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RESEARCH ARTICLE

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Evaluation of Egg Drop Syndrome Virus Fiber Protein as a Vaccine Candidate: In Silico Analysis, Expression, Purification and Its Stability

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ABSTRACT

The Egg Drop Syndrome Virus (EDSV), an avian adenovirus, triggers a sharp decline in both egg production and quality in infected chickens, leading to substantial economic losses in poultry industry. Previous studies have suggested that the EDSV fiber protein may serve as a candidate for subunit vaccine. The present research focused on the expression, purification, and thermal stability evaluation of recombinant fiber protein as a vaccine against EDSV. Using an in *silico* approach, we investigated the fiber protein structure and its expression in *Escherichia coli* (*E. coli*). We also evaluated the thermal stability of the expressed protein. The protein was expressed predominantly as soluble trimeric proteins in *E. coli* and purified using nickel-affinity purification method, yielding approximately 15 mg/L of purified protein. Structural analysis using immunological and bioinformatics tools Confirmed retention of the native trimeric conformation in the recombinant protein. Based on the thermal stability evaluation on this recombinant protein, the protein showed good thermal stability, highlighting its potential as a subunit vaccine candidate for a vaccine against EDSV.

Keywords

Egg drops syndrome virus, Fiber protein, In silico, Purification, Vaccines

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Number of References:: 41
Number of Pages: 12

Abbreviations

BSA: Bovine serum albumin
CAI: Codon adaptation index
CE: Conformational epitopes
EDSV: Egg drop syndrome virus
GRAVY: Grand average of hydropathicity
MFE: Minimum free energy
PBS: Phosphate-buffered saline
TMB: Tetramethylbenzidine

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Introduction

The Egg drop syndrome virus (EDSV), a member of the Atadenovirus genus [1], poses a significant threat to poultry health worldwide. First identified in the Netherlands in 1976 [2], this non-enveloped, double-stranded DNA virus causes substantial economic losses through its characteristic clinical manifestation: the production of thin-shelled, soft, or shell-less eggs in laying hens. The virus demonstrates broad host specificity, infecting not only chickens but also turkeys, geese, and quails, making it a particularly challenging pathogen to control [3]. The viral capsid of EDSV is comprises of 11 structural proteins, among which the fiber protein plays a pivotal role in viral-host interaction and cell entry [4]. This trimeric protein consists of three distinct domains: tail, shaft, and knob, with the knob domain being particularly crucial for initial host cell binding [5]. Due to its immunodominant nature and its essential function in viral pathogenesis, the fiber protein has emerged as an ideal candidate for recombinant vaccine development [6]. Current vaccination strategies rely on inactivated vaccines produced using duck eggs, these vaccines presents several limitations. Dependence on non-specific pathogen-free duck eggs carries inherent risks of pathogen contamination and viral spread, given the high susceptibility of ducks to various avian pathogens [7, 8]. These challenges highlight the urgent need for alternative vaccine production methods that can ensure both safety and efficacy. Subunit vaccines comprising viral surface protein components can circumvent these limitations [9, 10]. Advances in recombinant DNA technology have greatly expanded the potential for vaccine development, as evidenced by the successful commercialization of numerous recombinant viral vector vaccines against major avian diseases. As reported in a review by Hein et al, more than 15 commercially available recombinant viral vector vaccines have already been developed against key avian diseases, including Newcastle disease, infectious laryngotracheitis, infectious bursal disease, avian influenza, and Mycoplasma gallisepticum [11]. The potential of subunit vaccines, particularly those targeting key viral surface proteins like the fiber protein, offers a promising solution to the limitations of traditional vaccine production methods [12]. This study addresses current limitations in EDSV vaccine development through three primary objectives: 1) Comprehensive in silico analysis of the fiber protein structure to identify key immunogenic elements, 2) Development of an optimized expression and purification system for the recombinant fiber protein, and 3) Evaluation of the thermal stability properties of the purified protein. By combining computational biology with experimental validation, this research aims to establish a foundation for developing a safer, more effective alternative to current EDSV vaccines. The broader significance of this work extends beyond EDSV control, as the methodologies developed may be applicable to other avian adenoviruses. Furthermore, adopting recombinant production methods can significantly improve vaccine safety profiles while reduce production costs and complexity. Overall, this study aligns with global shift in modern in vaccinology toward rational, structure-based vaccine design and production.

Result

Physicochemical parameter evaluation

The molecular weight, the number of amino acids, and numbers of positively and negatively charged residues of the fiber protein were 28659.33 Da, 268, 15 and 16, respectively. The extinction coefficient of was 34630 M−1 cm−1 at 280 nm measured in water. The aliphatic index and Grand Average of Hydropathicity (GRAVY) values of the chimeric protein were 77.16 and 0.007, respectively. The bio computed half-life was 5.5 hours in mammalian reticulocytes (*in vitro*), 3 minutes in yeast (*in vivo*), and 2 minutes in *E. coli* (in vivo). Moreover, the instability index, and isoelectric point (pI) were 23.39, and 6.14, respectively (Table 1). The protein solubility, predicted using SOLpro was estimated to be 0.840895.

Secondary structures Evaluation

The secondary structural composition of the fiber protein, analysis using the GOR IV server, revealed 7.46% α-helix, 35.07% β-sheet, and 57.46% coiled-coil. According to GOR IV results, it can be seen that random coils are dominant in the fusion protein sequence, followed by extended strands and alpha helices (Fig. 1). Also, using the PSIPRED server, were shown the secondary structure and graphical representation of our protein.

3D structures prediction of the fiber protein and validation

Three modeling servers, Phyre2, I-TASSER and Galaxy, were used for 3D structure modeling. The evaluation of the results of different servers showed that the I-TASSER server is suitable for modeling the 3D structure of our protein. The scoring system of I-TASSER server is based on C-score, and the C-score range is usually between -5 and 2. Models with higher C-score (-1.23) were selected for further evaluations. (Fig. 2). Fiber protein refinement was done using GalaxyRefine server to optimize quality. Regarding the role of trimer structure in antigenicity and binding to host cell, the trimer structure of the mentioned construct was modeled using the Galaxy Web server. Ramachandran diagram showed that, 81.2 of residues

Table 1.
Parameters analyzed by the Expasy ProtParam server

Sequence length	Mw*	T pI	-R	+R	EC	II	AI	GRAVY
268	28659.33	6.14	16	15	34630	23.39	77.16	0.007

