 kDa.

after immunization. The individual liver samples were collected aseptically at day 5 post-challenge.

2.7. Detection of anti-EDSV neutralizing antibodies

The serum neutralization (SN) test checks the ability of antibodies produced against an antigen to prevent the penetration and propagation of a virus in cells. The SN test was performed as described by Fingerut et al. (2003). Briefly, serum samples were heat inactivated at 56 °C for 30 min. EDS virus at a concentration of $10^{6.5}$ $TCID_{50}$/ml was incubated with an equal volume of two-fold serial dilutions of pooled sera for 30 min at 37 °C. One hundred microlitres of the mixtures were inoculated into 12-day-old embryonated duck eggs, which were then incubated for 7 days. Viral propagation was evaluated by assessing the haemagglutination activity of the amniotic-allantoic fluid. The titer was the dilution of serum (expressed as its log2 value) that protected 50% of the eggs inoculated, calculated by the Reed-Muench method (Reed and Muench, 1938).

2.8. Detection of HI antibody response

The HI antibody titers of the serum samples were assessed in 96-well V-shaped microtiter plates according to the standard method as described (Fingerut et al., 2003). Briefly, 25 µl of EDSV, equivalent to 4 HA units, was mixed with an equal volume of 2-fold serially diluted serum samples, and incubated at room temperature for 10 min. The mixtures were then incubated with 25 µl of 0.8% chicken erythrocyte suspension at room temperature for 30 min. The negative control was also performed as described. The endpoint was determined as the highest dilution which caused the complete inhibition of haemagglutination.

2.9. Lymphocyte proliferation assay

PBMCs were isolated from the blood of vaccinated chickens using Ficoll-Paque PLUS (Invitrogen). After being washed three times with PBS buffer (pH 7.2), the PBMCs were resuspended at 2×10^6 cells/ml in DMEM medium supplemented with 10% FBS and seeded in triplicate in 96-well flat-bottom plates at 150 µl per well. The cells were then stimulated at 37 °C for 48 h with 50 µl of purified fiber protein (50 mg/ml), PBS, or cell stimulation cocktail (positive control). The absorbance of each well was measured at 450 nm with a reference wavelength at 650 nm. T lymphocyte proliferation was represented as the stimulation index (SI), which was the ratio of the mean reading of stimulated wells to unstimulated ones.

2.10. Cytokine production assay

The expression of the interferon IFN-γ, cytokines IL-2 and IL-4 in the culture supernatant of chicken PBMCs were measured using commercially available chicken IFN-γ, IL-2 and IL-4 (Bogoo, China) ELISA kits, respectively. All procedures were performed according to the manufacturer's instructions.

2.11. Quantitative RT-PCR for EDSV in tissues

Total DNA was extracted from liver tissues of challenged chickens, and penton of EDSV 127 (GenBank accession No. Y09598.1) was detected by RT-PCR as previously described (Qi et al., 2017). The fragment (194 bp) was amplified by a pair of primers (F: CGTTCGCCTAA TGACT; R: CTGCCTTCCAACTTTC) used for the PCR. Sterile water was used as the template of the negative controls. Quantitative RT-PCR was performed using a SYBR Green Master (Roche). A real-time PCR protocol was performed using the following cycling parameters: 95 °C for 10 min, and then 40 cycles of 95 °C for 10 s, 60 °C for 30 s. All samples were performed in triplicate. The viral dose was calculated according to the standard curve plotting Ct values against different dilutions of standard plasmid.

2.12. Statistical analyses

Data were presented as the mean ± standard error of mean (SEM). Comparisons and graphs were analyzed using GraphPad Prism version 7.0.

3. Results

3.1. Expression and purification of the EDSV Fiber protein

On the basis of protein folding analysis, the construct containing the entire knob domain and part of the shaft domain was generated. The recombinant fiber protein was expressed as the soluble fraction of the bacterial cell lysate. The molecular mass of the protein was approximately 30 kDa, as expected (Fig. 1A). It is shown that the fiber protein accounted for greater than 95% of the total protein amount. The recombinant protein was recognized specifically by anti-His antibodies (Fig. 1B). Adenovirus fibers is among the few oligomeric proteins that do not dissociate upon incubation with sodium dodecyl sulfate (SDS) at moderate temperatures (Hong and Engler, 1996). Indeed, the purified protein predominantly existed as trimeric form and was SDS-resistant at the ambient temperature (Fig. 1B). When boiled before SDS-PAGE, the trimer was dissociated into monomers.

