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Original Article

Design, in silico optimization, and generation of recombinant rabies virus glycoprotein: Paving the Way for Enhanced Vaccines

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Abstract

Rabies is a fatal disease that may be transmitted from animals to humans. It is caused by a virus called rabies virus (RABV), which is a kind of RNA virus belonging to the family *Rhabdoviridae* and the genus Lyssavirus. Current rabies vaccines, while effective, require multiple doses for adequate protection, which presents significant financial and logistical challenges, especially in low-resource settings. Additionally, the risk of reversion to virulence in live-attenuated vaccines limits their use. This study aims to design and optimize the rabies virus glycoprotein (G protein) using in silico methods to address these challenges and develop a more effective and accessible rabies vaccine. The nucleotide sequences of the rabies virus glycoprotein were acquired from GenBank (accession number LT839616) and optimized to improve expression in Chinese Hamster Ovary (CHO) cells using bioinformatics tools. PSIPRED was employed for secondary structure prediction, and SWISS-MODEL was used for 3D structure modeling. The optimized gene was synthesized and inserted into the pcDNA3 vector. CHO cells were transfected with the recombinant plasmid, and the presence of the expressed gene was confirmed using RT-PCR and Western blot analysis. Optimizing the codons significantly enhanced the synthesis of the G protein derived from the rabies virus in CHO cells. Structural analyses confirmed the stability and proper conformation of the protein. The gene was successfully subcloned into the pcDNA3 vector, and its expression in CHO cells was verified using RT-PCR and Western blot analysis, demonstrating the effective production of the recombinant glycoprotein. This study successfully utilized bioinformatics and experimental methodologies to optimize the rabies virus G protein, demonstrating its potential as a viable vaccine candidate. The results provide a strong foundation for developing an advanced rabies vaccine that is both effective and accessible, particularly in regions where rabies remains prevalent.

Keywords: Rabies virus, Rabies glycoprotein, Codon optimization, Eukaryotic expression system, Bioinformatics, In silico methods, Chinese Hamster Ovary (CHO) cells.

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1. Introduction

Rabies is a fatal zoonotic disease caused by the rabies virus (RABV), a single-stranded RNA virus belonging to the family *Rhabdoviridae* and the genus *Lyssavirus* [1, 2]. The disease primarily affects the central nervous system (CNS), leading to fatal encephalitis if left untreated [3, 4]. Despite the availability of post-exposure prophylaxis, which includes wound treatment, vaccination, and rabies-specific immunoglobulin [5], rabies remains a significant public health concern, particularly in low-resource regions where dog bites are the primary mode of transmission [6]. Globally, rabies causes approximately 59,000 deaths annually, with 95% of cases occurring in Africa and Asia, and an average of nine deaths per year reported in Iran [7-11].

The rabies virus genome encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large protein (L) [12, 13]. Among these, the glycoprotein (G protein) plays a critical role in viral entry into host cells by facilitating attachment to host receptors and inducing endocytosis [14-16]. Importantly, the G protein is the primary antigen that stimulates the immune response against the virus, making it a key target for vaccine development [17, 18].

Current rabies vaccines, which are primarily inactivated vaccines, require multiple injections for both pre-exposure and post-exposure immunization to achieve effective protection [19]. This requirement presents financial and logistical challenges, particularly in low-resource settings [20]. Although live-attenuated vaccines offer a more durable immune response, their use is limited due to the risk of reversion to virulence, which could lead to disease in vaccinated individuals [21-23]. Therefore, there is a pressing need for the development of more effective and safer rabies vaccines.

Recent advances in computational biology and bioinformatics have opened new avenues for vaccine development. In silico methods, such as homology modeling, molecular dynamics simulations, and docking studies, provide valuable insights into protein structure and function, aiding in the identification and optimization of vaccine candidates. The rabies virus G protein, given its crucial role in viral entry and immune system activation, is an ideal candidate for such computational optimization [17, 24].

The primary objective of this study is to utilize in silico methodologies to optimize the rabies virus G protein, enhancing its expression in mammalian cells and evaluating its potential as a vaccine candidate. This approach aims to address the challenges associated with current rabies vaccines by developing a more effective and accessible vaccine, particularly for regions where rabies remains a significant public health issue. By integrating computational and experimental approaches, this study seeks to pave the way for the development of an advanced rabies vaccine that could significantly reduce the global burden of this deadly disease.

