IRSTI 34.23.15; 34.27.19; 34.15.23 https://doi.org/10.26577/IJBCh20251821

G.A. Bayandy[1, 2], N.B. Baltakhozha[1, 2], Kh.E. Yerkinova[1, 2],
K.B. Nurpeis[2], G.N. Sailauova[2], I.T. Smekenov[1, 2],
A.K. Bissenbaev[1, 2*]

[1]Scientific Research Institute of Biology and Biotechnology Problems,
Al-Farabi Kazakh National University, Almaty, Kazakhstan
[2]Al-Farabi Kazakh National University, Almaty, Kazakhstan
*e-mail: amangeldy.bisenbaev@kaznu.kz
(Received 12 May 2025; received in revised form 26 December 2025; accepted 29 December 2025)

Production of the soluble form of the Rabies virus glycoprotein ectodomain as a subunit vaccine candidate

Abstract. Rabies remains a serious global public health concern. The envelope glycoprotein of the rabies virus (RABV-G) is the principal viral antigen and harbors critical neutralizing epitopes, making it a promising target for subunit vaccine development and diagnostic applications. Nevertheless, the efficient production of recombinant RABV-G with preserved antigenic and immunogenic properties remains technically demanding. In this work, we developed an optimized strategy for the expression and purification of recombinant RABV-G and assessed its antigenicity and immunogenicity. The rabies virus glycoprotein ectodomain (rRABV-GE) was successfully expressed in *Escherichia coli* Rosetta2(DE3)pLysS and purified in a biologically relevant form. The rRABV-GE gene was codon-optimized and cloned into the pET-28c(+) expression vector, resulting in recombinant protein expression largely in an insoluble state. Consequently, the protein was subjected to refolding and purification procedures. To improve protein solubility, hydrophobic loop regions were substituted with flexible linker sequences. Protein purification was performed under denaturing conditions using urea, followed by affinity chromatography. The identity and antigenicity of the purified protein were confirmed by Western blot analysis and indirect enzyme-linked immunosorbent assay (ELISA). ELISA testing was performed using rabbit sera collected from animals immunized with the commercial Purified Vero Cell Rabies Vaccine (RHABDOVAC-1). Collectively, these findings indicate that the optimized bacterial expression platform enables the production of biologically relevant rRABV-GE and provides a cost-effective approach for the development of rabies subunit vaccines and diagnostic reagents. In summary, the recombinant rRABV-G protein produced in this study appears to be a suitable diagnostic antigen for ELISA-based and other rabies virus immunodiagnostic uses.
Keywords: Rabies virus, glycoprotein ectodomain, antibody, ELISA, Rosetta2(DE3)pLysS, vaccine.

Introduction

Rabies is considered one of the most lethal viral infections, with an almost 100% fatality rate once clinical symptoms develop. The causative agent, rabies virus (RABV), is an RNA virus belonging to the family Rhabdoviridae and the genus Lyssavirus. RABV causes rabies, an acute infection of the central nervous system that progresses rapidly and is invariably fatal in the absence of timely intervention [1]. As a prototype neurotropic virus, RABV possesses a small, negative-sense single-stranded RNA genome of approximately 12 kb, which encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L). Among these, the viral glycoprotein (RABV-G) plays a pivotal role in pathogenesis by mediating viral attachment and entry through interaction with host cell receptors [2].

The G protein, with a molecular weight ranging from 62 to 67 kDa, is a type I transmembrane glycoprotein composed of 505 amino acids. It contains two to four potential N-glycosylation sites, although only one or two are glycosylated depending on the viral strain [3]. Structurally, the protein is divided into three domains: an ectodomain, a transmembrane region, and a cytoplasmic domain. The ectodomain consists of 439 amino acid residues and assembles

into a homotrimeric structure. The transmembrane domain spans approximately 20 amino acids (residues 460–480), while the cytoplasmic domain comprises 44 amino acids that extend into the cytoplasm of infected cells and interact with the matrix protein during viral assembly [4]. Importantly, the ectodomain is exposed on the surface of the virion and represents the only viral component involved in host cell receptor recognition, underscoring its essential role in viral infectivity and its significance as the primary target of virus-neutralizing antibodies [5].