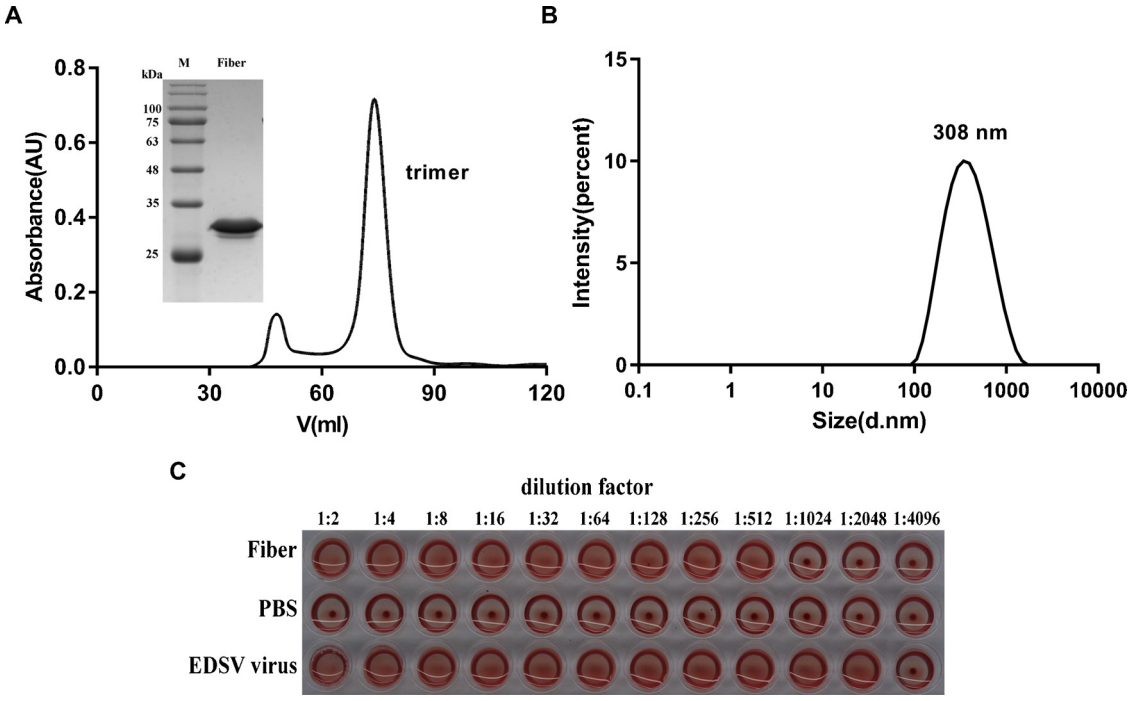

Fig. 2. Characterization of the recombinant fiber protein which exists predominantly as trimer. A: fiber protein was purified to high purity by a gel filtration column (Superdex 200 10/300 G L). The retention volume of 75 ml corresponds to the trimeric form of fiber protein. SDS-PAGE analysis of the elution fraction corresponds to the peak (Inset). B: Dynamic light scattering data for the fiber protein. The hydrodynamic molecular radius of fiber protein by DLS is 308 nm. C: Assessment of fiber protein function by hemagglutination assay. The fiber protein or viruse was diluted in a two-fold serial dilution. PBS was included as the negative control.

3.2. Characterization of the EDSV fiber protein

The homo-trimer is a critical feature of the fiber protein and plays an important role in physiological processes. We observed that the recombinant fiber protein existed predominantly as a trimer in gel filtration analysis with a retention volume of about 75 ml (Superdex 200 10/300 G L column, Fig. 2A). Further, the size of the protein was analyzed by dynamic light scattering (DLS), which gave a single species with a hydrodynamic radius of 308 nm (Fig. 2B). The fiber trimer was monodispersed (Fig. 2B) and no large aggregates were observed, showing that the trimer is the predominant species in solution. To evaluate the biological activity of the protein, an equal volume of recombinant protein and the EDSV virus were subjected to hemagglutination assay (HA) assay under the same conditions. The result showed that the purified recombinant protein had the highest HA titer (1:512) at minimum total protein amount of 0.016 μg (Fig. 2C).