2. Materials and Methods

2.1. Sequence Retrieval and Analysis

The rabies virus glycoprotein (G protein) nucleotide and protein sequences were retrieved from the GenBank database at the National Center for Biotechnology Information

(NCBI), specifically under the accession number LT839616 (35009_RABV_CVS). To improve expression efficiency in Chinese Hamster Ovary (CHO) cells, these sequences were optimized using the Integrated DNA Technologies (IDT) tool. This optimization process involved modifying codon usage bias, decreasing GC content, and removing potential inhibitory motifs. The resulting optimized sequences were then stored in FASTA format for subsequent analyses.

2.2. Epitope Prediction and Analysis

The secondary structure of the optimized rabies virus glycoprotein was predicted using the PSIPRED v4.0 server (http://bioinf.cs.ucl.ac.uk/psipred/). PSIPRED was chosen for its high accuracy and reliability in secondary structure prediction, which has been validated across a wide range of protein structures. The tool utilizes neural networks trained on large datasets of known protein structures, making it particularly robust for predicting alpha-helices, beta-sheets, and random coils within protein sequences. This tool provides crucial information for understanding the protein's overall folding and functional domains, which is essential for subsequent stages of vaccine development (**Table 1**).

Table 1. Primer sequences and expected PCR product length**.**

Forward (5'→3')	AGTCAAGAAAAGAGAGGAAT
Reverse (5'→3')	CCGTTTGTACACATCGGGGA
Length (nt)	450

2.3. Disulfide Bond Prediction

The potential formation of disulfide bonds within the optimized G protein was predicted using the CYS_REC tool (http://www.softberry.com/berry.phtml?topic=cys_rec&group=programs&subgroup=propt). This tool identifies cysteine residues within the protein sequence and predicts the likelihood of disulfide bond formation between pairs of cysteines, which is critical for the stability and proper folding of the protein.

2.4. 3D Structure Prediction and Analysis

The three-dimensional structure of the optimized rabies virus glycoprotein was predicted through homology modeling. The sequence was submitted to the SWISS-MODEL (https://swissmodel.expasy.org/) server to create a 3D model based on existing structures of similar glycoproteins. The resulting structure was visualized and analyzed with PyMOL and Chimera software. To evaluate the stereochemical quality of the protein model, Ramachandran plot analysis was conducted using the RAMPAGE server. Molecular docking between the final structure of the protein and the known antibodies, after preparation and energy minimization of each target protein was performed using the ClusPro 2.0 server (https://cluspro.org). TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) was used to identify potential transmembrane helices. TMHMM uses a hidden Markov model to predict the topology of membrane proteins and distinguishes between soluble and membrane proteins with high accuracy [25]. SWISS-MODEL was selected due to its

widespread use and proven accuracy in generating reliable 3D protein models. The tool is well-regarded in the scientific community for its ability to produce high-quality structural predictions, which are essential for understanding the protein's stability and function in the context of vaccine development.

2.5. Vector Construction

The optimized G protein gene, which included the identified epitopes, was synthesized by a commercial gene synthesis provider (GeneArt, Thermo Fisher Scientific) and inserted into the pcDNA3 eukaryotic expression vector. This cloning process utilized specific restriction enzymes (*EcoRI* and *XhoI*) to incorporate the gene into the vector's multiple cloning site. The resulting recombinant plasmid, named pcDNA3-RVGP, was confirmed through restriction enzyme digestion and sequencing.

2.6. Cell Culture and Transfection

The CHO cells were cultured in DMEM with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified environment with 5% CO2. As directed by the manufacturer, Lipofectamine 2000 (Thermo Fisher Scientific) was used for transfection. CHO cells were seeded at 5×10^5 per well in 6-well plates and transfected with 2 µg pcDNA3-RVGP plasmid DNA. Stable transfectants were selected using G418 sulfate (800 µg/mL).

2.7. Expression and Detection of RVGP

2.7.1. RT-PCR Analysis

The Qiagen RNeasy Mini Kit was employed to extract RNA from both transfected and non-transfected CHO cells. Agarose gel electrophoresis was implemented to confirm the RNA's quality. The Applied Biosystems High-Capacity cDNA Reverse Transcription Kit was employed to perform the reverse transcription. The cDNA obtained was then used as a template for PCR amplification using primers specific to RVGP (**Table 1**). The PCR findings were confirmed and assessed using a UV transilluminator after agarose gel electrophoresis.

2.7.2. Western Blot Analysis

CHO cell lysates, both transfected and non-transfected, were produced using Radioimmunoprecipitation Assay (RIPA) buffer that included protease inhibitors. The Bradfor vd test was used to quantify the protein content. A gel electrophoresis technique called SDS-PAGE was used to separate an equivalent quantity of proteins. The proteins were then transferred to PVDF membranes using a semi-dry transfer device at 20V for 45 minutes. Next, the membrane was stained with Ponceau S solution to verify the effectiveness of the transfer and examined using conventional imaging equipment.