Antibodies interact with RABV-G primarily through their heavy chain and bind to a bipartite conformational epitope on the viral protein to achieve neutralization. These structures provide valuable insights for vaccine and therapeutic development. The purified ectodomain of RABV-G (RABV-GE), produced in eukaryotic cells, has demonstrated effectiveness as a subunit vaccine in animal models [6]. Currently available rabies vaccines are costly, technically demanding to manufacture, and require intensive immunization schedules and boosters to induce and sustain protective immunity. Given that RABV-GE is the most immunologically relevant component of rabies vaccines, a system was established for the production of a subunit vaccine based on a soluble form of RABV-GE obtained from *Escherichia coli* [7].

In the present work, expression conditions for the recombinant ectodomain of the rabies virus G protein were optimized in *Escherichia coli* strain Rosetta2(DE3)pLysS. Additionally, a purification protocol was optimized for isolating the protein from the insoluble fraction using urea-based denaturation and affinity chromatography on a HisTrap column [8].

Materials and methods

Plasmid Construction

The nucleotide sequence of the ectodomain of the rabies virus glycoprotein (RABV-GE; NCBI accession number GQ918139.1) was synthesized with the substitution of two fusion loops by flexible linkers (GGSGG) and the addition of a C-terminal c-Myc epitope. Prior to synthesis, the gene sequence was optimized in terms of codon usage and GC content for efficient expression in *Escherichia coli* using the GenSmart Codon Optimization tool (https://www.genscript.com/tools/gensmart-codon-optimization). The optimized gene was synthesized by GeneCust (Dudelange, Luxembourg) and cloned into the pBluescript II SK(+) vector.

The sequence of the G protein ectodomain (1357 bp, rRABV-GE) was subcloned from the pBluescript II SK(+) vector into the pET-28c(+) expression vector using the *Nco*I and *Xho*I restriction sites, thereby introducing a sequence encoding a C-terminal 6×His tag to the gene (resulting in C-terminal 6×His-tagged proteins). Plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, #K0503) according to the manufacturer's instructions. *E. coli* DH5α (Promega, USA) was used as the cloning host for propagation of the expression constructs. Plasmids from positive colonies were analyzed using polymerase chain reaction (PCR) with gene-specific primers Dir_NcoI_G-ecto (ACCGACCATGGGGAAATTTCCCATATATACG) and Rev_XhoI_G-ecto (ATTACCTCGA GCAGGTCCTCTTCAGAGATC), synthesized by Macrogen (South Korea), as well as by restriction digestion with *Nco*I and *Xho*I enzymes (Thermo Fisher Scientific, USA). PCR amplification was performed on a thermocycler (Eppendorf, model AG 22331, Germany) under the following conditions: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s; followed by a final extension at 72 °C for 5 min.

Expression of the rRABV-GE protein in bacterial cells and its chromatographic purification

The constructed expression plasmid, pET-28c/rRABV-GE, produces the corresponding protein carrying C-terminal 6×His and c-Myc tags. The expression strain *E. coli* Rosetta2(DE3)pLysS (Invitrogen, Thermo Fisher Scientific, USA) was prepared and transformed according to the standard protocol [9].

The rRABV-GE protein was obtained through heterologous expression in *E. coli* Rosetta2(DE3) pLysS cells carrying the expression plasmid encoding the rRABV-GE gene. After transformation, bacterial cultures were grown at 37 °C until reaching a mid-logarithmic phase (OD600 ≈ 0.6). To optimize protein expression and folding, the incubation temperature was subsequently reduced to 4, 16, or 30 °C and maintained for 30 min prior to induction. Protein expression was then initiated by the addition of IPTG (isopropyl β-D-1-thiogalactopyranoside; Thermo Fisher Scientific, USA) at final concentrations ranging from 0.05 to 0.5 mM, followed by continued cultivation for 16 h. As additional components, 1% glucose (Applichem, Germany) and 3% C_2H_5OH (Applichem, Germany) were added prior to induction.