3.3. SAXS study of fiber protein in solution

SAXS has emerged as a powerful technique to study biological macromolecules in solution. It can provide molecular information on morphology of proteins in solution (Arai et al., 2004; Svergun, 2010). The SAXS results gave masses of 118 kDa corresponding to the recombinant fiber protein at the concentrations of 5 mg/ml. Within the inherent error of the technique, these results unequivocally indicated that the protein is a trimer in solution (Table 1). In addition, while the overall parameters clearly demonstrate the presence of large particles consisting of several protein molecules in solution, the Porod (Fig. 3B) as well as Kratky plots (Fig. 3C) are characteristic to rather compact particles with definite shapes. Consequently, the trimeric structure model of the EDSV fiber protein fits the molecular envelop well, which is consistent with previous solved crystal structures of adenovirus fiber proteins (El Bakkouri et al., 2008; Seiradake et al., 2006; Xia et al., 1994), showing again that the trimer is the predominant species in solution. Taken together, these results suggest the recombinant protein maintain the native conformation and functions of the fiber.

3.4. Antibody response in the chickens

To evaluate the prophylactic immunogenicity of EDSV fiber protein, antibody responses were measured using the neutralization assay and HI assay every week after immunization. As shown in Fig. 4, the EDSV neutralization assay and HI assays were detected in all the protein immunized groups after a single vaccination. From 2 μg to 30 μg, higher doses of the fiber protein induced higher and more persistent antibody titers, and gradually increased with time. Interestingly, Neutralizing titers in sera from the 30 μg group were significantly higher than those from the other dose groups. Moreover, 30 μg group showed highest neutralizing antibody titers of 11 log2 against EDSV, which was similar to that of inactivated vaccine immunization sera (Fig. 4A). There were no significant differences in EDSV neutralizing titers among the other recombinant fiber-immunized groups.

The HI assay is commonly used in EDSV research since it is a good predictor of the protective efficacy of vaccines. As shown in Fig. 4B, in

Table 1
SAXS parameters for fiber.

Sample	mg/mL	Guinier		Real space			Mass, kDa	
		Rg,	I(0)	Rg,	I(0)	Dmax	SAXS, kDa	Observed, kDa
Fiber	5	64.30	5.0e4	65.05	5.0e4	259	121	30

