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Designing a vaccine candidate against egg drop syndrome virus: an *in silico* approach

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Abstract

Today, a combination of immunological and bioinformatics tools has become common for vaccine design, making vaccine production affordable. Considering the importance of recombinant protein purification for vaccine production in a cost-effective way, our study aimed to *in silico* design a fusion protein vaccine against egg drop syndrome virus (EDSV) with a higher isoelectric point for more affordable purification. The *in silico* design of fusion protein, including egg white lysozyme and fiber protein as an antigen from egg drop syndrome virus, was performed. In addition to isoelectric point changing, lysozyme probably helps the antigenicity by increasing the size of the antigen. Also, lysozyme can act as a preservative. The physicochemical characteristics, stability, secondary and tertiary structure, epitope prediction, antigenicity, and mRNA structure were analyzed using computational and bioinformatics tools. The results showed that the isoelectric point of the gene construct was 8.87, which can be purified by ion exchange chromatography. Validation of the Ramachandran plot showed that the predicted model was accurate and suitable. The tertiary structure of the fusion protein was modeled as well, and its trimer structure, being required for immunogenicity, was preserved. The antigenicity of the target construct was also suitable. Protein was stable and hydrophilic based on aliphatic index and grand average of hydropathicity, which can be a good candidate for a vaccine. After experimental studies, this fusion protein may be used as a vaccine against EDSV.

Introduction

Egg drop syndrome virus (EDSV) is a member of the *Atadenovirus* genus of the *Avian adenovirus* family. The EDSV is a non-enveloped virus with double-stranded DNA.[1] The outbreak of EDSV was first reported in the Netherlands in 1976.[2] In EDS, laying hens produce low-quality eggs with clinical symptoms, including thin-shelled, soft, or shell-less eggs.[3] The EDSV infects healthy laying birds, flocks of turkeys, geese, and quails.[4] The organization of the complete genome sequence of EDSV is similar to that of other adenoviruses.[5] The capsid of the virus consists of 11 proteins,[6] among which the fiber protein plays a role in the interaction of the virus with cells of the host.[7] Fiber protein is composed of three domains, including tail, shaft, and knob.[8] The knob domain plays a role in the initiation of binding, causing the virus entry to the cell,[7,9] which could be a good candidate for a recombinant vaccine against adenoviruses causing infectious diseases in chickens.[10] The inactivated EDS vaccine was developed in 1977.[11] The need to develop new vaccines that are cost-effective and provide adequate immunity against avian adenovirus infections seems essential. The fiber protein encoded by the genome of all adenoviruses is the main capsid protein and is highly immunogenic.[12] Today, the EDSV vaccine is prepared using duck eggs and is widely used. Due to the lack of access to duck-specific pathogen-free duck eggs, non-specific commercial duck eggs are used to produce vaccines against EDSV.[13] Due to the sensitivity of ducks to avian pathogens,[14] this practice has a significant risk of spreading pathogens and should be avoided. The use of subunit vaccines that are prepared from parts of the surface proteins of the virus can overcome these limitations.[15-17] Recombinant technology has made it possible to make new vaccines. Efficient immunization by recombinant vaccines has been reported for several pathogenic viruses. A review by Hein *et al.*, has shown that more than 15 recombinant viral vector

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vaccines against Newcastle disease, infectious laryngotracheitis, infectious bursal disease, avian influenza, and *Mycoplasma gallisepticum* have been developed and are commercially available.[18]

In this study, we proposed adding egg lysozyme to fiber protein and designing a new fusion protein as a vaccine candidate against EDSV for several reasons. First, the fiber protein contains important epitopes and its antigenicity has been demonstrated in previous studies.[13,19] Second, lysozyme can help protein purification by ion exchange method by changing isoelectric point (pI) of the fusion protein. Third, lysozyme can act as a preservative in the vaccine composition. Fourth, this fusion protein can help the immune response by increasing the size of the antigen. On the one hand, the goal of this study was to utilize bioinformatics tools for the purpose of developing a new recombinant fusion protein, which will function as a subunit vaccine against EDSV, which contains the complete sequence of egg white lysozyme in the N-terminal region and Knob fragment, and 60 amino acids of the fiber protein shaft region located in the C-terminal region. Previous studies have shown that fiber protein is the best vaccine candidate.[13,19] Also, considering that the effective fiber protein as a vaccine candidate, after expression using the *Escherichia coli* expression system, has limitations due to the presence of inclusion bodies,[13] in this study, the gene construct was optimized for expression in the eukaryotic system.

Materials and Methods

Sequence selection and design of construct. In the present study, the amino acid sequence of the fiber protein, partial A0A482J640, was retrieved from UniProt Knowledgebase data at https://www.uniprot.org/ in FASTA (FAST-All) format. In addition, the amino acid sequence of lysozyme (P00698) was retrieved from UniProt Knowledgebase data at in FASTA format. The knob domain and part of the shaft from fiber proteins and the entire lysozyme sequence were joined together to create a new construct.

***In silico* analysis of signal peptide (SP).** Considering that lysozyme is secretory, in addition to the lysozyme SP, three others SPs were also investigated in terms of their ability to secrete the target protein. The amino acid residues of SP were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) and UniProt (https://www.uni-prot.org/) databases. The significant characterization of the SPs was investigated by several major computational and predicting tools, namely Signal Peptide Prediction version 4.1., Signal-CF, and Phobius.

Prediction of physicochemical properties. The physicochemical parameters of the fusion protein were predicted using the ProtParam tool available in https://web.expasy.org/protparam/.[20] The ProtParam was used to predict various physicochemical properties of the designed fusion protein, including molecular weight, theoretical pI, number of amino acids, amino acid composition, atomic composition, chemical formula, extinction coefficients, estimated half-life, aliphatic index, instability index, and grand average of hydropathicity.