All protein purification procedures were performed at 4 °C. To obtain the recombinant rRABV-

GE protein under native (non-denaturing) conditions, bacterial cells were harvested by centrifugation at 6000 rpm for 7 min at 4 °C using a centrifuge (Eppendorf, model 5417 R, Germany). The resulting cell pellets were disrupted by ultrasonication using an OmniRuptor 4000 (Omni International, USA) in lysis buffer composed of 50 mM sodium phosphate (pH 7.6; Sigma-Aldrich, USA), 100 mM NaCl (Applichem, Germany), 20 mM imidazole (Applichem, Germany), 5% glycerol (Applichem, Germany), 1 mM EDTA (pH 8.0; Applichem, Germany), 10 mM β-mercaptoethanol (Sigma-Aldrich, USA), 1 mM DTT (Sigma-Aldrich, USA), and 2% Triton X-100 (Sigma-Aldrich, USA), supplemented with a complete protease inhibitor cocktail (Roche, Switzerland). EDTA was included to suppress proteolytic activity, while DTT and β-mercaptoethanol were used to maintain reducing conditions. The non-ionic detergent Triton X-100 facilitated efficient solubilization of membrane-associated and cytoplasmic proteins while preserving their native conformation [10,11].

Following cell disruption, the lysates were clarified by centrifugation at 11,000 × g for 60 min at 4 °C using a centrifuge (Eppendorf, model 5804 R, Germany). The supernatant was subsequently loaded onto a 1 mL HisTrap HP affinity column (Cytiva, USA) pre-charged with Ni^{2+} ions (Sigma-Aldrich, USA). Protein elution was carried out using a linear imidazole (Applichem, Germany) gradient ranging from 20 to 500 mM.

For purification of inclusion bodies, cell pellets obtained from 1 L of bacterial culture were resuspended in 20 mL of 50 mM sodium phosphate buffer (pH 7.6) containing 20 mM NaCl and lysed by ultrasonic homogenization at 40% amplitude. The lysate was centrifuged at 14,000 rpm for 20 min at 4 °C using a centrifuge (Eppendorf, model 5417 R, Germany), and the resulting pellet was resuspended in 20 mL of buffer containing 1 M urea (Applichem, Germany) and 1% Triton X-100, followed by centrifugation. This washing step was repeated three times to remove contaminating soluble proteins.

For denaturation and reduction, the purified inclusion body pellets were dissolved in 100 mL of 50 mM sodium phosphate buffer (pH 7.6) containing 8 M urea, 500 mM NaCl, 20 mM imidazole, and 5 mM β-mercaptoethanol. The suspension was incubated for 16 h at room temperature under constant agitation to ensure complete denaturation and reduction. Insoluble material was removed by centrifugation at 12,000 rpm for 20 min using a centrifuge (Eppendorf, model 5417 R, Germany), and the clarified supernatant was applied to a Ni^{2+}-charged HisTrap HP column. Bound proteins were eluted in a single step using 500 mM imidazole.

Protein purification and fraction collection were performed using an ÄKTA Start FPLC system (Cytiva, USA). The column was equilibrated for 5 min, protein sample (20 mL) was loaded, washed for 15 min, and eluted in 7 mL fractions, collecting 500 µL per fraction. The overall flow rate during purification was 0.5 mL/min. The purity and homogeneity of the recombinant protein were assessed by SDS-PAGE. Purified protein samples were stored at −20 °C in a buffer containing 50% glycerol.

Refolding of rRABV-GE expressed in E. coli Rosetta2(DE3)pLysS

For refolding of the denatured purified rRABV-GE protein, gradient dialysis was performed using a Cellu Sep cellulose membrane (Membrane Filtration Products, USA) with a molecular weight cut-off of 14 kDa at 4°C. The dialysis buffer, containing 100 mM NaCl, 50 mM sodium phosphate buffer (pH 7.6), 0.5 M arginine (Applichem, Germany), 20% glycerol, and urea, was gradually reduced from 6M to 0M in a stepwise manner, with the buffer changed every 3 hours. After overnight incubation, the protein was collected into a 1.5 mL Eppendorf tube and stored on ice [12,13].