*Mw, Molecular weight; T pI, Theoretical Isoelectric point; -R, Number of negative charged residues; +R, Number of positive charged residues; EC, Extinction coefficient at 280 nm; II, Instability index; AI, Aliphatic index; GRAVY, Grand Average Hydropathy

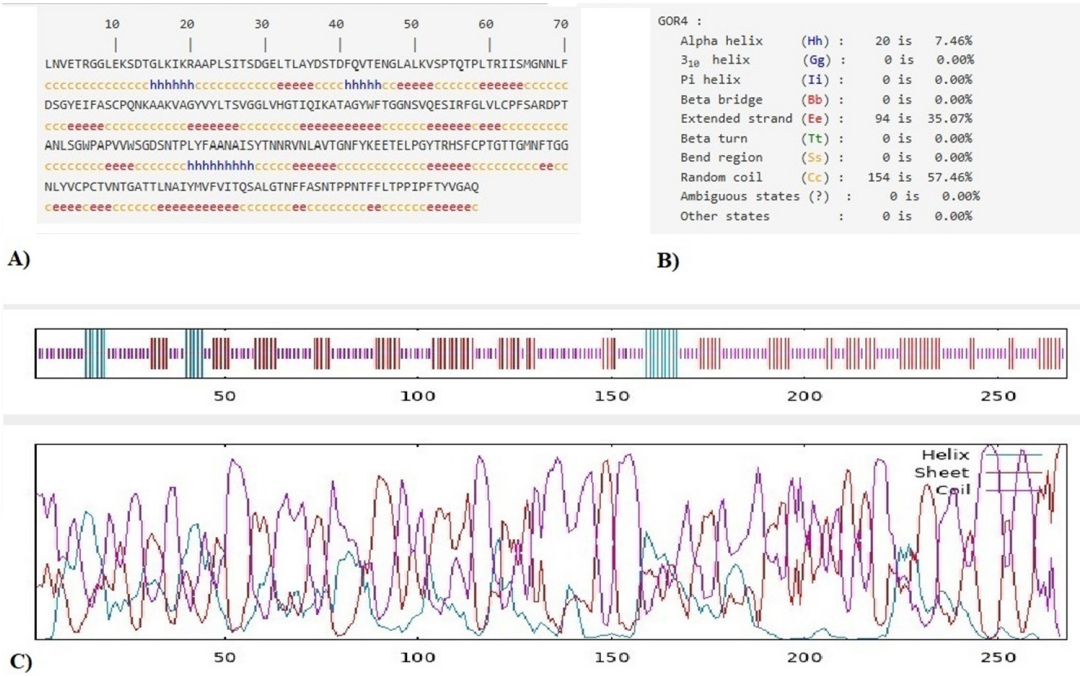

Figure 1.
Fiber protein secondary structure predicted by GOR4 server. (A) The composition of the secondary structure in percent (B) fiber protein secondary structure map (h: alpha-helix; e: extended strand; c: coil). (C) diagram of fiber protein secondary structure (the blue color represents the alpha-helices; the red color represents beta strands, and the purple color was the coils)

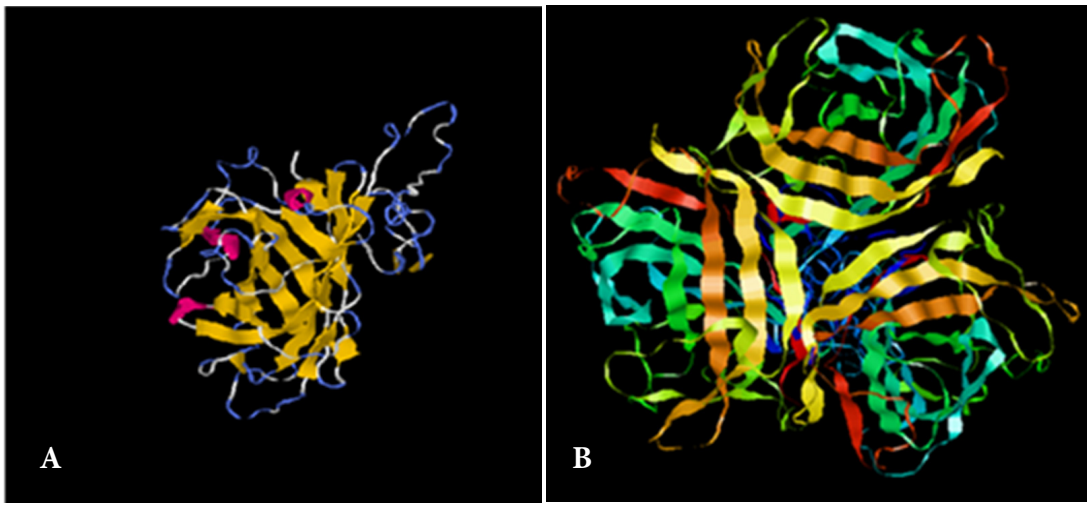

Figure 2.
Tertiary structure prediction. A probabilistic structural model for fiber protein by online servers: A) Monomer prediction by I-TASSER server; B); prediction of trimer structure by Galaxyhomomer

were in favored regions, 15.7 in allowed regions, 1.3 in generously allowed regions and 1.8% in disallowed regions. In order to determine the potential errors in the 3D structures, the analysis of the models has been done by ProSA-webserver (Wiederstein & Sippl, 2007). ProSA z-score of input model was -4.38 (Fig. 3).