Secondary structure prediction of the chimeric protein. In this study, the amino acid sequence was used as an input for secondary structure prediction. The Garnier-Osguthorpe-Robson IV (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html) and PSI-blast based secondary structure PREDiction (http://bioinf.cs.ucl.ac.uk/psipred/) were used to calculate the probability of the α-helix, β-sheet, and random coil at each amino acid position, and the initial prediction with the highest probability.[21]

Prediction of the chimeric protein three-dimensional (3D) structures. Various servers, including Protein Homology/AnalogY Recognition Engine, Iterative Threading ASSEmbly Refinement (I-TASSER), and Galaxy web servers, were used to model the 3D structure.[22-24] Finally, according to the results of the above servers, the I-TASSER server (http://zhanglab.ccmb.med. umich.edu/I-TASSER/) was used as a server for modeling the protein structure. To analyze the accuracy of predicted structures, I-TASSER uses a confidence score called C-score. Also, the Galaxy server (https://galaxy.seoklab. org/) was used to model the protein trimer structure. The designed protein was also evaluated and validated by Ramachandran plot available in https://saves.mbi.ucla. edu/ and Protein Structure Analysis (ProSA)-web, being available in https://prosa.services.came.sbg.ac.at/prosa.php.[25]

Antigenicity prediction. Antigenicity evaluation of the recombinant protein was performed using VaxiJen version 2.0, which is available at (https://www.jenner.ac.uk/VaxiJen) and can predict antigenicity based solely on physical and chemical properties of antigens.[26] Threshold scores greater than 0.50 were considered to indicate possible antigens. The ANTIGENpro server was used to evaluate antigenicity (https://scratch.proteomics.ics.uci.edu/).

Epitope prediction. Due to the fact that this vaccine candidate is used against EDSV in chickens and the selected lysozyme was taken from chickens, only fiber protein was evaluated for epitope prediction.

B-cell epitopes prediction. Both continuous and discontinuous B-cell epitopes were predicted for fiber (knob + shaft) protein sequences using the Ellipsoid and Protrusion server (http://tools.iedb.org/ellipro/).[27] The Ellipsoid and Protrusion server scoring is based on a score of 0.00 to 1.00 and a cut-off of 0.50, where a score higher than 0.50 is considered an epitope and a score less than 0.50 is considered a non-epitope. The support vector machine tri-peptide (SVMTriP) server (http://sysbio.unl.edu/SVMTriP/prediction.php), which is a method to predict antigenic epitopes using support vector machine to

integrate tri-peptide similarity and propensity, and antigen B-cell prediction (ABCpred) server (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html), to predict B-cell epitopes in an antigen sequence using an artificial neural network, were used.

Major histocompatibility complex (MHC) class I and class II binding prediction. The NetMHCcons (network-based on MHC consensus alleles) method, is a consensus method, was used by NetMHCcons 1.1 server (https://services.healthtech.dtu.dk/services/NetMHCcons-1.1/), for prediction MHC I binding affinity. The threshold for strong binding peptides was determined as a half-maximal inhibitory concentration value of less than 2.00 nM, and cut-off of 50.00 nM was set for weak binders; thus, peptides with half-maximal inhibitory concentration< 2.00 nM were considered as strong binders and those with half-maximal inhibitory concentration< 50.00 nM as weak binders.[28] Fiber protein sequences (shaft + knob) were submitted to the NetMHCcons 1.1 server. The NetMHCII-pan 4.0 server was used to predict the binding of peptides to MHC-II molecules (https://services.healthtech.dtu.dk/services/NetMHCIIpan-4.0/). The fiber protein was cleaved into peptides with a length of 15 amino acids in the server. Among these peptides, only the peptides that were strong binders (%rank < 2.00) for MHC alleles were selected.[29] In this study, based on previous studies, the allele HLA*B 40:06 was used for MHC-I and the allele DRB1:1482 was used for MHC-II.[30] Using MHCcluster software, Thomsen *et al*., have shown that human MHC-I/II alleles are the best substitute for the homologues in chicken.[31]

mRNA structure prediction. The RNA fold server (http://rna.tbi.univie.ac.at//cgibin/RNAWebSuite/RNAfold.cgi) was used to analyze the minimum free energy (MFE), a loop-based energy model presented by Zuker and Stiegler, for the secondary structures formed by RNA molecules.[32]

Optimization of the synthetic gene for expression of the recombinant protein. Reverse translation and codon optimization for the mentioned protein fusion were done using Java Codon Adaptation Tool online software (https://www.jcat.de/). [33] The NEB Cutter Server (New England Biolabs) was used to find the presence of common restriction enzyme sites in the nucleotide sequence of the designed construct (http://www.labtools.us/nebcutter-v2-0/). Considering that origin of the eukaryotic cells is diverse, it seems essential to evaluate the compatibility of the constituent genes in these cells, as well as the potential for the expression of the desired genes in these cells. In this study, the nucleotide sequence of the fusion protein was optimized for expression in the eukaryotic system (in general). Parameters, such as codon adaptation index and guanine-cytosine content, were also analyzed.

Results

Sequence selection and design of construct. In this study, the complete sequence of egg lysozyme was attached to the knob domain and part of the shaft from fiber proteins. The His-tag (histidine-tagged), also called 6xHis-tag, was used for purification and also the thrombin cleavage site was used for experimental studies (Fig. 1).