The PVDF membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature to avoid nonspecific binding. The membrane was then incubated overnight at 4°C with a Rabies Virus Glycoprotein Polyclonal Antibody at 1:500 according to the manufacturer's instructions.

After three TBST washes, the membrane was incubated with 1:5000 HPP-coupled secondary antibodies at room temperature for 1

hour. The membrane was washed three times in TBST to remove binding secondary antibodies.

3. Results and Discussion

3.1. Sequence Retrieval and Optimization

The nucleotide and protein sequences of the rabies virus glycoprotein (G protein) were successfully obtained from the GenBank database with the accession number LT839616. After being retrieved, these sequences were refined to enhance their expression in Chinese Hamster Ovary (CHO) cells. The optimization method included altering the codon use to align with the host's preferences, decreasing the GC concentration, and deleting putative inhibitory motifs. The improved sequences were saved in FASTA format and used for future analysis (**Figure 1**). This optimization process is crucial as it directly addresses one of the study's primary objectives: improving the expression efficiency of the G protein in a mammalian system. Similar approaches have been employed in previous studies, such as the work by Pei et al. (2019).

The study demonstrated that codon optimization can significantly enhance the expression and immunogenicity of the rabies virus G protein. By aligning our results with these findings, we further validate the effectiveness of our optimization strategy and underscore the novelty of our approach in applying these techniques to the development of a more effective rabies vaccine.

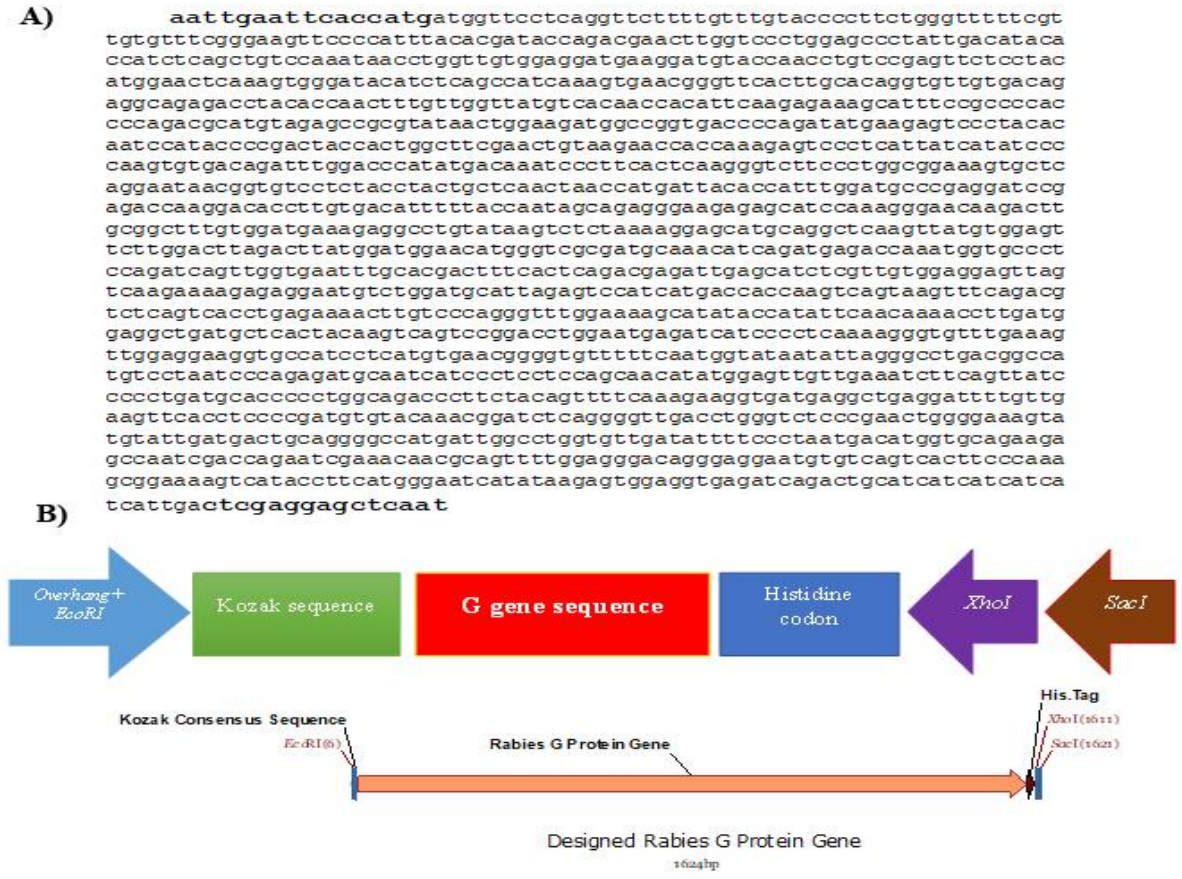

Figure 1. Design and Optimization of the Recombinant Rabies Virus Glycoprotein Gene; A) Nucleotide sequence of the optimized rabies virus glycoprotein (G protein) gene. **B)** The schematic illustration of the designed recombinant gene.