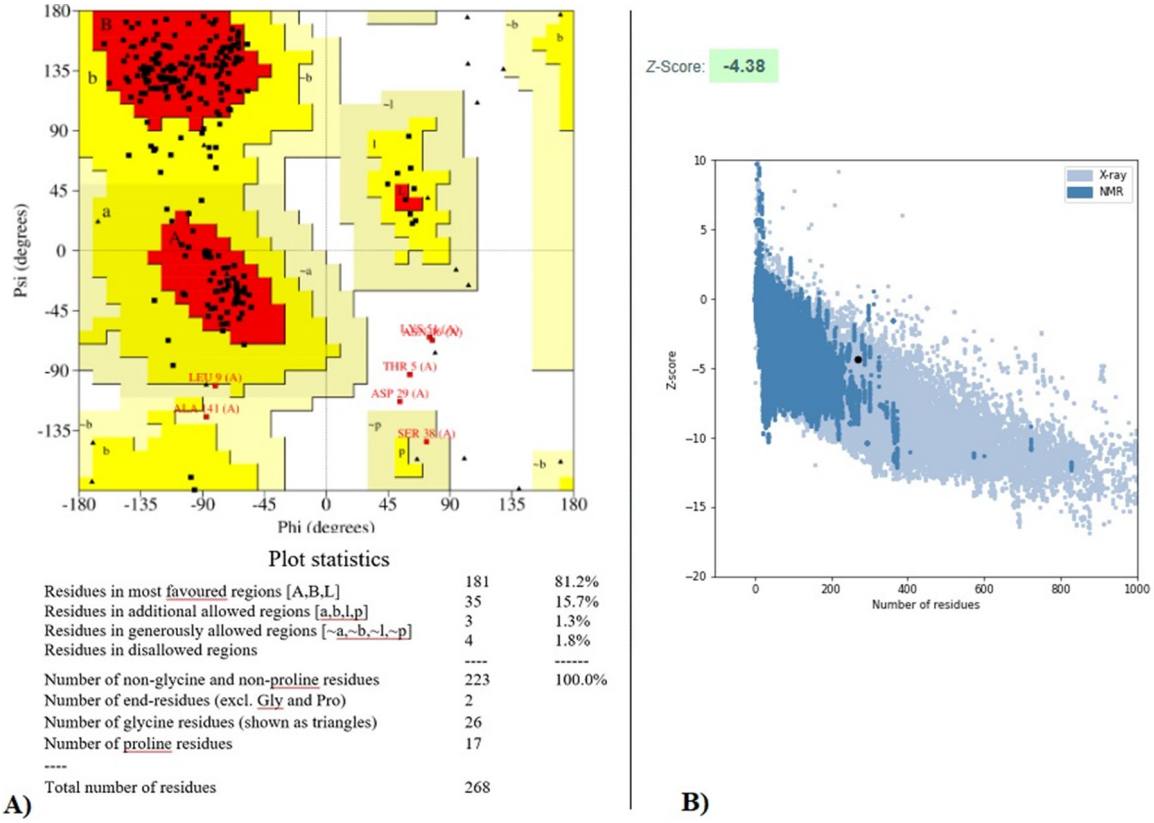

Figure 3.
Model stability was evaluated based on Ramachandran plot for fiber protein: A) Ramachandran plots for trimer structure fiber protein by PROCHEC; B) Fiber protein structure analysis by ProSA- web server

Antigenicity evaluation

Antigenicity prediction using the ANTIGENpro and Vaxijen servers predicted high antigenicity probabilities of 93% and 100%, respectively. These results suggest that, engineered fiber protein is highly antigenic.

Table 2.
Linear B-cell epitope of fiber protein predicted using the SVMTriP server

Rank	Location	Epitope	Score
1	91 – 110	YVYLTSVGGLVHGTIQIKAT	1
2	173 – 192	RVNLAVTGNFYKEETELPGY	0.807
3	131 – 150	LCPFSARDPTANLSGWPAPV	0.605

Prediction of B-cell epitopes

Linear epitope prediction results of the target protein were predicted using the Ellipro and SVMTriP servers, and are shown in the Tables 2 and 3. B-cell epitopes were computationally ranked by prediction scores obtained from ABCpred's neural network model (Table 4). Prediction scores directly correlate with epitope probability (threshold= 0.5), with all shown

Table 3.
Linear B-cell epitope of fiber protein predicted using the ElliPro server

No.	Start	End	Peptide	Residues No.	Score
1	35	59	AYDSTDFQVTENGLALKVSPTQTPL	25	0.79
2	236	254	TQSALGTNFFASNTPPNTF	19	0.79
3	218	226	TVNTGATTL	9	0.784
4	200	208	TGTTGMNFT	9	0.737
5	135	141	SARDPTA	7	0.663
6	110	124	TAGYWFTGGNSVQES	15	0.661
7	165	192	NAISYTNNRVNLAVTGNFYKEETELP-GY	28	0.646
8	68	75	NLFDSGYE	8	0.597
9	9	24	LEKSDTGLKIKRAAPL	16	0.591
10	153	158	SGDSNT	6	0.567
11	26	29	ITSD	4	0.548

Table 4.
Predicted Epitope by ABCpred Prediction Server

Rank	Sequence	Start position	Score
1	GTTGMNFTGGNLYVCP	201	0.93
2	GNSVQESIRFGLVLCP	118	0.92
3	SGDSNTPLYFAANAIS	153	0.9
3	LSGWPAPVVWSGDSNT	143	0.9
4	ANAISYTNNRVNLAVT	164	0.89
5	GNLYVCPCTVNTGATT	210	0.88
5	KATAGYWFTGGNSVQE	108	0.88
6	GELTLAYDSTDFQVTE	30	0.87
7	VSPTQTPLTRIISMGN	52	0.86
7	NFFASNTPPNTFFLTP	243	0.86
7	TQSALGTNFFASNTPP	236	0.86
8	SGYEIFASCPQNKAAK	72	0.85
8	VETRGGLEKSDTGLKI	3	0.85
8	HGTIQIKATAGYWFTG	102	0.85
9	RIISMGNNLFDSGYEI	61	0.83
10	PFSARDPTANLSGWPA	133	0.8

peptides exceeding this cutoff value. Discontinuous epitope were also identified using the Ellipro server and are shown in Table 5. Predicted conformational epitopes including: conformational epitopes 1 (CE1) and conformational epitopes 2 (CE2), conformational epitopes 3 (CE3), conformational epitopes 4 (CE4), which having score greater than 0.5. Three discontinuous epitopes were identified with substantial surface accessibility: CE1 (68 residues, 68.5% exposed), CE2 (35 residues, 65.9% exposed), and CE3 (55 residues, 63.7% exposed). The significant solvent accessibility of these regions (scores > 0.63) supports their inclusion in vaccine design.

MHC-I binding prediction analysis

The MHC-I processing tool identified 259 potential nonamer peptides from the fiber protein that may bind to MHC-I and trigger immune responses. Among these, three peptide fiber protein sequence, i.e., QESIRFGLV and GELTLAYDS and TENGLALKV, were determined, which are likely to be to bind with MHC-I alleles, with percentage rank ≤ 2 (Table 6).

MHC-II binding prediction analysis

For MHC class II binding prediction, the fiber protein sequence was segmented into 254 overlapping 15-mer peptides using NetMHCIIpan 4.0 server. The peptide LTRIISMGNNLFDSG was identified as a strong MHC-II binder, with an optimal peptide binding core of IISMGNNLF (Table 7).