Physicochemical parameters evaluation. The pI and molecular weight of the protein were calculated as 8.87 and 46.19 kDa, respectively. Aliphatic and grand average of hydropathicity indices were defined 77.42 and – 0.100, respectively. According to the results obtained from the ProtParam server, the half-lives of the fusion protein were 30 hr (mammalian reticulocytes; *in vitro*), > 20 hr (yeast; *in vivo*), and > 10 hr (*E. coli*; *in vivo*). Our fusion protein based on the aliphatic index was stable at different temperatures, and a low grand average of hydropathicity index indicated that our protein was considered a hydrophilic protein with 426 amino acid, theoretical isoelectric point = 8.87, extinction coefficient at 280 nm = 72,725, instability index= 22.68, aliphatic index = 77.42, and grand average hydropathy= – 0.10. Also in this fusion protein, 25 and 34 amino acids had negative and positive charged, respectively. Proteins with an instability index higher than 40.00 may be unstable, but our fusion protein was stable with an instability index of 22.68.

***In silico* analysis of signal peptides (SPs).** Four SPs amino acid sequences were selected for *in silico* analysis, which are used for protein expression, and the SP of egg lysozyme had the highest D-score (Fig. 2).

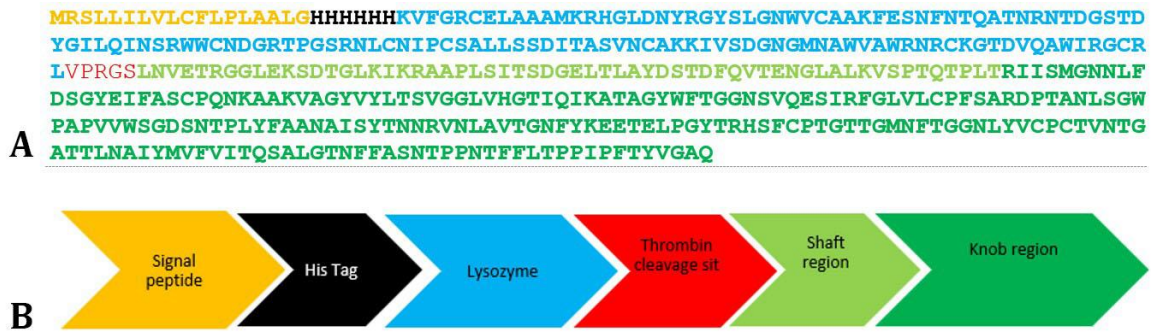
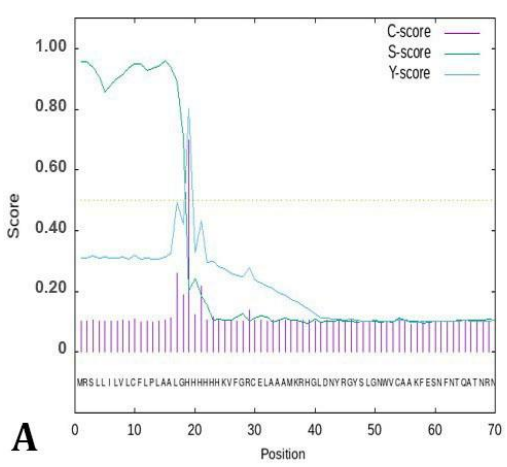
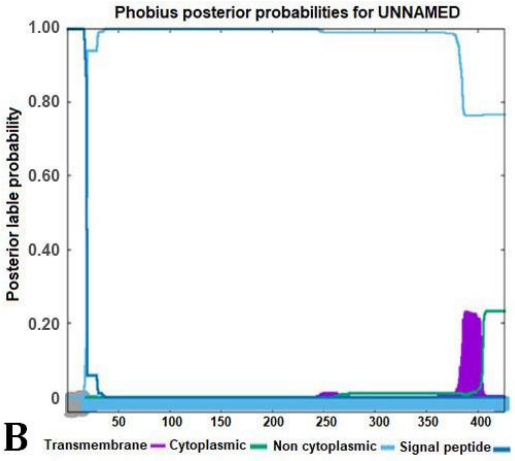


Fig. 1. Amino acid sequence and schematic design of the fusion protein. **A)** Amino acid sequences, and **B)** Schematic design of the designed gene construct. Amino acid residues of the fusion protein are shown by different colors.

Secondary structures evaluation. The results obtained from Garnier-Osguthorpe-Robson IV server showed that the secondary structure in our protein contained 10.56% α-helix, 32.16% β-sheet, and 57.28% coiled-coil. According to Garnier-Osguthorpe-Robson IV results, it can be seen that random coils are dominant in the fusion protein sequence, followed by extended strands and alpha helices (Fig. 3). Also, using the PSI-blast based secondary structure PREDiction server, the secondary structure and graphical representation of our protein were shown (Fig. 4).



A



B

C

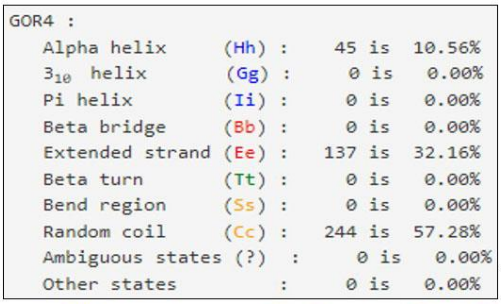
Sequences of four signal peptides and their sources	
Signal peptides	**Source**
MEFGLSWVFLVALFRGVQC	Antibody
MGVKVLFALICIAVAEA	*Gaussia luciferase*
MKWVTFISLLFLFSSAYS	Human albumin
MRSLLILVLCFLPLAALG	Egg lysozyme

D

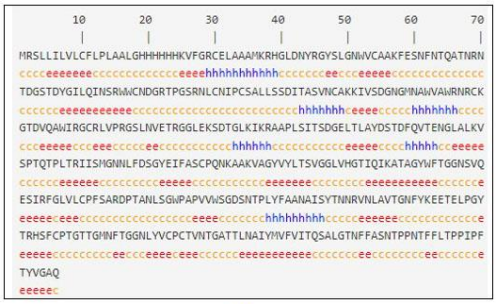
Prediction of UNNAMED

ID	UNNAMED			
FT	SIGNAL	1	18	
FT	REGION	1	3	N-REGION.
FT	REGION	4	14	H-REGION.
FT	REGION	15	18	C-REGION.
FT	TOPO_DOM	19	426	NON CYTOPLASMIC.