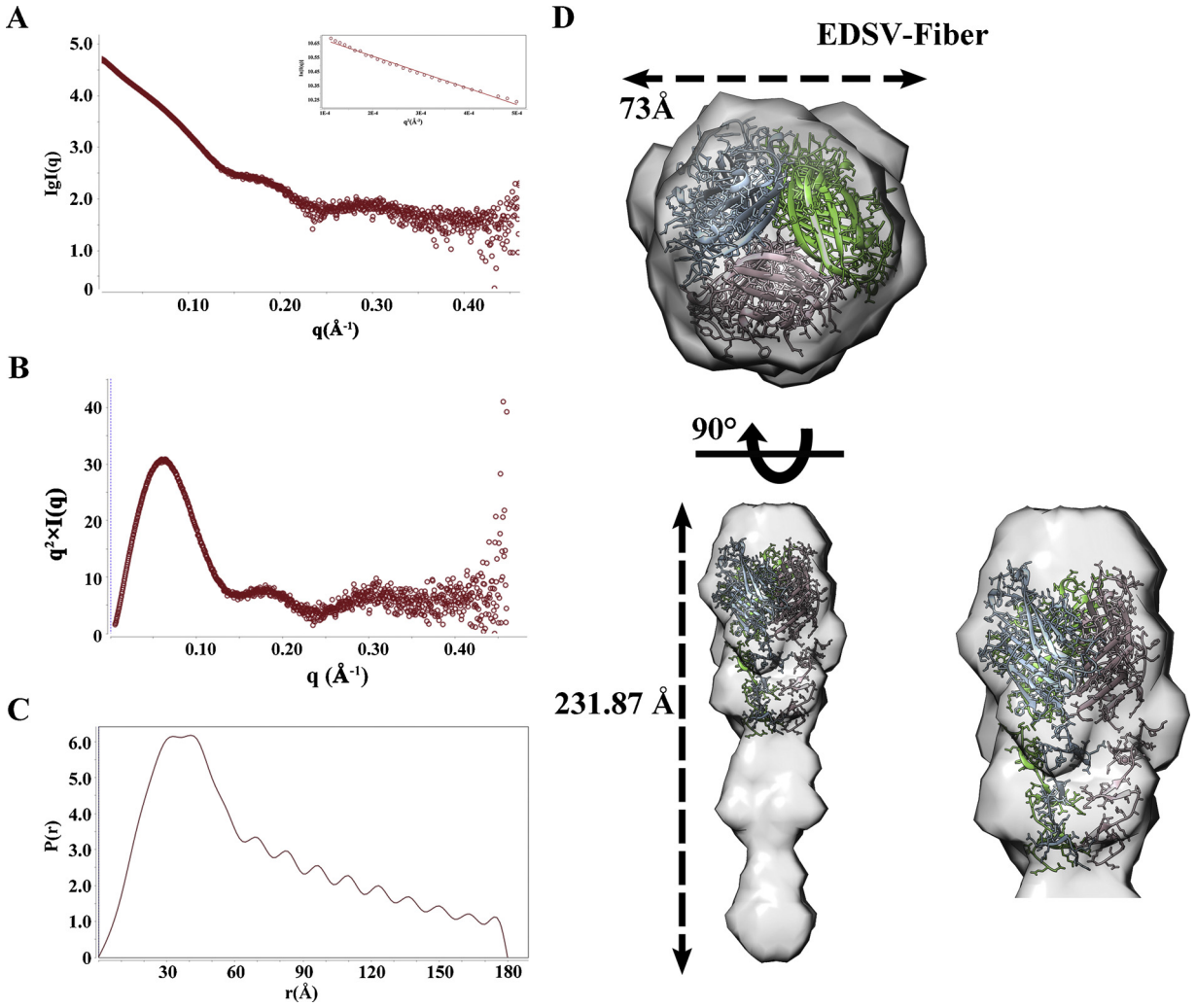

Fig. 3. SAXS data and model of the fiber protein reveals s a protein. A. Experimental Small-Angle X-ray Scattering curve (sphere) of the fiber protein in solution. The Guinier region and the corresponding linear fitting are shown in the inset. B. Pair-distance distribution function derived from the experimental curve. C. Kratky plot calculated from the experimental data. Shaft-knob shaped curves indicate compact structures. D. Superimposition of the experimental envelope (shown as a surface) onto the homology model fiber trimer, represented as stick.

the dose-response study, all vaccinated groups had similar highest HI titers at 4 weeks post-vaccination, with a gradual rise that was related to the dose. And the HI titers maintained to at least 16 weeks. As expected, neutralization antibody and HI antibody levels in the serums of the PBS were negative before challenge.

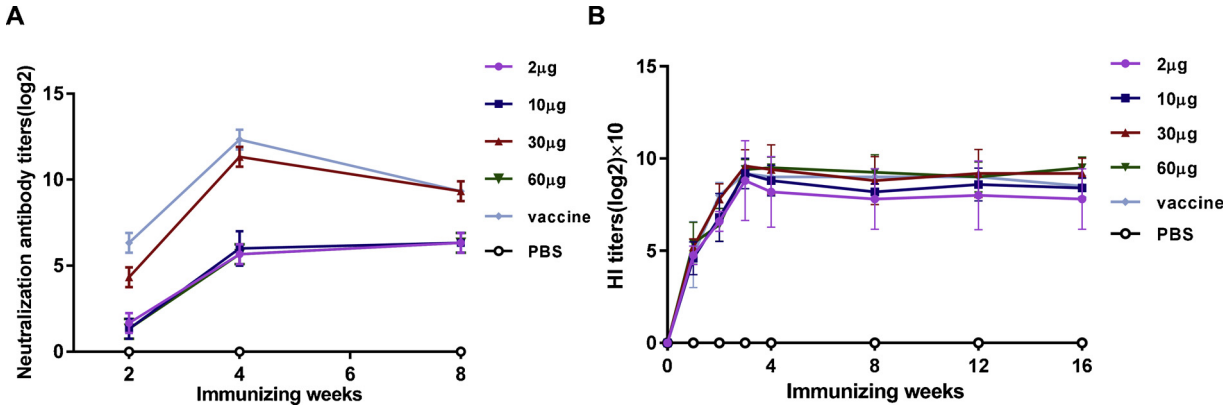

Fig. 4. EDSV specific immune response in chickens. Groups of chickens (n = 9) were immunized with 2 μg, 10 μg, 30 μg and 60 μg of fiber protein, inactivated EDSV and PBS. Blood samples were collected at each week after the first immunization for virus neutralization antibody (A) and hemagglutination inhibition antibody (B). The titers of antibodies are expressed as mean ± SEM.