Prediction of mRNA structure

The predicted mRNA secondary structure, analyzed using the RNAfold server, exhibited a minimum free energy of -255.60 kcal/mol, indicating that the gene structural sequence can form a stable RNA sec-

Table 5.
Conformational B-cell epitope of fiber protein vaccine predicted using the ElliPro server

No.	Peptide regions and residues number	Residues No.	Score
1	A:N67, A:N68, A:L69,A:F70, A:D71, A:S72, A:G73, A:Y74, A:E75, A:Q82, A:N83, A:K84, A:T110, A:A111, A:G112, A:Y113, A:W114, A:F115, A:T116, A:G117, A:G118, A:N119, A:S120, A:V121, A:Q122, A:E123, A:S124, A:I125, A:A177, A:V178, A:T179, A:G180, A:N181, A:F182, A:Y183, A:K184, A:E185, A:T187, A:E188, A:P199, A:T200, A:G201, A:T202, A:T203, A:G204, A:M205, A:N206, A:F207, A:T208, A:T236, A:Q237, A:S238, A:A239, A:L240, A:G241, A:T242, A:N243, A:F244, A:F245, A:A246, A:S247, A:N248, A:T249, A:P250, A:P251, A:N252, A:T253, A:F254	68	0.685
2	A:F134, A:S135, A:A136, A:R137, A:D138, A:P139, A:T140, A:A141, A:N165, A:A166, A:I167, A:S168, A:Y169, A:T170, A:N171, A:N172, A:R173, A:N175, A:L189, A:P190, A:G191, A:Y192, A:T218, A:V219, A:N220, A:T221, A:G222, A:A223, A:T224, A:T225, A:L226, A:N227, A:G266, A:A267, A:Q268	35	0.659
3	A:N2, A:T5, A:R6, A:G7, A:G8, A:L9,A:E10, A:K11, A:S12, A:D13, A:T14, A:G15, A:L16, A:K17, A:I18, A:K19, A:R20, A:A21, A:A22, A:P23, A:L24, A:S25, A:I26, A:T27, A:S28, A:D29, A:G30, A:E31, A:L32, A:T33, A:A35, A:Y36, A:S38, A:T39, A:D40, A:F41, A:Q42, A:V43, A:T44, A:E45, A:N46, A:G47, A:L48, A:A49, A:L50, A:K51, A:V52, A:S53, A:P54, A:T55, A:Q56, A:T57, A:P58, A:L59, A:T60	55	0.637
4	A:V151, A:S153, A:G154, A:D155, A:S156, A:N157, A:T158	7	0.548

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Table 6.
MHC-I binding epitopes predicted by the NetMHCcons 1.1 server

Allele	Start	Length	Peptide	1-log50k (aff)	Affinity (nM)	% Rank
HLA-B40:06	29	9	GELTLAYDS	0.247	3441.22	2.00
HLA-B40:06	43	9	TENGLALKV	0.330	1407.46	1.50
HLA-B40:06	121	9	QESIRFGLV	0.287	2244.37	1.50

Table 7.
MHC-II binding epitopes predicted by the NetMHC 4.0 server.

Allele	Start	Peptide	Core Sequence	Score EL	PR*
DRB1_1454	13	DTGLKIKRAAPLSIT	LKIKRAAPL	0.367271	3.55
DRB1_1454	14	TGLKIKRAAPLSITS	IKRAAPLSI	0.340573	4.11
DRB1_1454	15	GLKIKRAAPLSITSD	IKRAAPLSI	0.419096	2.66
DRB1_1454	58	PLTRIISMGNNLFDS	IISMGNNLF	0.393941	3.04
DRB1_1454	59	LTRIISMGNNLFDSG	IISMGNNLF	0.464146	2.10
DRB1_1454	60	TRIISMGNNLFDSGY	IISMGNNLF	0.358547	3.73
DRB1_1454	101	VHGTIQIKATAGYWF	IQIKATAGY	0.336206	4.22
DRB1_1454	102	HGTIQIKATAGYWFT	IQIKATAGY	0.370812	3.48
DRB1_1454	116	TGGNSVQESIRFGLV	VQESIRFGL	0.370514	3.48
DRB1_1454	117	GGNSVQESIRFGLVL	VQESIRFGL	0.367819	3.54

ondary structure. (Fig. 4).

Gene expression and protein purification

Codon optimization of the fiber protein gene was performed using the Java codon adaptation tool (JCAT) for expression in a eukaryotic system. The optimized gene sequence consisted of 804 nucleotides, encoding a 268 amino acid protein. CAI value of 0.99, and CG-content of 53%, suggest the possibility of expression of protein in eukaryotic system (Fig. 5). The optimized construct was cloned into the pET and then transformed into *E. coli* SHuffle cells, a strain engineered for enhanced disulfide bond formation