3.2. Secondary Structure Prediction

The PSIPRED v4.0 server (http://bioinf.cs.ucl.ac.uk/psipred/) was used to predict the secondary structure of the optimized rabies virus glycoprotein. This program employs neural networks that have been trained using known protein structures to forecast the positioning of alpha-helices, beta-sheets, and random coils inside the protein sequence. Secondary structure information is crucial for comprehending the protein's complete folding and functional domains (**Figure 2**).

The results of Protparam analysis showed a theoretical pI of 8.43, indicating the basic nature of the vaccine construct, with a negatively charge of 29 at pH 7. The multi-epitope vaccine showed good solubility in water (**Table 2**).

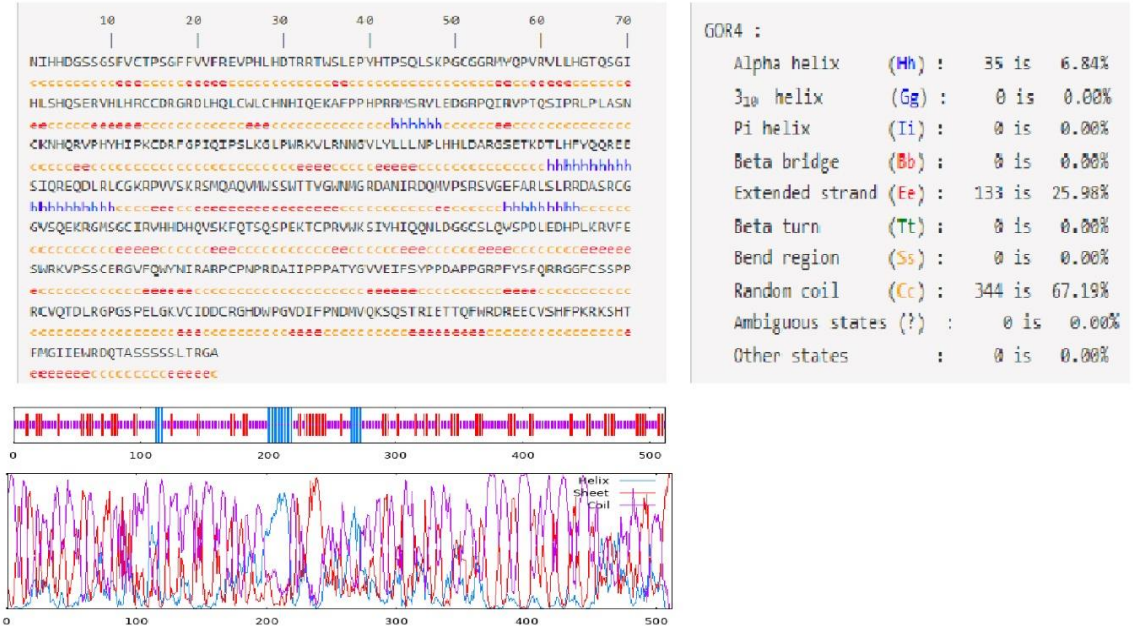

Figure 2. Secondary structure of the rabies glicoprotein. The structure of the vaccine comprises 6.84% alpha helix, 25.98% extended strand, and 67.19% random coil.

Table 2. Computation of diverse physical and chemical properties for the given protein sequence. Multiple physical and chemical properties were computed for the given protein sequence. The criteria include molecular weight, theoretical isoelectric point (pI), amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index, and total hydropathic average (GRAVY). The findings demonstrated that the engineered protein exhibits thermal stability, has an appropriate half-life, and displays hydrophilic properties**.**

Parameter	Value
Number of amino acids	308
Molecular weight	35.4
Theoretical pI	8.43
Half-life	30 hours (mammalian reticulocytes, in vitro), >20 hours (yeast, in vivo), >10 hours (Escherichia coli, in vivo).
Instability index	37.33
Aliphatic index	80.47
GRAVY	-0.267
Cysteine	6 (Ar positions: 12, 23, 90, 229, 234, 270)

3.3. Disulfide Bond Prediction

Disulfide bond prediction using the CYS_REC tool identified several potential disulfide bridges within the glycoprotein structure. These bonds are important for maintaining the stability and proper folding of the protein, contributing to its functional integrity (**Table 1** and **Figure 3**).