Fig. 2. Sequences of four signal peptides and their analysis by Signal Peptide Prediction version 4.1 (SignalP4.1) and Phobius prediction for the fusion protein of recombinant. **A)** Prediction by SignalP4.1, **B)** Prediction by Phobius, **C)** Sequences of four signal peptides and their sources, and **D)** Different fusion protein regions, predicted by Phobius server. Signal peptide = 'YES' cleavage site between positions 18 and 19, ALG-HH D = 0.863, D-cutoff = 0.450, and Networks = SignalP-noTM.



A



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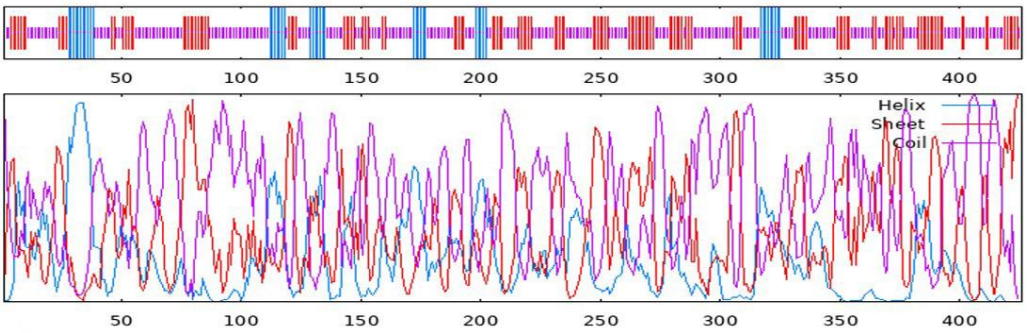


C

Fig. 3. Fusion protein secondary structure predicted by GOR4 server. **A)** The composition of the secondary structure in percent, **B)** Fusion protein secondary structure map (h = alpha-helix, e = extended strand, and c = coil), and **C)** Diagram of fusion protein secondary structure. The blue color represents the alpha-helices, the red color represents beta strands, and the purple color represents the coils.

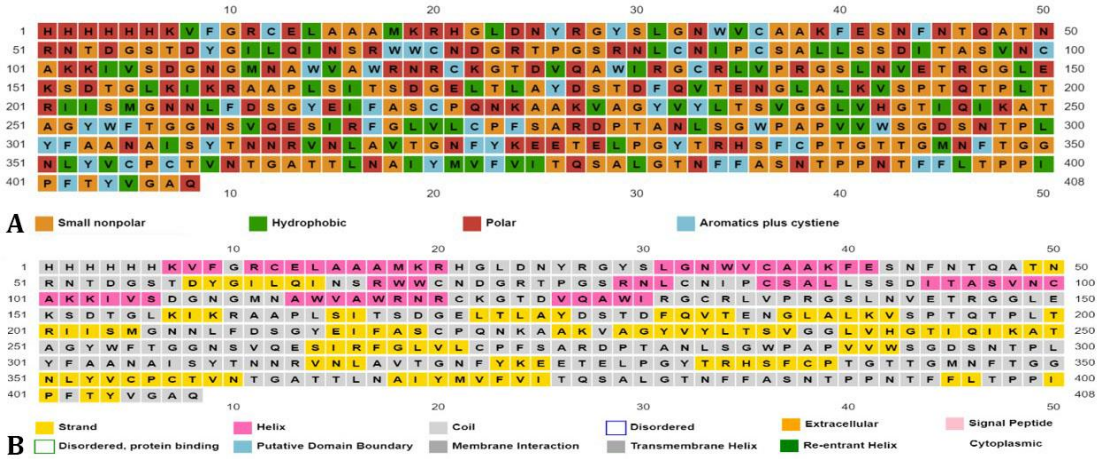


Fig. 4. Secondary structure and graphical representation predicted by PSI-blast based secondary structure PREDiction. **A)** Types of amino acids, and **B)** Types of secondary structures. The pink blocks represent the alpha-helices, the yellow blocks represent beta strands, and the black thread-like structures are the coils.

Prediction and validation of the chimeric protein three-dimensional structures. The Protein Homology/ AnalogY Recognition Engine, I-TASSER, and Galaxy servers were used for 3D structure modeling. The evaluation of the results of different servers showed that the I-TASSER server was suitable for modeling the 3D structure of our protein. The scoring system of I-TASSER server is based on C-score. The C-score range is usually between – 5 and 2; models with higher C-score were selected for further evaluations (Fig. 5). Fusion protein refinement was done using GalaxyRefine server in order to achieve higher quality (Fig. 5). Regarding the role of trimer structure in antigenicity and binding to the host cell, the trimer structure of the mentioned construct was modeled by the Galaxy web server. The evaluation of the Ramachandran diagram showed that, 96.00%, 2.60%, and 0.90% of residues were located in favored, allowed, and outlier regions, respectively (Fig. 6). In order to determine the potential errors in the 3D structures, the analysis of the models was done by ProSA-web. The ProSA Z-score on the input model was -5.81 (Fig. 6).

Antigenicity evaluation. Based on the results obtained from ANTIGENpro and Vaxijen servers, the thresholds of 0.40 were 0.93 and 0.57%, respectively. The results obtained from the above servers showed that, with a high probability, our fusion protein was antigenic.