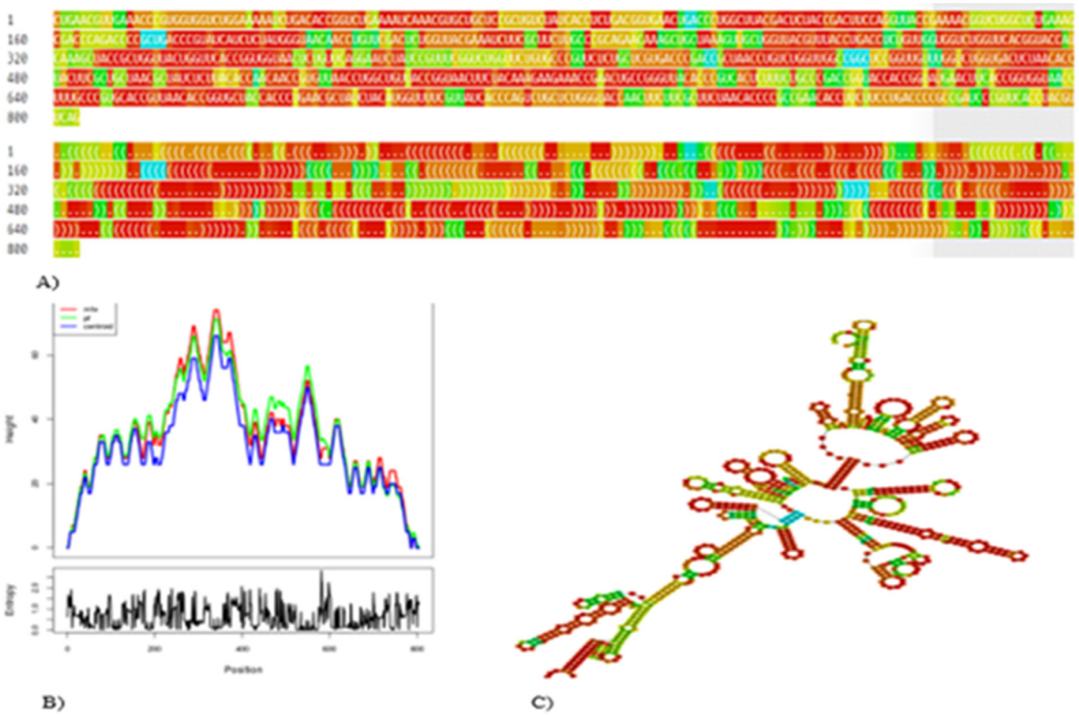
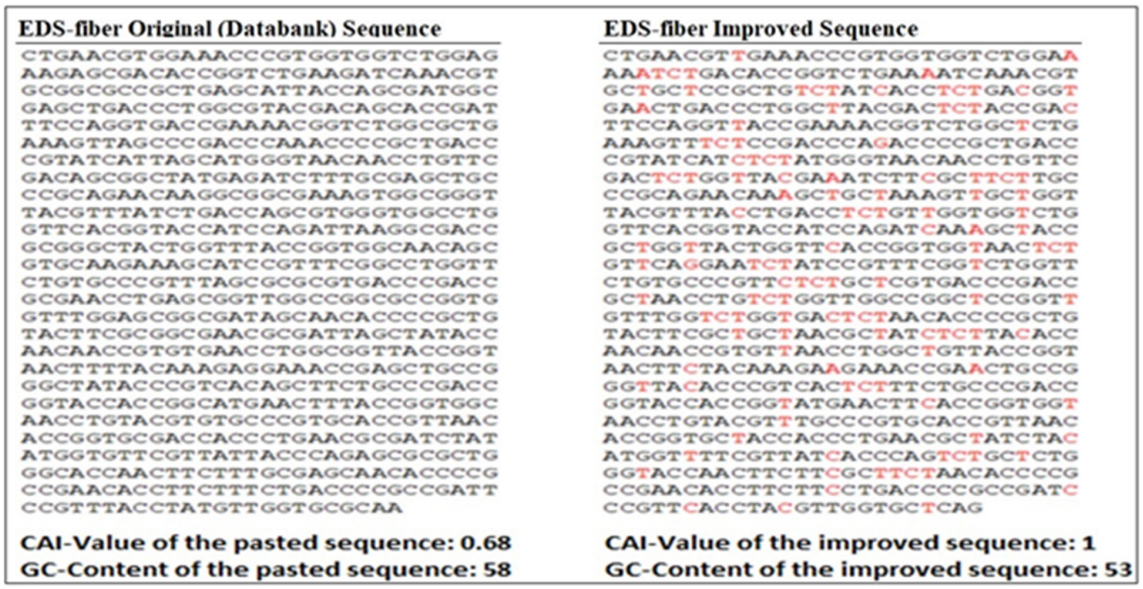


Figure 4.
Predicted mRNA secondary structure by RNA fold server: A) Evaluation of the minimum free energy. The optimal secondary structure with the minimum free energy is indicated in the dot-bracket notation, which is colored by the positional entropy. The shown interactive drawing represents the RNA structure with the minimum free energy, colored based on the probabilities of base pairing. [((())) stem; . loop; ,,,,,, internal loop]; B) The mountain plot of the MFE structure, RNA structures thermodynamic ensemble, and the centroid structure. Plateaus correspond to loops in the mountain plot (with hairpin loops representing peaks), while slopes represent helices. (The red, green and blue lines referred to the minimum free energy (MFE), the probability of the pair (PF) and, the centroid algorithm, respectively. Also, the positional entropy was indicated below; C) The mRNA secondary structure.


EDS-fiber Original (Databank) Sequence
CTGAACGTGGAAACCCGTGGTGGTCTGGAG
AAGAGCGACACCGGTCTGAAGATCAAACGT
GCGGCGCCGCTGAGCATTACCAGCGATGGC
GAGCTGACCCTGGCGTACGACAGCACCGAT
TTCCAGGTGACCGAAAACGGTCTGGCGCTG
AAAGTTAGCCCGACCCAAACCCCGCTGACC
CGTATCATTAGCATGGGTAACAACCTGTTC
GACAGCGGCTATGAGATCTTTGCGAGCTGC
CCGCAGAACAAGGCGGCGAAAGTGGCGGGT
TACGTTTATCTGACCAGCGTGGGTGGCCTG
GTTCACGGTACCATCCAGATTAAGGCGACC
GCGGGCTACTGGTTTACCGGTGGCAACAGC
GTGCAAGAAAGCATCCGTTTCGGCCTGGTT
CTGTGCCCGTTTAGCGCGCGTGACCCGACC
GCGAACCTGAGCGGTTGGCCGGCGCCGGTG
GTTTGGAGCGGCGATAGCAACACCCCGCTG
TACTTCGCGGCGAACGCGATTAGCTATACC
AACAACCGTGTGAACCTGGCGGTTACCGGT
AACTTTTACAAAGAGGAAACCGAGCTGCCG
GGCTATACCCGTCACAGCTTCTGCCCGACC
GGTACCACCGGCATGAACTTTACCGGTGGC
AACCTGTACGTGTGCCCGTGCACCGTTAAC
ACCGGTGCGACCACCCTGAACGCGATCTAT
ATGGTGTTCGTTATTACCCAGAGCGCGCTG
GGCACCAACTTCTTTGCGAGCAACACCCCG
CCGAACACCTTCTTTCTGACCCCGCCGATT
CCGTTTACCTATGTTGGTGCGCAA
CAI-Value of the pasted sequence: 0.68
GC-Content of the pasted sequence: 58
EDS-fiber Improved Sequence
CTGAACGTTGAAACCCGTGGTGGTCTGGAA
AAATCTGACACCGGTCTGAAAATCAAACGT
GCTGCTCCGCTGTCTATCACCTCTGACGGT
GAACTGACCCTGGCTTACGACTCTACCGAC
TTCCAGGTTACCGAAAACGGTCTGGCTCTG
AAAGTTTCTCCGACCCAGACCCCGCTGACC
CGTATCATCTCTATGGGTAACAACCTGTTC
GACTCTGGTTACGAAATCTTCGCTTCTTGC
CCGCAGAACAAAGCTGCTAAAGTTGCTGGT
TACGTTTACCTGACCTCTGTTGGTGGTCTG
GTTCACGGTACCATCCAGATCAAAGCTACC
GCTGGTTACTGGTTCACCGGTGGTAACTCT
GTTCAGGAATCTATCCGTTTCGGTCTGGTT
CTGTGCCCGTTCTCTGCTCGTGACCCGACC
GCTAACCTGTCTGGTTGGCCGGCTCCGGTT
GTTTGGTCTGGTGACTCTAACACCCCGCTG
TACTTCGCTGCTAACGCTATCTCTTACACC
AACAACCGTGTTAACCTGGCTGTTACCGGT
AACTTCTACAAAGAAGAAACCGAACTGCCG
GGTTACACCCGTCACTCTTTCTGCCCGACC
GGTACCACCGGTATGAACTTCACCGGTGGT
AACCTGTACGTTTGCCCGTGCACCGTTAAC
ACCGGTGCTACCACCCTGAACGCTATCTAC
ATGGTTTTCGTTATCACCCAGTCTGCTCTG
GGTACCAACTTCTTCGCTTCTAACACCCCG
CCGAACACCTTCTTCCTGACCCCGCCGATC
CCGTTCACCTACGTTGGTGCTCAG
CAI-Value of the improved sequence: 1
GC-Content of the improved sequence: 53
A)