3.4. 3D Structure Prediction and Analysis

The three-dimensional structure of the optimized G protein was successfully predicted using the SWISS-MODEL server. The homology modeling process generated a 3D model, which was subsequently visualized and analyzed using PyMOL and Chimera software.

According to the results of the TMHMM program, all recombinant gene-expressed amino acids are likely outside the cell membrane. The Ramachandran diagram also showed protein structural angle limits. A Ramachandran plot showed that most residues in the protein model were stereochemically good (**Figure 4**). The successful prediction of the 3D structure of the G protein is a significant milestone in the study, aligning with our hypothesis that structural optimization would lead to a stable and functional vaccine candidate. This result is consistent with previous research that utilized homology modeling for vaccine development, such as the study by Zhang et al. (2013), which demonstrated the importance of accurate structural modeling in predicting the immunogenicity of viral proteins.

By comparing our results with those studies, we highlight the innovative aspects of our approach, particularly in the context of optimizing the rabies virus G protein for enhanced vaccine efficacy.

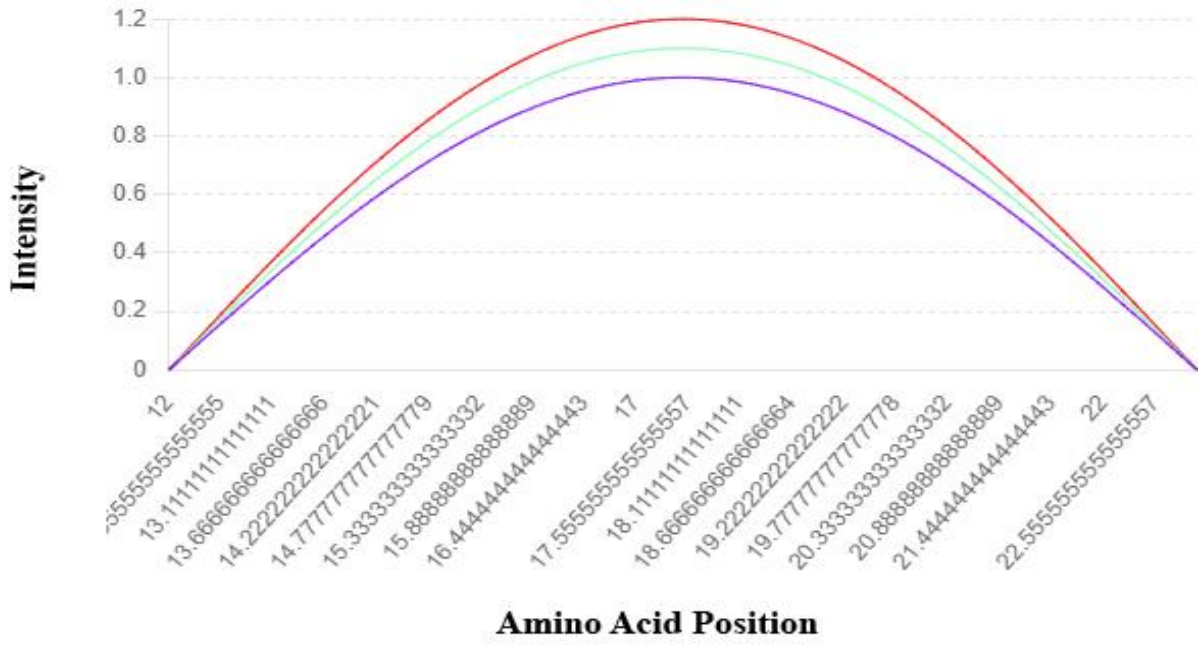

Figure 3. Schematic Representation of Disulfide Bonds in Protein Sequence. Each curved line represents a disulfide bond between two cysteines.