Western Blot Analysis of Purified Proteins

Two micrograms of each purified protein were resolved by SDS–PAGE on a 10% polyacrylamide gel and subsequently transferred onto a PVDF membrane using a Bio-Rad Mini Trans-Blot Cell (Bio-Rad, USA), in accordance with the manufacturer's instructions. Membranes were blocked for 1.5 h at room temperature in 1× TBS (50 mM Tris–HCl (Applichem, Germany), pH 7.5, 20 mM NaCl) containing 5% non-fat dry milk and 0.1% Tween-20 (Sigma-Aldrich, USA), with gentle agitation using a shaker (Biosan, OS-20, Latvia).

Following blocking, the membranes were incubated overnight at 4 °C with anti-Myc monoclonal antibody (Abcam, UK) diluted 1:5000 in blocking buffer supplemented with 0.1% Tween-20. The membranes were then washed five times for 5 min each with 1× TBS containing 0.1% Tween-20 and subsequently incubated for 1 h at room temperature with the appropriate horseradish peroxidase–conjugated anti-mouse IgG secondary antibody (Abcam, UK). After a second series of five 5-minute washes, the membrane was treated with the chemiluminescent substrate for 60–120 seconds in the dark and the signal was captured on Kodak X-Omat film (Kodak, USA).

Preparation of Anti-RABV-G Polyclonal Antibody

Polyclonal antibodies were produced in rabbits by subcutaneous injection of the RHABDOVAC 1 vaccine (Antigen, Almaty, Kazakhstan), emulsified in an equal volume of complete Freund's adjuvant (Invitrogen, Thermo Fisher Scientific, USA). Subsequent booster injections were performed at two-week intervals using the same dose emulsified in incomplete Freund's adjuvant (Invitrogen, Thermo Fisher Scientific, USA). A pre-immune serum sample was collected prior to the first injection to serve as a negative control. One week after the final (fourth) injection, blood was collected for isolation of the immune serum. The animal study protocol was approved by the Local Ethics Committee of the LLP Scientific and Production Enterprise «Antigen», Almaty, Kazakhstan (protocol code #14, 25 November 2024).

The antibody was raised against the RABV vaccine. For primary immunization, rabbits were subcutaneously injected with 1 mL of RHABDOVAC-1 vaccine at five sites on the dorsal surface. Booster immunizations were administered three times at 14-day intervals, with each dose consisting of 1 mL of the vaccine. One week after the final booster, blood samples were collected, and 3 mL of the resulting antiserum was subjected to ammonium sulfate (Applichem, Germany) precipitation at 50% saturation. The precipitated proteins were recovered by centrifugation, dissolved in purified water, and dialyzed against 10 mM potassium phosphate buffer (pH 7.0; Applichem, Germany).

The dialyzed immunoglobulin fraction was subsequently loaded onto a Protein A–agarose affinity column (Pierce, Thermo Fisher Scientific, USA) pre-equilibrated with the same buffer. After thorough washing, bound antibodies were eluted using 100 mM glycine buffer (pH 3.0). Fractions containing IgG were pooled, and the pH was immediately adjusted to 7.0 with 1.0 M Tris base (Applichem, Germany). The purified antibody preparation was stored at 4 °C until further use. To generate a high-specificity immune response, a polyclonal antibody was raised in rabbits against the ectodomain of the rabies virus glycoprotein (RABV-G), which is a critical viral antigen involved in host cell recognition and membrane fusion. This domain was selected due to its surface exposure and immunogenicity, making it an ideal target for antibody development.

Determination of Antiserum Titer by ELISA

The production of antigen-specific antibodies was confirmed using an indirect enzyme-linked immunosorbent assay (ELISA) performed according to standard immunological procedures. Microtiter plates were coated with viral antigens and blocked with bovine serum albumin (BSA; Sigma-Aldrich, USA) to minimize non-specific binding. Detection was achieved using horseradish peroxidase (HRP)–conjugated goat anti-rabbit secondary antibodies (Abcam, USA). All measurements were carried out in triplicate to ensure reproducibility.