Prediction of B-cell epitopes. Analysis of linear B-cell epitopes of fiber protein predicted using by different online-servers. Results showed target protein when analysis by Ellipsoid and Protrusion server contains 11 epitopes (with score > 0.548). But its analysis by SVMTriP and ABCpred servers were showed 3 and 10 epitopes, respectively (Table 1). Results of discontinuous epitope prediction by Ellipsoid and Protrusion server are shown in Table 2. Predicted conformational epitopes (CE) include CE1, CE2, CE3, and CE4, having scores greater than 0.50. The first predicted CE, CE1, shows a peptide length of 68 residues. The score for this epitope is 0.685, signifying that 68.50% of the amino acids are exposed to the surface. So, the second discontinuous epitope, CE2, shows a peptide length of 35 residues, and 65.90% of the total 35 amino acid residues are on the outer surface of the structure. The CE3 has a peptide length of 55 residues, and 63.70% of the total 55 amino acid residues are on outer surface of the structure. The higher surface exposure recommends these CE as promising candidates for vaccine development.

Major histocompatibility complex-I binding prediction. The MHC- I binding prediction and MHC-I processing tool retrieved 259 nonamers from fiber protein. Three peptide fiber protein sequences (QESIRFGLV, GELTLAYDS, and TENGLALKV) were determined, being likely bound with MHC-I alleles, with percentage rank of ≤ 2.00 (Table 3).

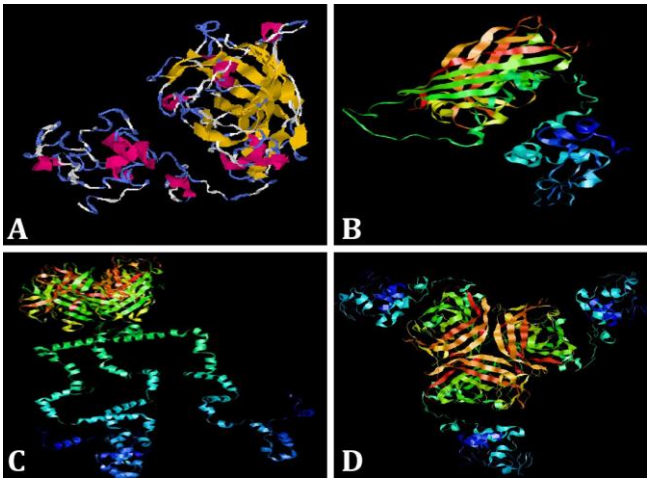


Fig. 5. Tertiary structure prediction; a probabilistic structural model for lysozyme- fiber protein by online servers. **A)** Monomer prediction by Iterative Threading ASSEmbly Refinement server, **B)** Monomer prediction by GalaxyRefine, **C)** Prediction of trimer structure by GalaxyGemini, and **D)** Prediction of trimer structure by Galaxyhomomer.

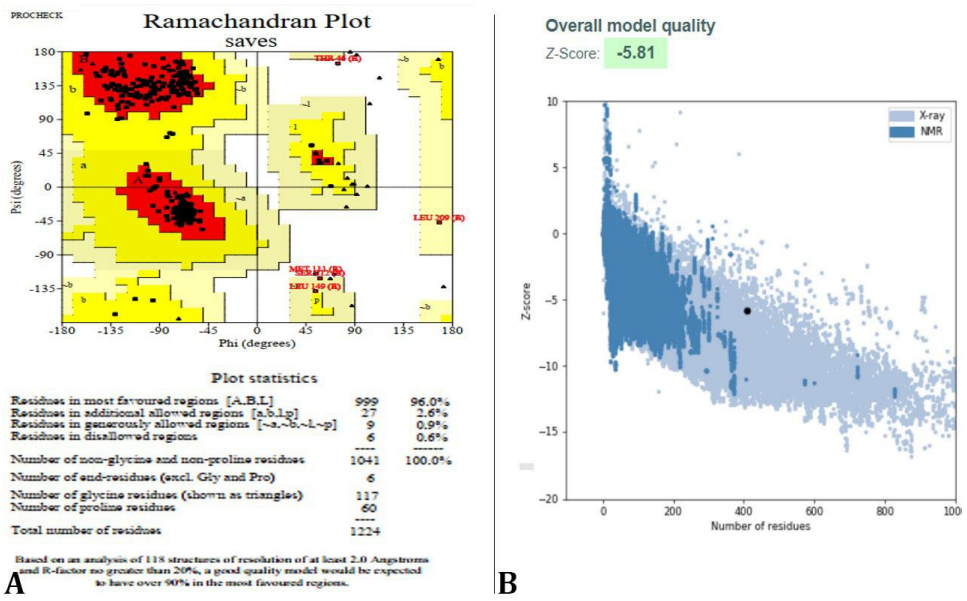


Fig. 6. Model stability evaluation based on Ramachandran plot for fusion protein of lysozyme and fiber. **A)** Ramachandran plots for trimer structure fusion protein by PROCHEC (Protein Checker), which checks the stereochemical quality of a protein structure (https://www.ebi.ac.uk/thornton-srv/software/ PROCHECK/), and **B)** Protein structure analysis by Protein Structure Analysis -web server.

Table 1. Linear B-cell epitopes of fiber protein predicted using by SVMTriP, Ellipsoid and Protrusion, and ABCpred servers.