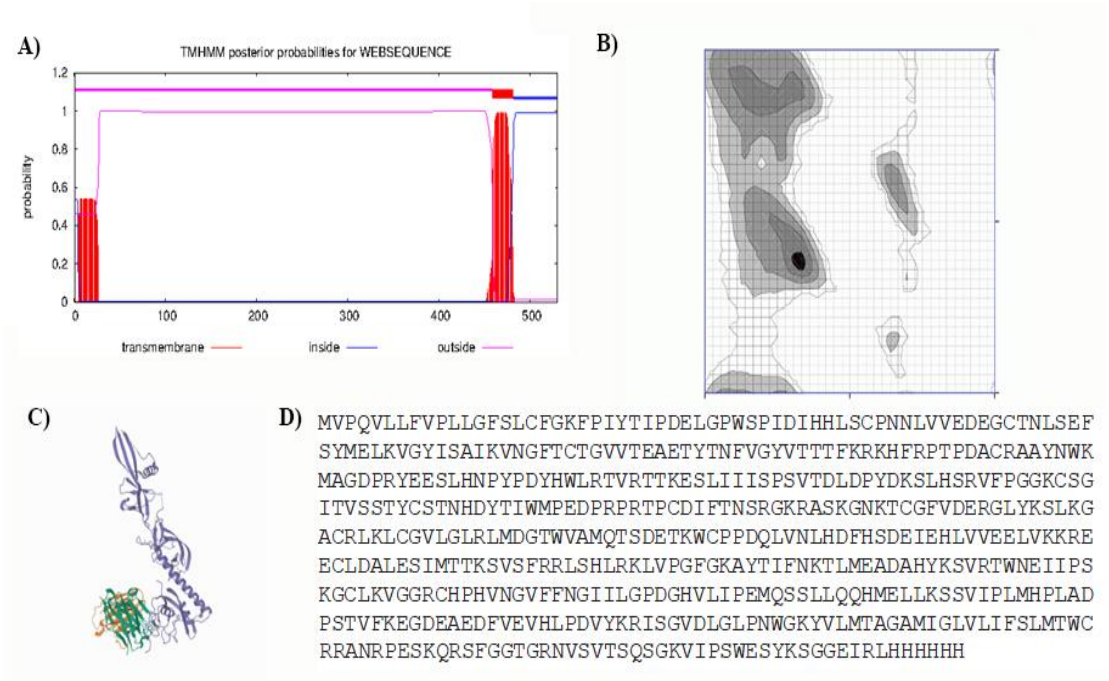

Figure 4. Topology and Structural Analysis of the Protein Sequence. A) The protein sequence TMHMM posterior probability. The figure indicates the likelihood of transmembrane regions (red), within (purple), and outside (blue) the membrane along sequence length; **B)** The Ramachandran diagram; Based on the analysis of 118 structures with a resolution of at least 2 angstroms and an R factor of less than 20%, a quality model is predicted in the desired areas in more than 90% of cases. **C)** The protein structural model with a graph showing amino acid residue distribution in three dimensions. The model shows domains and their locations; **D)** Protein amino acid sequence.

3.5. Vector Construction

The optimized G protein gene was synthesized and successfully cloned into the pcDNA3 eukaryotic expression vector using *EcoRI* and *XhoI* restriction enzymes. The recombinant plasmid, designated as pcDNA3-RVGP, was confirmed through restriction enzyme digestion and sequencing, ensuring the correct insertion and orientation of the G protein gene (**Figure 5**).

3.6. Expression and Detection of RVGP

3.6.1. RT-PCR Analysis

RNA extracted from both transfected and non-transfected CHO cells was of high quality, as confirmed by agarose gel electrophoresis. Reverse transcription followed by PCR amplification using RVGP-specific primers produced the expected bands in transfected cells, confirming the presence of RVGP mRNA (**Figure 6A**).

3.6.2. Western Blot Analysis

Protein lysates from both transfected and non-transfected CHO cells were subjected to SDS-PAGE and transferred to PVDF membranes.

The presence of the RVGP was detected using a specific polyclonal antibody against the rabies virus glycoprotein. The transfected CHO cells showed a distinct band corresponding to the RVGP, while no such band was observed in non-transfected cells. This confirmed the successful expression of the recombinant glycoprotein in CHO cells (**Figure 6B**).