Antiserum titers were determined by indirect ELISA using 96-well polystyrene microplates (Thermo Fisher Scientific, USA). Each well was coated with 1 μg of whole RABV diluted in 100 μL of 1× phosphate-buffered saline (PBS, pH 7.4; Thermo Fisher Scientific, USA) and incubated overnight at 4 °C. Plates were washed three times with 1× PBS containing 0.05% Tween-20 (PBST) and then blocked with 100 μL of 3% BSA in PBST for 1 h at 37 °C, followed by two additional washing steps.

Serial dilutions of antiserum (anti-RABV-G and anti-rRABV-GE), ranging from 1:500 to 1:128,000, were added to the antigen-coated wells (100 μL per well) and incubated for 2 h at room temperature. After four washes with PBST, 100 μL of HRP-conjugated goat anti-rabbit IgG antibody (1:30,000) was added to each well and incubated for 1 h at 37 °C.

Following extensive washing, 100 μL of freshly prepared tetramethylbenzidine (TMB; Thermo Fisher Scientific, USA) substrate was added and allowed to react at room temperature. The enzymatic reaction was terminated by the addition of 1 M sulfuric acid (Sigma-Aldrich, USA), and absorbance was measured at 450 nm using a microplate reader (DAS, Italy). Each assay included both positive and negative control sera, and all samples were analyzed in triplicate.

Results and discussion

Construction of a Recombinant Design for Expression of the G Protein Ectodomain

The production of a soluble, homogeneous, and properly folded extracellular domain of the rabies virus glycoprotein (RABV-G) is a critical prerequisite for its downstream application in diagnostics and immunological studies. Previous studies by Yang et al. demonstrated that the predicted fusion loops within the extracellular domain of RABV-G, located between amino acid residues 73–79 and 117–125, are enriched in hydrophobic amino acids, which significantly contributes to protein aggregation and heterogeneity when expressed recombinantly in *E. coli* [14,15].

In agreement with these findings, the relatively short length of these fusion loops (17–19 amino acids) allows substitution of hydrophobic residues without disturbing the overall folding or antigenicity of the protein [15]. Based on this rationale, we substituted the hydrophobic amino acids in the fusion loop regions with the Gly-Gly-Ser-Gly-Gly linker prior to gene synthesis (Figure 1). This strategy has been previously reported to enhance solubility and promote proper folding of RABV-GE [15], and thus was expected to improve the biochemical properties of the recombinant protein in our system.

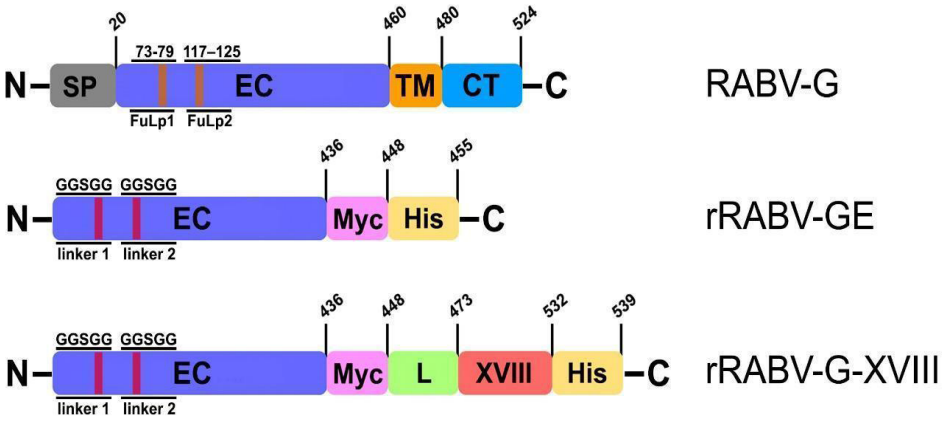

Figure 1 – Recombinant construct of the RABV-GE gene. Full sequence of RABV-G: SP = signal peptide, EC = ectodomain, TM = transmembrane domain, CT = cytosolic domain. In rRABV-GE, the hydrophobic residues at positions 73–79 and 117–125 were replaced with the Gly-Gly-Ser-Gly-Gly linker

Following codon optimization for *E. coli*, the redesigned rRABV-GE gene was successfully synthesized and cloned into the pET-28c(+) expression vector, yielding the pET-28c-rRABV-GE construct containing a C-terminal 6×His tag and c-Myc epitope (Figure 2). Restriction digestion and PCR analysis confirmed correct insertion of the target gene, indicating successful construction of the expression plasmid. These results demonstrate the feasibility of generating a genetically stable construct suitable for high-level expression in bacterial systems.