Rank	Residual location	Amino acid sequences in the epitopes	Scores
A) Predict epitopes in SVMTriP server			
1	91 - 110	YVYLTSVGGLVHGTIQIKAT	1.00
2	173 - 192	RVNLAVTGNFYKEETELPGY	0.807
3	131 - 150	LCPFSARDPTANLSGWPAPV	0.605
B) Predict epitopes in Ellipsoid and Protrusion server			
1	35-59	AYDSTDFQVTENGLALKVSPTQTPL	0.790
2	236-254	TQSALGTNFFASNTPPNTF	0.790
3	218-226	TVNTGATTL	0.784
4	200-208	TGTTGMNFT	0.737
5	135-141	SARDPTA	0.663
6	110-124	TAGYWFTGGNSVQES	0.661
7	165-192	NAISYTNNRVNLAVTGNFYKEETELPGY	0.646
8	68-75	NLFDSGYE	0.597
9	9-24	LEKSDTGLKIKRAAPL	0.591
10	153-158	SGDSNT	0.567
11	26-29	ITSD	0.548
C) Predict epitopes in ABCpred server			
1	201-216	GTTGMNFTGGNLYVCP	0.93
2	118-133	GNSVQESIRFGLVLCP	0.92
3	153-168	SGDSNTPLYFAANAIS	0.90
3	143-158	LSGWPAPVVWSGDSNT	0.90
4	164-179	ANAISYTNNRVNLAVT	0.89
5	210-125	GNLYVCPCTVNTGATT	0.88
5	108-123	KATAGYWFTGGNSVQE	0.88
6	30-45	GELTLAYDSTDFQVTE	0.87
7	52-67	VSPTQTPLTRIISMGN	0.86
7	243-258	NFFASNTPPNTFFLTP	0.86
7	236-251	TQSALGTNFFASNTPP	0.86
8	72-87	SGYEIFASCPQNKAAK	0.85
8	3-18	VETRGGLEKSDTGLKI	0.85
8	102-117	HGTIQIKATAGYWFTG	0.85
9	61-76	RIISMGNNLFDSGYEI	0.83
10	133-148	PFSARDPTANLSGWPA	0.80

A higher score of the peptide means a higher probability of being an epitope. All these peptides were above the threshold value chosen.

Table 2. Conformational B-cell epitope of fiber protein vaccine predicted using the Ellipsoid and Protrusion server.

No.	Peptide regions and residues number	Residues number	Score
CE1	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
CE2	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
CE3	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
CE4	A:V151, A:S153, A:G154, A:D155, A:S156, A:N157, A:T158	7	0.548

Table 3. Major histocompatibility complex-I binding epitopes predicted by the NetMHCcons 1.1 server.

Allele	Residual location	Peptides	1-log50k (aff)	Affinity (nM)	Rank (%)
HLA-B40:06	29-37	GELTLAYDS	0.247	3441.22	2.00
HLA-B40:06	43-51	TENGLALKV	0.330	1407.46	1.50
HLA-B40:06	121-129	QESIRFGLV	0.287	2244.37	1.50

Major histocompatibility complex-II binding prediction. To MHC- II binding prediction, fiber protein was cleaved into 254, 15-mer peptides using NetMHC 4.0 server. The LTRIISMGNNLFDSG were considered to be strong MHC-II binders. The optimal peptide binding core of this peptide was IISMGNNLF (Table 4).

Prediction of mRNA structure. Based on the results of the RNA fold server, the MFE of the RNA structure was –501.20 kcal mol^{-1}, and it can be concluded that the gene structural sequence can form a stable RNA secondary structure. The MFE of the thermodynamic ensemble was –518.05 kcal mol^{-1} (Fig. 7).

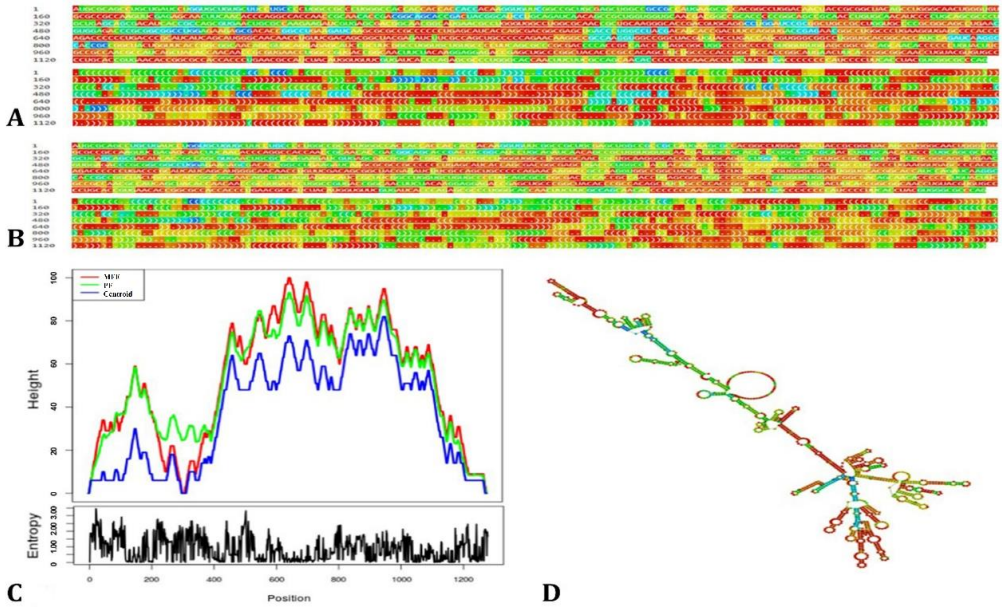


Fig. 7. Predicted mRNA secondary structure by RNA fold server. **A)** Evaluation of the minimum free energy (MFE): The optimal secondary structure with the MFE is indicated in the dot-bracket notation, which is colored by the positional entropy, **B)** The shown interactive drawing represents the RNA structure with the minimum free energy, colored based on the probabilities of base pairing. [((())) stem; …. loop; „„„ internal loop], **C)** 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 are referred to the MFE, probability of the pair (PF), and centroid algorithm, respectively. Also, the positional entropy is indicated below, and **D)** The mRNA secondary structure of fusion protein.