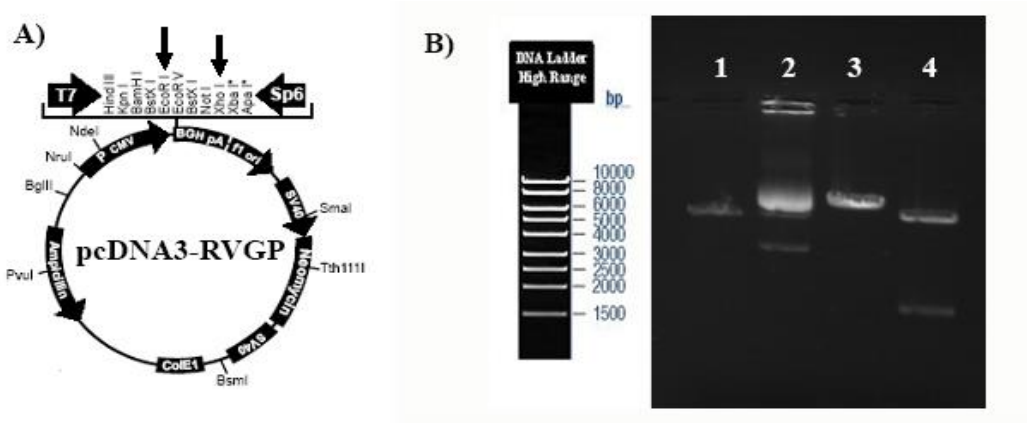

Figure 5. Construction and Verification of the Recombinant pcDNA3-RVGP Plasmid. A) Schematic representation of the pcDNA3-RVGP plasmid. Using *EcoRI* and *XhoI* restriction sites, the optimized rabies virus glycoprotein (RVGP) gene is inserted into the pcDNA3 vector's multiple cloning site (MCS). B) Agarose gel electrophoresis analysis of the recombinant pcDNA3-RVGP plasmid. Lane 1: pcDNA3 is linearized (with *XhoI*); Lane 2: pcDNA3; Lane 3: The linearized pcDNA3-RVGP using *XhoI*; Lane 4: The effect of *EcoRI* and *XhoI* digestion on pcDNA3-RVGP, further confirming the correct orientation and integrity of the inserted gene.

The objective of this work was to create and enhance the rabies glycoprotein (G protein) for the development of a novel rabies vaccine. By using bioinformatics and experimental validation, we effectively produced the G protein in mammalian cells and assessed its structural stability and immunogenicity. When compared with recent advances in rabies vaccine development, our study stands out due to its comprehensive approach combining in silico optimization and experimental validation. For example, while previous studies have focused on codon optimization or structural modeling in isolation, our research integrates these approaches to enhance both the expression and stability of the G protein in CHO cells. This dual approach not only ensures high yields of the target protein but also maintains its functional integrity, which is crucial for its role as a vaccine antigen.

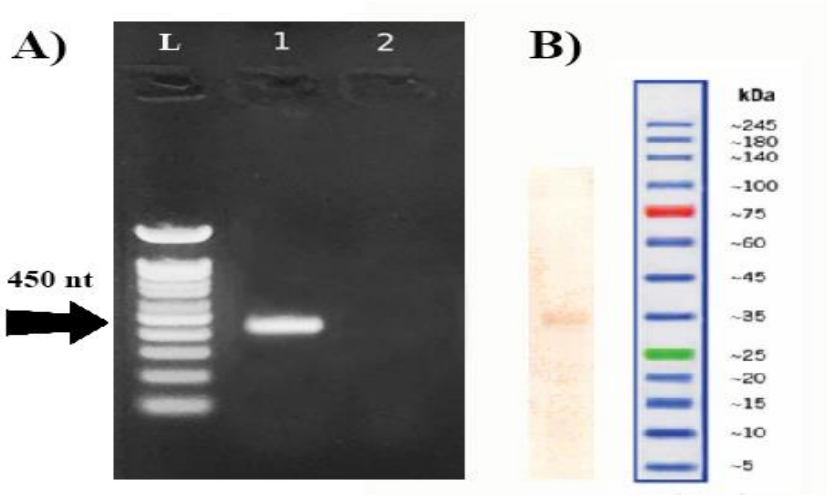

Figure 6. A) Agarose gel electrophoresis of RT-PCR Analysis, indicating successful amplification of the target DNA fragment. Lane L: DNA ladder (100bp) with indicated size of 450 nucleotides (nt). Lane 1: Negative control. Lane 2: Sample showing a band at approximately 450 nt. B) Western Blot analysis of protein samples. The band at approximately 35 kDa corresponds to the protein of interest, confirming its expected size.

The findings of this study have significant implications for the real-world application of rabies vaccines, particularly in low-resource settings where rabies remains a substantial public health challenge. The optimized G protein, developed through a combination of in-silico methods and experimental validation, offers a potentially more cost-effective and accessible solution compared to current vaccine options. This is especially relevant in regions where the financial burden of multiple-dose vaccine regimens limits widespread immunization. The improved stability and expression of the G protein in CHO cells could lead to the production of a more efficient and durable vaccine that requires fewer doses, thereby reducing both the logistical and economic barriers to rabies prevention.