Index	Description	Range	Value
GC3	GC Content at the Third Position of Synonymous Codons	0~1	0.76
GC	GC Content		0.59
GC1	GC Content at the First Position of Synonymous Codons		0.51
CAI	Codon Adaptation Index	0~1	0.91
CFD	Codon Frequency Distribution	0~1	0
FOP	Frequency of Optimal Codons	0~1	0.74
CBI	Codon Bias Index	-1~1	0.61

B)

Figure 5.
Optimization of the synthetic gene for expression of the recombinant protein: A) Optimization by Jcat; B) Analysis by GenRCA Rare Codon Analysis Tool.

in cytoplasmic proteins. Following induction, SDS-PAGE analysis revealing a prominent band at approximately 30 kDa, consistent with the predicted molecular weight of the monomeric fiber protein subunit (Fig. 6).

Thermal stability evaluation

SDS-PAGE analysis of the recombinant fiber protein under different treatment conditions revealed distinct oligomeric states (Figs. 6A, 7). Under native (non-boiled) conditions, the protein migrated as

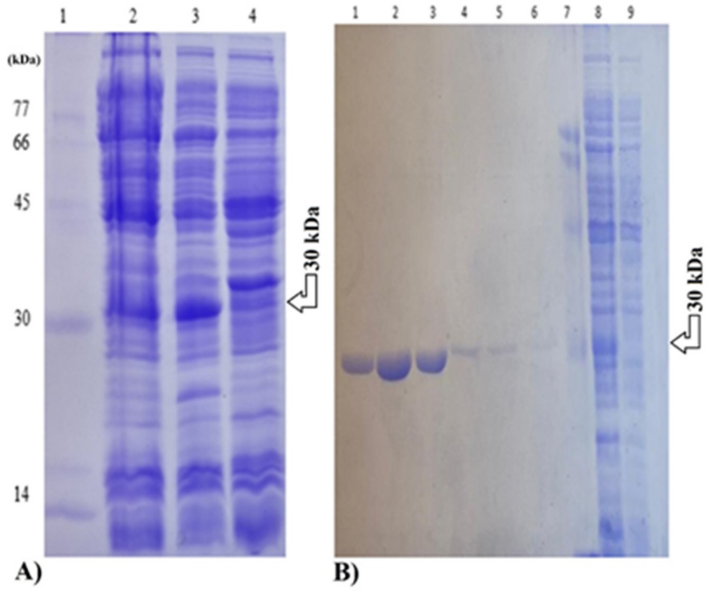


Figure 6.
Protein pattern of the LB crude extract and purified fiber protein in the SDS-PAGE: A) SDS-PAGE analysis of level of expression of fiber protein in LB broth, Lane1= Protein molecular weight marker (77-14 kDa), lane2= Soluble part of supernatant containing fiber protein, Lanes3 and 4= Pellet of E. coli shuffle including pET-fiber not and induced with IPTG respectively, grown in LB broth; B) Expression and purification of fiber protein (PET28-fiber) as a candidate vaccine in E. coli cells by SDS-PAGE, Lane 7= Protein molecular weight marker; lane 9= Non induced, lane8= Induced E. coli cells bearing fiber protein-PET28 plasmid, lane 1 to 6= Elution

two predominant bands corresponding to molecular weights of 30 kDa (monomeric form) and 65 kDa (trimeric form), with the trimeric form representing the primary conformation. This trimeric structure demonstrated remarkable stability, maintaining its oligomeric state even in the presence of SDS at room temperature. Upon thermal denaturation by boiling prior to electrophoresis resulted in complete dissociation of trimers, yielding exclusively monomeric 30 kDa band. Notably, pre-incubation at 4°C and 37°C, prior to analysis did not affect these migration patterns, suggesting temperature-independent stability of the protein's oligomeric states under non-denaturing conditions. Parallel ELISA results further confirmed that the purified recombinant fiber protein maintained strong antigenic properties after storage at both temperatures (4°C and 37°C), as shown in the Figure 7B.