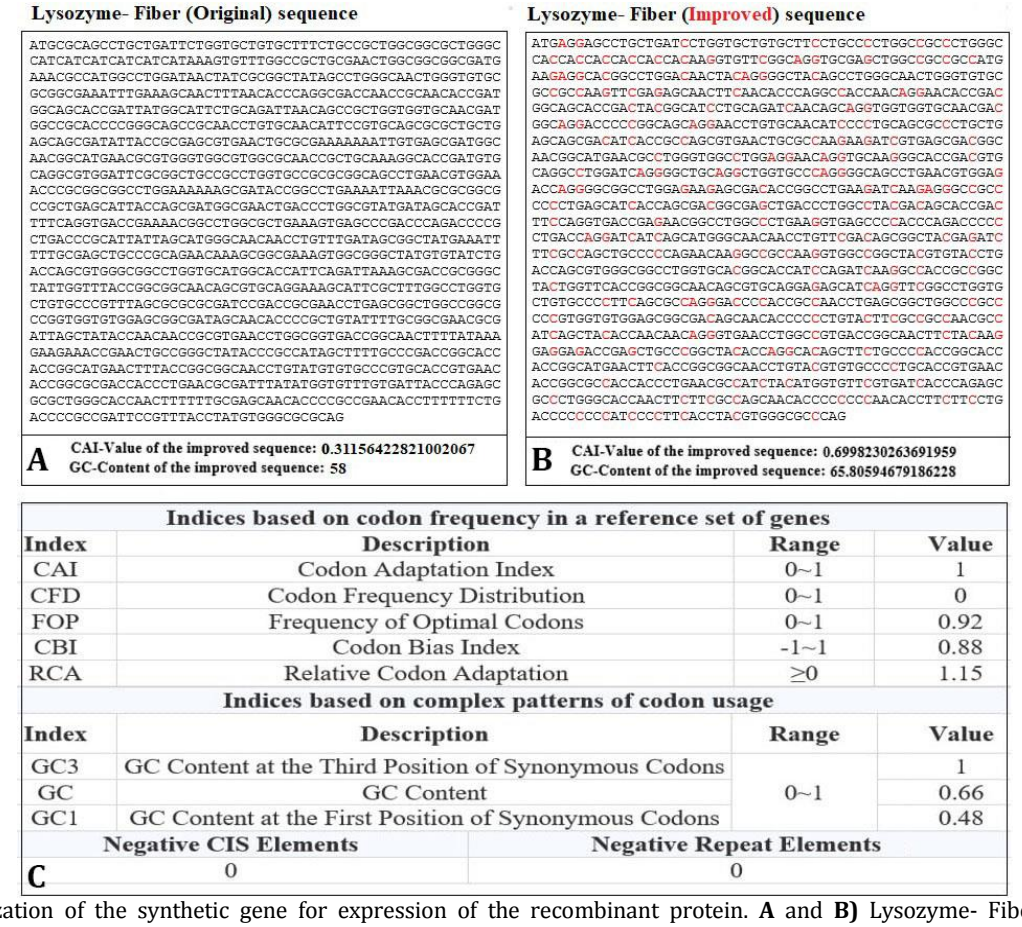
Table 4. Major histocompatibility complex-II binding epitopes predicted by the NetMHC 4.0 server.

Alleles	Residual location	Peptides	Core sequence	Score Eluted ligand	Percentile rank
DRB1_1454	13-27	DTGLKIKRAAPLSIT	LKIKRAAPL	0.367271	3.55
DRB1_1454	14-28	TGLKIKRAAPLSITS	IKRAAPLSI	0.340573	4.11
DRB1_1454	15-29	GLKIKRAAPLSITSD	IKRAAPLSI	0.419096	2.66
DRB1_1454	58-72	PLTRIISMGNNLFDS	IISMGNNLF	0.393941	3.04
DRB1_1454	59-73	LTRIISMGNNLFDSG	IISMGNNLF	0.464146	2.10
DRB1_1454	60-74	TRIISMGNNLFDSGY	IISMGNNLF	0.358547	3.73
DRB1_1454	101-15	VHGTIQIKATAGYWF	IQIKATAGY	0.336206	4.22
DRB1_1454	102-116	HGTIQIKATAGYWFT	IQIKATAGY	0.370812	3.48
DRB1_1454	116-130	TGGNSVQESIRFGLV	VQESIRFGL	0.370514	3.48
DRB1_1454	117-131	GGNSVQESIRFGLVL	VQESIRFGL	0.367819	3.54

Optimization of the synthetic gene for expression of the recombinant protein. Codon adaptation and *in silico* cloning of fusion protein was performed by Java Codon Adaptation Tool in order to codon optimization in eukaryotic expression system. The optimized codon sequence length for our construct with 426aa was 1,278 nucleotides. The codon adaptation index value for optimized nucleotide sequence was 0.99, and guanine-cytosine content of the sequence was 67.35%, showing the possibility of expression of fusion protein in the eukaryotic systems (Fig. 8).

Discussion

Vaccine production is time-consuming and expensive. Researchers are able to design more efficient vaccines due to recent advances in immunology and computer science.[34-37] Of course, there are shortcomings related to *in silico* design, including the accuracy of prediction and translation under *in vivo* and *in vitro* conditions, which need to be improved. In the present study, bioinformatics and immunology approaches were used to design a fusion protein consisting of lysozyme sequence and fiber protein



Indices based on codon frequency in a reference set of genes			
Index	**Description**	**Range**	**Value**
CAI	Codon Adaptation Index	0~1	1
CFD	Codon Frequency Distribution	0~1	0
FOP	Frequency of Optimal Codons	0~1	0.92
CBI	Codon Bias Index	-1~1	0.88
RCA	Relative Codon Adaptation	≥0	1.15
Indices based on complex patterns of codon usage			
Index	**Description**	**Range**	**Value**
GC3	GC Content at the Third Position of Synonymous Codons	0~1	1
GC	GC Content		0.66
GC1	GC Content at the First Position of Synonymous Codons		0.48
Negative CIS Elements		**Negative Repeat Elements**	
0		0	