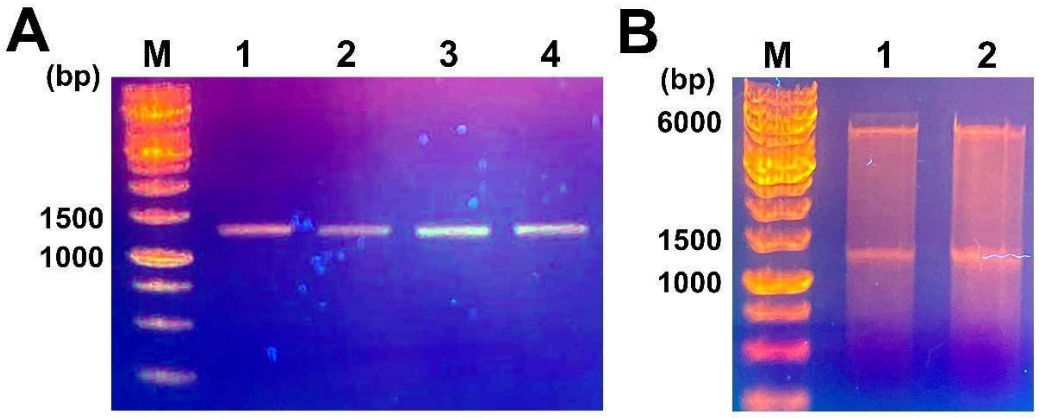

Figure 2 – Analysis of constructed recombinant plasmids pET-28c-rRABV-GE in *E. coli* strain DH5α. A) PCR analysis; B) Restriction analysis. M – 1 kb GeneRuler marker; 1–4 – recombinant plasmid clones

Expression of the Recombinant rRABV-GE Protein in the E. coli Expression Strain

The recombinant rRABV-GE protein was expressed using a T7 RNA polymerase–based expression system in *E. coli* Rosetta2(DE3)PLysS cells. This strain was selected due to its ability to supply tRNAs for codons rarely used in *E. coli*, thereby enhancing the expression efficiency of heterologous eukaryotic proteins. The presence of the pLysS element further minimized basal expression, reducing potential toxicity and proteolytic degradation prior to induction [16].

To improve the solubility and yield of rRABV-GE, expression conditions were systematically optimized by varying IPTG concentration and induction temperature, parameters known to critically influence recombinant protein folding in *E. coli*. Lower induction temperatures (16 °C and 4 °C) are frequently employed to reduce the rate of translation, thereby allowing nascent polypeptide chains more time to fold correctly and decreasing the likelihood of aggregation into inclusion bodies [17,18]. Similarly, reducing IPTG concentration can moderate transcriptional strength and alleviate metabolic burden on host cells, often resulting in improved solubility of heterologous proteins [19]. In the present study, IPTG concentrations ranging from 0.05 to 0.5 mM and induction temperatures of 4 °C, 16 °C, and 30 °C were evaluated. SDS-PAGE analysis demonstrated robust expression of a protein with an apparent molecular mass of approximately 50 kDa, consistent with the predicted size of rRABV-GE (50.8 kDa). The absence of this band in non-induced samples confirmed tight regulation of expression and IPTG-dependent induction (Figure 3). These results indicate that codon optimization and host strain selection were sufficient to achieve high-level expression of rRABV-GE.

Despite these modifications, rRABV-GE remained predominantly localized in the insoluble fraction, with no significant increase in soluble protein yield observed. This outcome suggests that aggregation of rRABV-GE is not primarily driven by excessive expression rates, but rather by intrinsic structural features of the protein (Figure 3).