Table 2
The T-lymphocyte proliferation of chickens.

	fiber				vaccine	PBS
	2 μg	10μg	30 μg	60 μg		
SI	1.061109	1.08903	1.239636	1.174558	1.112048	1.044435

3.5. Lymphocyte proliferation assay

To evaluate the impact of the recombinant protein on cellular immune response in chickens, fiber-specific lymphocyte proliferative responses were measured at 14 dpi (Table 2). The levels of lymphocyte proliferation of the protein immunized groups were marginally higher than the negative control groups, indicating the recombinant protein induced T-cell responses.

3.6. Fiber protein induces cytokine production

To analyze the concentration of cytokine in the supernatants of PBMCs from chickens, the cell culture supernatants were detected by ELISA kits after stimulation with protein for 72 h. The amount of IL-4 released by the stimulated PBMCs reflected T helper cell type 2 (Th2) responses of the vaccinated groups. Cytokines IL-2 and IFN-γ is associated with T helper cell type 1 (Th1) responses and cell-mediated immunity. The production of IL-2, IFN-γ and IL-4 were significantly increased in all protein vaccinated groups with dose-dependent correlation. In addition, the secretion level of IL-2 from chickens vaccinated with 30 μg fiber protein was markedly higher than other protein vaccinated groups (Fig. 5), and positively correlated with the neutralization antibody. Taken together, the cytokine concentration of the protein immunized groups was significantly higher than the PBS group, while it was similar to the inactivated vaccine immunized group.

3.7. Fiber protein confers full protection against EDSV challenge

The chickens in all groups described above were challenged with EDSV subcutaneously at 4 weeks after immunization. The liver of the chickens was collected and RT-PCR was performed to measure the virus dose. As shown in Fig. 6, the PBS control group had showed a remarkably higher viral dose of approximately up to 8×10^5 at day 5 post infection than other groups. A significantly lower virus gene quantity was detected in the fiber protein groups and vaccine group compared with those in PBS group (Fig. 6). The groups inoculated with the protein showed a positive correlation with protection against the EDSV challenge, similar to the group inoculated with inactivated vaccine. These results indicated that the chickens immunized with the EDSV recombinant fiber protein could be protected against the EDSV infection.

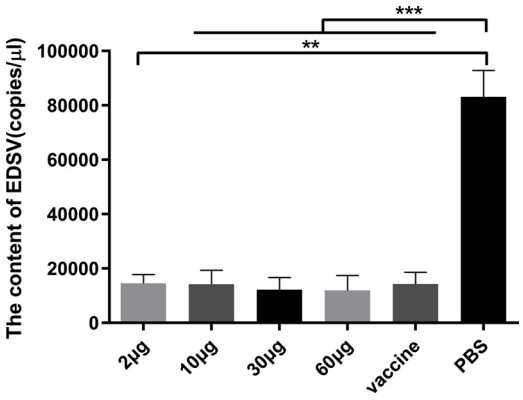

Fig. 6. Protection challenged with EDSV in chickens. All of the chickens (n = 5) were challenged with 100 μl of $10^{6.5}$ $TCID_{50}$/ml of the EDSV virus at 4 weeks after immunization. Livers were isolated and the genomes were extracted to measure the content of EDSV using quantitative real-time PCR. Date are shown as mean ± SEM, statistical differences between antigen groups and PBS groups were measured by one-way ANOVA, indicated by asterisk. The results are shown as the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The lowest dose of fiber that was able to provide protective immunity was found to be 2 μg/bird.

4. Discussion

Previous work about EDSV subunit vaccine represented that the truncated fiber protein containing the knob and part of shaft region was produced as inclusion bodies in *E. coli*, which required dialysis and refolding (Fingerut et al., 2003; Gutter et al., 2008). In a more recent work, to improve the protein solubility, the α-helical coiled coils instead of the triple β-spiral motif of the shaft region was used to stabilize the trimeric knob domain (Harakuni et al., 2016). On the basis of previous studies, we proposed that the improvement of protein solubility and stability could be achieved at the same time by proper construction of the fiber protein. After screening different truncation of the shaft region, consequently, we demonstrated that the knob domain with the adjacent 60 amino acids of the shaft region, could be expressed in the soluble fraction in *E. coli* (Fig. 1B) and maintains the trimeric conformation. The expression levels of the soluble fiber proteins after nickel-affinity purification was 126 mg/L, while the EDSV fiber proteins with coiled-coil fusion were 15–81 mg/L (Harakuni et al., 2016). Our data support that the shaft region is important to the trimerization of the fiber, and thus is required to produce a stable trimer antigen. Meanwhile, the knob domain equipped with proper length of shaft sequence is beneficial, particularly in conferring high water solubility, and therefore high level of soluble protein expression has been