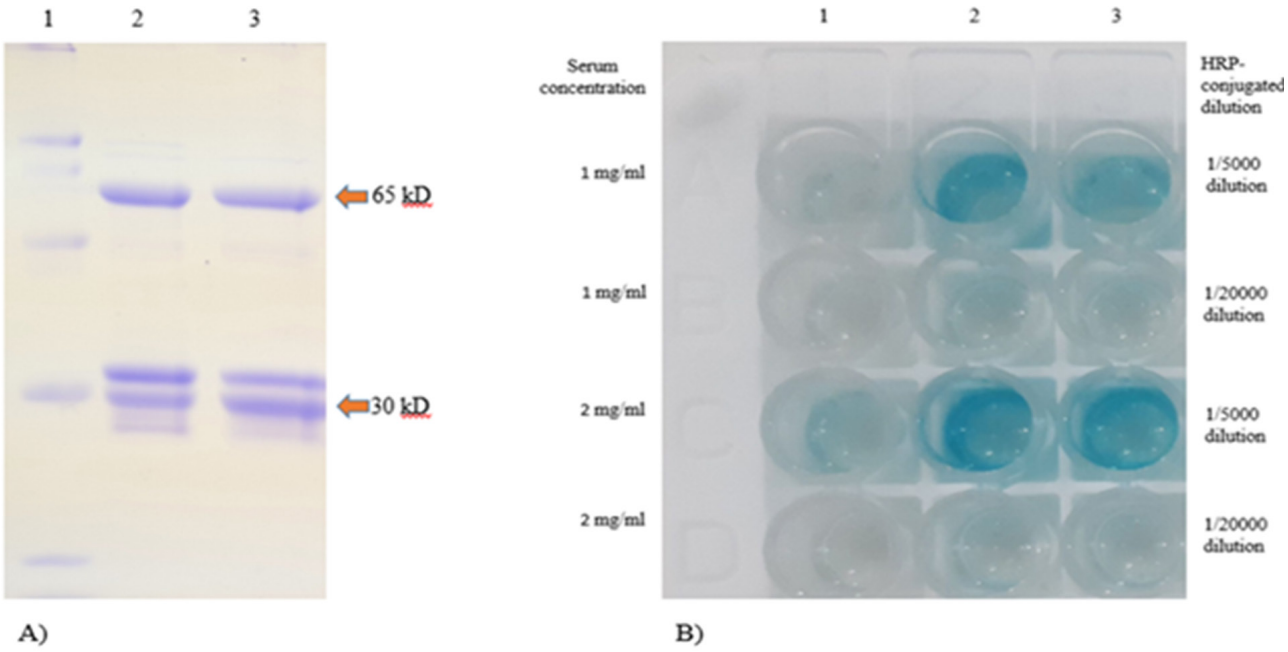


Figure 7.
Thermal stability evaluation by SDS-PAGE and ELISA. A) SDS-PAGE of purified fiber protein: lane 1, Protein molecular weight marker (77-14 kDa), lane2; Protein fiber stored in 4° C, lane3; Protein fiber stored in 37° C. B) ELISA assay in order to evaluate antigenicity. lane1; negative control, lane2; Protein fiber stored in 4°C that coated as antigen, lane3; Protein fiber stored in 37° C that coted as antigen.

Discussion

Developing effective vaccines against avian pathogens remains a major challenge due to the time-consuming and costly nature of traditional vaccine production methods [13]. Recent advances in computational immunology and structural biology have enabled more rational vaccine design approaches. In this study, we combined comprehensive bioinformatics analyses and experimental characterization of the EDSV fiber protein as a potential vaccine candidate. Our findings are consistent with the emerging trends in epitope-based vaccine design, building upon successful examples such as immunoinformatics approach for MARV and cross-protective vaccine for Mpox and EBOV. These studies collectively highlight the transformative potential of computational tools in identifying stable, immunogenic vaccine candidates while significantly reducing development timelines [14-16]. Our study provide crucial insights for vaccine development, one of the key outcomes of our work is the confirmation of trimeric stability through both computational modeling using the Galaxy server [17] and experimental validation via SDS-PAGE, with the protein maintaining structural integrity at physiological conditions (37°C for 14 days). This stability was further supported by favorable physicochemical parameters, including an aliphatic index of 77.16 and instability index of 23.39. The design of our construct, strategically incorporating both the knob domain and partial shaft region (268amino acid), was guided by previous studies demonstrating their critical role in maintaining proper trimer formation and antigenicity [7, 18].

Preservation of conformational epitopes (CE1-CE3), each exhibiting more than 63% surface exposure, underscores potential of this protein to elicit a strong and protective immune responses. From a production standpoint, the recombinant protein demonstrated several advantageous characteristics. The *E. coli* Shuffle [19] strain facilitated efficient expression with high yield, producing both monomeric (30 kDa) and trimeric (65 kDa) forms. The mRNA stability (MFE= -255.60 kcal/mol) and retained antigenicity post-temperature challenge (as validated by ELISA) further support the practical utility of this vaccine candidate. These results are particularly encouraging given the well-documented challenges of maintaining adenovirus fiber protein stability during production and storage. However, several important questions and limitations remain that should be addressed in future. The compatibility of this antigen with various adjuvants requires systematic evaluation, as adjuvant selection can significantly impact both immunogenicity and stability [20]. Additionally, comprehensive in vivo studies in chicken models will be essential to confirm the protective efficacy suggested by our in silico and in vitro results. Long-term storage stability and large-scale production optimization must also be thoroughly assessed to support commercial applications [10]. Structural refinement through techniques like cryo-EM could provide atomic-level insights into the trimer conformation and potential stabilization strategies [21]. This study makes significant contributions to multiple aspects of vaccine development. It demonstrates how integrating computational predictions with experimental approaches can streamline rational vaccine design, particularly for veterinary applications. By focusing on thermal stability, we address two critical limitation in vaccine distribution, especially relevant for resource-limited settings. Moreover, the design framework established here for EDSV could be readily adapted for other avian pathogens, potentially transforming poultry vaccine development pipelines [22]. Despite promising results, we acknowledge certain limitations. The absence of in vivo immunogenicity data remains a key gap. Likewise, the effects of adjuvants on both stability and immunogenicity remain to be characterized, and scale-up production parameters need optimization. Nevertheless, our multidisciplinary approach exemplifies how modern computational tools can accelerate and improve vaccine development while maintaining rigorous scientific standards [14]. By combining robust bioinformatics predictions with systematic experimental confirmation, this study establish a strong foundation for developing an effective EDSV vaccine while minimizing animal use in preliminary testing stages.

Conclusion

In this study, we investigated the EDSV fiber protein using a comprehensive in silico and experimental method. The result of structural and immunological analyses indicate that this recombinant protein possesses strong potential as a preventive vaccine candidate. Its demonstrated stability, antigenicity, and expression efficiency support its feasibility for further vaccine development. Although more validations are needed, to fully establish its protective efficacy, the EDSV fiber protein can be used as a vaccine candidate against EDSV.

Materials and Methods

Sequence selection and design of construct

The amino acid sequence of the partial fiber protein A0A482J640, comprising the knob domain and a segment of the shaft region, was obtained from the UniProt Knowledgebase (https://www.uniprot.org/) in FASTA format.

Prediction of physicochemical properties

Various physicochemical parameters of the designed fiber protein were predicted using the ProtParam tool. Parameters assessed included molecular weight, theoretical isoelectric point (pI), amino acid composition and count, atomic composition, chemical formula, extinction coefficients, estimated half-life, aliphatic index, instability index, and grand average of hydropathicity (GRAVY). Additionally, protein solubility was evaluated using the SOLpro (http://scratch.proteomics.ics.uci.edu/) and Protein-Sol (https://protein-sol.manchester.ac.uk/) servers.