Optimizing the sequence of the rabies virus G protein for expression in CHO cells is a crucial step in enhancing the effectiveness of the vaccination. This procedure included modifying the codon use to align with the preferences of the host, decreasing the GC content, and eliminating putative inhibitory motifs, consequently augmenting the effectiveness of expression. These findings are consistent with previous studies that have highlighted the importance of codon optimization in improving protein expression across various systems. For instance, Angov et al. (2008) demonstrated the significant enhancement of recombinant protein expression through codon optimization, a result that aligns closely with our observations [26]. Similarly, Gustafsson et al. (2004) underscored the benefits of codon optimization in heterologous protein expression, further validating the approach used in our study [27]. The experiments corroborate the results of the present study, demonstrating that codon optimization resulted in enhanced production of the rabies G protein in CHO cells.

Utilizing the PSIPRED v4.0 server for secondary structure prediction and the CYS_REC program for disulfide bond prediction provided valuable information on the stability and functional integrity of the G protein. These investigations verify that the protein retains a stable structure, which is essential for its ability to provoke an immune response. The predicted secondary and tertiary structures of the G protein are in line with the structural characteristics reported in previous studies, such as those by Dietzschold et al. (1983), which identified critical structural features of the rabies virus glycoprotein that correlate with immunogenicity [28]. Our study not only confirms these findings but also extends them by demonstrating that these structural features are preserved and potentially enhanced through bioinformatics optimization.

The accurate prediction of the improved G protein's three-dimensional structure was achieved by the use of the SWISS-MODEL service. Subsequent analysis utilizing PyMOL and Chimera software further confirmed the structural accuracy and correct folding of the protein. The Ramachandran plot analysis validates the stereochemical integrity of the protein model, revealing that the majority of residues are situated in favorable areas. This finding is crucial for ensuring the proper functioning of the G protein as a vaccine antigen. Previous research, such as that by Navid et al. (2016), emphasized the importance

of structural integrity in the development of effective vaccine candidates, particularly in ensuring that the antigen can elicit a robust immune response [29]. Our study builds on this by providing a detailed structural analysis that confirms the suitability of the optimized G protein for use in a vaccine.

In terms of real-world application, the optimized G protein's potential for use in a recombinant vaccine offers several advantages over traditional rabies vaccines. The use of a eukaryotic expression system, such as CHO cells, not only improves the quality and yield of the protein but also reduces the risk of adverse reactions compared to vaccines produced in bacterial systems. This is particularly important in low-resource settings, where access to high-quality vaccines is often limited, and where the logistical challenges of delivering multiple vaccine doses are significant. The potential to produce a single-dose, highly effective vaccine could be transformative in such regions, contributing to the global effort to eliminate rabies.

Furthermore, the combination of bioinformatics tools and experimental validation used in this study provides a model for the development of other viral vaccines. By demonstrating the effectiveness of this approach, our study contributes to the broader field of vaccine development, where similar strategies could be applied to other pathogens with significant public health impacts.

Future research should prioritize investigating the enhanced effectiveness of the G protein in stimulating a protective immune response by performing trials on animal models. Additionally, improving adjuvants and delivery methods to increase the immune response to the G protein, as well as conducting clinical trials to assess the safety and efficacy of the improved rabies G protein vaccine in human participants, will be crucial next steps.

4. Conclusion

This study successfully used bioinformatics and experimental methods to optimize the structure and function of the rabies G protein, demonstrating its potential as a viable candidate for a vaccine. The findings provide a strong foundation for the creation of an advanced rabies vaccine that is both effective and readily accessible. This work provides a significant addition to the global endeavors aimed at eradicating rabies and improving public health outcomes on a global scale. It does this by integrating bioinformatics, molecular modeling, and experimental validation. Unlike similar inquiries, this research highlights many significant advancements:

- The study employs an extensive computational optimization method that targets many elements of the sequence to enhance the expression efficiency in CHO cells. Previous studies often concentrate on certain optimization aspects, resulting in inefficient expression efficiency.
- The use of secondary structure prediction, disulfide bond prediction, and three-dimensional modeling improves our understanding of the protein's stability and function, resulting in a comprehensive structural analysis. This thorough process ensures the production of a vaccine candidate that is of exceptional quality.

- The use of the pcDNA3 eukaryotic expression vector and CHO cells in protein expression is a well-established and dependable method that ensures a significant quantity and high caliber of the recombinant protein. This technology surpasses the bacterial expression systems used in previous studies since it reduces the likelihood of protein misfolding and results in greater production yields.

Therefore, future research should prioritize A) investigating the enhanced effectiveness of the G protein in stimulating a protective immune response by performing trials on animal models, B) Improving adjuvants and delivery methods to increase the immune response to the G protein, and C) Conducting clinical trials to assess the safety and efficacy of the improved rabies G protein vaccine in human participants.

Conflict of interest

The authors declare to have no conflict of interest.

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