C

Fig. 8. Optimization of the synthetic gene for expression of the recombinant protein. **A** and **B)** Lysozyme- Fiber (Original and Improved) sequences, which optimization by JCat (Java Codon Adaptation Tool), and **C)** Optimization results of GenRCA (gene rare codon analysis) Tool; CAI: Codon adaptation index; GC: Guanine-Cytosine; Optimized nucleotides in the Lysozyme- Fiber (Improved) sequence showed red color font.

against EDSV. In previous studies, Harakuni *et al*., have demonstrated that the knob domain lacking the shaft sequence but fused to a trimeric coiled coil (chicken cartilage matrix protein) is a promising candidate subunit vaccine for the prophylaxis of EDS in chickens. [38] Song *et al.*, have suggested the truncated fiber protein as an effective single dose, long lasting, and rapidly effective vaccine to protect against EDSV.[19] Although previous studies have shown that the fiber protein has a good antigenicity,[13,19,38] its purification by nickel affinity chromatography on an industrial scale is not cost-effective. Different studies have shown that the fiber protein has three main domains, the antigenicity of the knob part has been shown, and the antigenicity of the fiber protein has been found to be related to its trimer structure.[13,19] Song *et al*., have reported that the shaft region plays an important role in forming the trimer structure in fiber protein and thus, is required to produce a stable trimer antigen. They showed that the knob domain with 60 amino acids adjacent to the shaft region played a role in the solubility and structure maintenance of the trimer structure of the protein expressed in *E. coli*.[19] For this reason, these two parts of the fiber protein, the knob and part of the shaft, must be present in our gene construct, which the *in silico* results showed that was preserved in this study after modeling the trimer structure. We analyzed the fiber protein of adenovirus, using bioinformatics tools, and evaluated the protective efficacy of its epitope regions in fusion with lysozyme as vaccine candidates for providing protection against EDSV in chickens. It is important to note that the knob and shaft region (268 amino acids at the C-terminal end of fiber protein) provided protection when used as a subunit vaccine in previous studies.[13,19] In the present study, 3D structural analysis of lysozyme and fiber protein by means of the online server I-TASSER revealed an appropriate spatial structure. Also, Galaxy web server results showed that the trimer structure of protein fusion was formed. Validation of the structure using the Ramachandran plot to evaluate the quality of the experimental structures and predict the biological function of the protein is another important parameter in protein structural prediction. In this study, due to the fact that the presence of the linker would reduce the allowed areas in the Ramachandran plot, the linker was not used. According to the obtained results, based on the high percentage of residues in favorable areas and low percentage in unfavorable areas, our desired protein was considered a good model. Physicochemical parameters of chimeric proteins should also be considered in vaccine design .For example, the aliphatic index reflects the thermal stability of a protein. Herein, our fusion protein was thermostable with an aliphatic index of 77.42, and this index is determined based on the volume occupied by the side chains of aliphatic amino acids, such as alanine, valine, isoleucine, and leucine. Also, according to the instability index which was 22.68, our fusion protein was stable. The stability of mRNA structure is also important, which often depends on the MFE, representing the stability of the hairpin structure of secondary stem-loop of mRNA, and the RNA molecule with a lower MFE is more stable.[39] The precursor mRNA sequences of our gene construct showed an MFE of – 501 kcal mol^{-1}. Low protein expression in the host is a limitation of antigen design.[40] Therefore, optimizing the codon compatibility index in the present study can improve the chimeric gene expression level. The main goal of this study was to design a fusion protein that can be purified by ion exchange chromatography. Since purification by affinity method is not economical, in this study, lysozyme was used for purification by ion exchange chromatography. After designing the structure, the pI was changed to 8.87, which can be useful for ion exchange purification. Different methods of liquid chromatography are used to separate biological molecules.[41] Ion exchange chromatography is one of the most powerful types of liquid chromatography and is still widely used to separate molecules with different charges.[42] Several advantages of ion exchange chromatography, including the analysis of a large number of molecules, industrial scale production with low cost, and high levels of purification of the desired molecule, have made it an efficient method in purification.[43] Lysozyme has a molecular weight of 14.00 kDa and a pI of 10.50 - 11.00. The high pI of lysozyme allows efficient and cost-effective extraction.[44] Lysozyme can also act as a preservative. Egg white lysozyme is the most solved and most stable protein among other lysozymes, being currently used with anti-bacterial and anti-viral properties as a pharmaceutical ingredient in medicine and veterinary medicine, as well as food industry, but it does not have a toxic or lytic effect on the membrane of animal and yeast cells.[45,46] Another point that was desired in this study was that increasing the size of the antigen can help the immune response. In this study, lysozyme can increase the size of fiber protein antigen, whose antigenicity was previously proven. The size of the antigen particles is considered an important parameter for the generation of immune response but with varied results.[47,48] Studies have shown that larger particles also generate antibody response.[49] The vaccine subunit designed in previous studies, included the knob domain and a part of the shaft region, was produced as inclusion bodies in *E. coli*, required dialysis and refolding.[13] Hence, we designed the gene construct for expression in a eukaryotic system. In this study, we introduced a novel fusion protein as a candidate vaccine against EDSV. Our bioinformatics results showed that this protein was a stable chimeric protein, and it can be used as a candidate vaccine against EDSV. Also, due to the increase in pI, it can be purified by an ion exchange method. Because of the limitations of *in silico* methods for predicting physico-chemical properties, structures, and immunogenicity,[50] the

protective efficacy of the designed recombinant vaccine candidate must be confirmed in an experimental study.

In this study, chimeric protein, including fiber protein and lysozyme, was investigated by *in silico* method. Based on the structural and immunological results, this protein fusion can be used as a preventive vaccine; although, more experimental studies are needed, and if confirmed using experimental studies, it can be used as a vaccine candidate against EDSV in the future.

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Conflicts of interest

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

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