Secondary structure prediction of fiber protein

Secondary structure elements of the fiber protein were performed using GOR IV and PSIPRED servers. Both algorithms calculated probabilities for α-helix, β-sheet, and random coil at each amino acid position. The conformation with the highest probability structure was chosen as the predicted conformation [23, 24].

3D structures prediction

Protein 3D structure prediction was performed using three computational servers, Phyre2, I-TASSER, and GalaxyWeb, [25-27]. Among these, I-TASSER server, which provides a confidence

score (C-score) for model evaluation, was chosen for final modeling based on comparative results. The Galaxy server generated the trimer structure. Model quality was verified through Ramachandran plot analysis and ProSA-web evaluation [28, 29].

Antigenicity prediction

Antigenicity Evaluation of the designed fiber protein was performed using VaxiJen version 3.0, (https://www.ddg-pharmfac.net/vaxijen3/home/) and ANTIGENpro servers. Both tools predict antigenicity based solely on Physicochemical properties of antigens [30].

B-cell epitopes prediction

Computational prediction of B-cell epitopes, both continuous (linear) and discontinuous (structural) was performed using multiple servers to ensure cross-validation. The EliPro server scoring was used to identify linear and structural epitopes based on a score, ranging from 0 to 1 and a cutoff of 0.5, where scores higher than 0.5 were considered antigenic [31]. Additional epitope prediction was conducted using SVMTriP [32] and ABCpred servers. . The ABCpred Prediction Server predict B cell epitopes in a protein sequence [33], using artificial neural network.

MHC class I and II binding prediction

The NetMHCcons 1.1 server was employed to predict MHC class I binding affinities. The threshold for strong binding peptides was determined as an IC50 value of less than 2 nM, and cutoff of 50 nM has been set for weak binders, that is, peptides with IC50< 2 nM were considered as strong binders and those with IC50 < 50 nM as weak binders [34]. Fiber protein sequences (shaft + knob) were submitted to the NetMHCcons 1.1 server. The NetMHCIIpan 4.0 algorithm was employed to identify potential MHC-II binding epitopes. The fiber protein was cleaved into peptides with a length of 15 amino acids in the server. Among these peptides, only those peptides that were strong binders (%Rank < 2) for MHC alleles were selected [35]. In this study, based on previous studies the allele (HLA*B 40:06) was used for MHC class I and the allele (DRB1:1482) was used for MHC class II [36]. Using MHCcluster software, Thomsen et al showed that human MHC-I/II alleles are the best substitute for its homologues in chicken [37].

mRNA structure prediction

To evaluate transcript stability, the RNAfold server was used to analyze the minimum free energy (MFE), which is a loop-based energy model presented by Zucker et al., for the secondary structures formed by RNA molecules [38].

Optimization of the synthetic gene

Codon optimization and reverse translation were conducted using the Java Codon Adaptation Tool (JCat) online software. Optimization parameters were set to maximize the codon adaptation index (CAI) for cloning and expression in *E. coli* K12. [39]. The NEB Cutter Server (New England Biolabs) was used to identify and confirm the presence of common restriction enzyme sites in the nucleotide sequence of the designed construct (http://www.labtools.us/nebcutter-v2-0/). Parameters such, codon adaptation index (CAI), and GC content were also analyzed and codon adaptation index (CAI), and GC content are also evaluated.

Protein expression and purification protocol

Following gene synthesis by ShineGene Co., the fiber protein gene was subcloned into the pET-28a vector (Novagen, 2023) to create recombinant pET-fiber plasmids. This constructs were subsequently transformed into *E. coli* SHuffle strain competent cells [19] using the heat shock method [40]. Transformed colonies were selected through overnight growth at 37°C in LB medium supplemented with 50 µg/mL kanamycin. For protein expression, starter cultures were diluted 1:100 into fresh LB-kanamycin medium and grown at 37°C with shaking until reaching mid-log phase (OD600= 0.4-0.6), typically requiring 3-5 hours. Protein expression was then induced by adding 0.5 mM, IPTG [41], followed by overnight incubation at 17°C with 150 rpm shaking to enhance proper protein folding. After induction, following centrifugation at 5,000 ×g for 10 min, cell pellets were collected and resuspended in lysis buffer for subsequent protein extraction. Initial expression analysis was performed by resolving whole cell lysates on 12.5%, SDS-PAGE gels. The recombinant protein was subsequently purified under native conditions using Ni-NTA Sepharose Fast Flow resin (ARG Biotech, Iran) to isolate the His-tagged fusion protein."

Thermal stability of the recombinant protein

Thermal stability analysis of the purified recombinant protein was assessed under two storage conditions: 1-incubation at 37°C for 14 days to simulate physiological temperature stress, and 2- refrigeration at 4°C as a stability control. Protein integrity was assessed through 12.5%, SDS-PAGE under both conditions. Antigenic properties were examined using a standardized ELISA protocol with the following steps: 96-well microplates were coated overnight at 4°C with 1 µg/mL of recombinant fiber protein in carbonate-bicarbonate coating buffer (100 mM $NaHCO_3$, pH 9.6). After four washes with PBS, plates were blocked for 1 hour with 1%, bovine serum albumin (BSA) in PBS-T (PBS containing 0.05%, Tween 20). Serial dilutions (1 mg/mL and 2 mg/mL) of chicken immune serum (containing EDSV-specific antibodies from inactivated vaccine immunization) were added and incubated for 1 hour at room temperature. Post-washing with PBS (3×5 min), plates were incubated with horseradish peroxidase-conjugated goat anti-chicken IgG secondary antibody for 60 minutes at 25°C with gentle shaking (Razi Biotech Co. Iran) at two working dilutions (1:5000 and 1:20000 in PBS-T). Tetramethylbenzidine (TMB) substrate (0.01% 3,3',5,5'-Tetramethylbenzidine, 99% Water, 0.1% Hydrogen peroxide, pH 3.3-4.0), which is a common substrate for horseradish peroxidase (HRP) and suitable for enzyme-linked immunosorbent assay (ELISA), was added for color development. Then, this reaction was stopped with 2M, H_2SO_4, and absorbance was measured at 450 nm using a microplate spectrophotometer.

Authors' Contributions

We confirmed that all authors were involved in writing this article. AHC as a supervisor of the Ph.D. candidate, in the conceptualization, and methodology; JN as a Ph.D. candidate, performed the experiment and data collection, analysis and interpretation of results, writing- original draft preparation; AM Managed the preparation of laboratory tools, experimental data collection and analysis.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript..

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