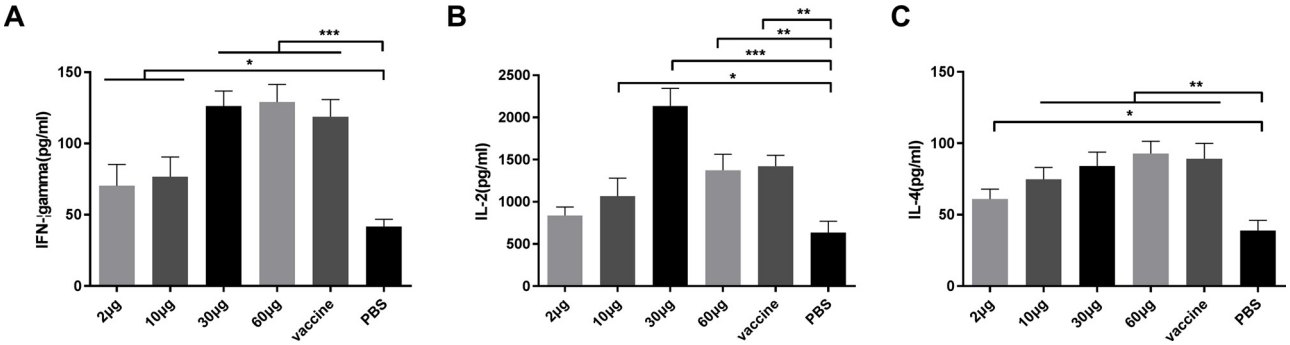

Fig. 5. Analysis of cytokines in the supernatants of PBMCs from chickens. Lymphocytes isolated from PBMCs from chickens (n = 5) at 42 days after the prime vaccination were stimulated with EDSV for 72 h, the supernatants were collected to detect the concentrations of Th2-type cytokine of IL-4 (C) and Th1-type cytokine of IFN-γ (A) by ELISA method. Date are shown as mean ± SEM, statistical differences between antigen groups and PBS groups were measured by one-way ANOVA, indicated by asterisk. The results are shown as the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

achieved.

In order to obtain further understanding of the folding and assembly of the truncated fiber protein, we used systematic approaches to analyze the sample. It is shown that the recombinant fiber protein forms trimers in solution, as determined by gel filtration, DLS and nonreducing SDS/PAGE. Further, the SAXS provides three-dimensional low-resolution structures of the fiber protein, indicating that the recombinant fiber protein assembled into trimers with a similar size and shape to the solved structure of CELO long fiber knob (Guardado-Calvo et al., 2007). Thus, these results support the suggestion that the fiber protein produced here self-assembled properly and restored its native trimeric conformation.

The fiber protein generated from *E. coli* was used to immunize the chickens. High titers of neutralization antibody and HI antibody against EDSV were detected, which were similar to the group inoculated with inactivated vaccine. Another interesting observation is that 30 μg fiber protein induced higher antibody titers than 60 μg, which might be caused by immunological paralysis induced by the high vaccination dose in chickens. The copy numbers of EDSV in fiber protein immunized groups were significantly lower than that in the PBS control group, suggesting that the recombinant fiber protein generated from *E. coli* could effectively protect chickens from EDSV infection. Moreover, the minimum immune dose of this subunit vaccine was also investigated. The lowest dose of the truncated fiber protein that could provide protection against EDSV challenge and elicit protective immunity was found to be 2 μg/bird.

In this study, we analyzed the proliferation of lymphocytes and Cytokine production assay to determine if fiber protein induced T-cell responses. Stimulation indices for the immunized groups were marginally higher than the negative control groups. Levels of IL-2, IL-4, and IFN-γ in animals vaccinated with fiber protein were elevated. indicating that the protein could induce strong cellular immune responses in treated animals. These results suggested that fiber protein could increase T-cell immunity via affecting T lymphocytes to control EDSV infection.

In conclusion, the recombinant fiber protein obtained in this study maintained the stable trimer structure and elicited rapid and long-lasting immune protection in SPF chickens. Considering all the factors, particularly the limitations faced by currently employed inactivated vaccines and the development of next-generation immunization strategies, we conclude that the recombinant fiber protein is an eligible candidate for the development of an effective and safe subunit vaccine.

Author contributions

GZ and GX designed and directed the project. YS and QW designed and performed the experiments. HF, YW, YS, LB, YC and RD contributed to data interpretation and manuscript preparation. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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