Rabies virus glycoprotein is a highly structured viral envelope protein that requires correct disulfide bond formation and glycosylation for native folding and stability [20]. The reducing environment of the *E. coli* cytoplasm is unfavorable for disulfide bond formation, and the absence of eukaryotic post-translational modifications further compromises proper folding. Similar observations have been reported for other viral glycoproteins expressed in bacterial systems, where temperature and IPTG optimization alone was insufficient to prevent inclusion body formation [15,21]. Thus, although modulation of induction parameters is a widely applied strategy for enhancing soluble expression, our results indicate that such approaches are insufficient for rRABV-GE, reinforcing the necessity of downstream refolding strategies or alternative expression platforms for producing functionally folded protein.

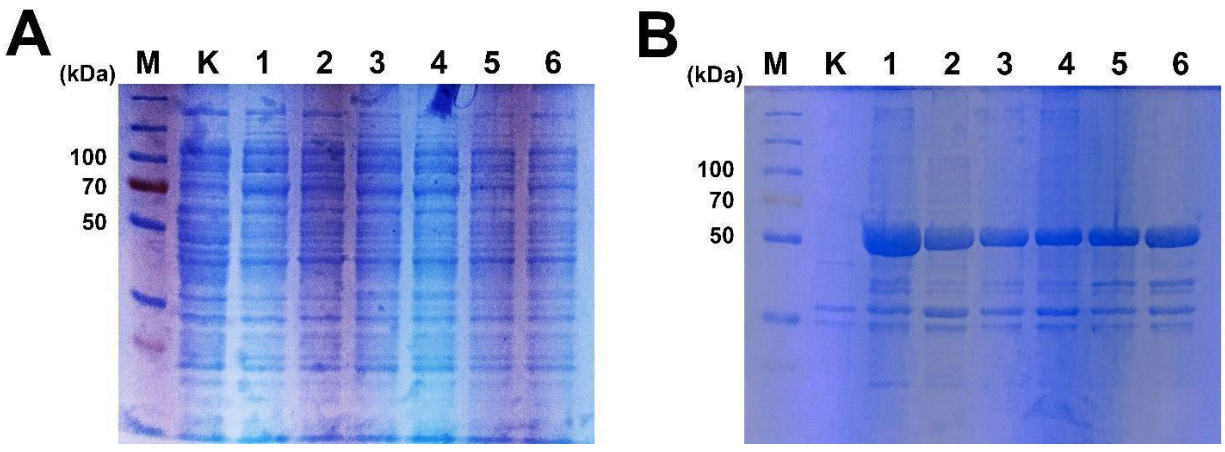

Figure 3 – Optimization of rRABV-GE protein induction in *E. coli* Rosetta2(DE3)PLysS cells. A) Proteins from cell extract; B) Proteins from membrane fraction. M – Marker PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa; K – Control, untransformed cells; 1 – Induction with 0.5 mM IPTG at 30°C, overnight (ON); 2 – Induction with 0.5 mM IPTG at 16°C, overnight (ON); 3 – Induction with 0.5 mM IPTG at 4°C, overnight (ON); 4 – Induction with 0.05 mM IPTG, 1% glucose, and 3% ethanol at 30°C, overnight (ON); 5 – Induction with 0.1 mM IPTG, 1% glucose, and 3% ethanol at 30°C, overnight (ON); 6 – Induction with 0.5 mM IPTG, 1% glucose, and 3% ethanol at 30°C, overnight (ON)

Purification, Refolding, and Immunoreactivity of rRABV-GE

Recombinant rRABV-GE was purified from inclusion bodies under denaturing conditions using 8 M urea, followed by nickel affinity chromatography. The use of urea effectively disrupted aggregated protein structures, enabling solubilization and purification of the target protein [13,22]. Subsequent refolding via stepwise dialysis with decreasing urea concentrations allowed gradual restoration of native-like conformation while minimizing aggregation.

The inclusion of arginine and glycerol in the refolding buffer proved essential for stabilizing the protein during renaturation, consistent with previous studies demonstrating their protective effects against misfolding and precipitation [23,24]. SDS-PAGE analysis confirmed high purity of the refolded protein (Figure 4), and the stability of rRABV-GE during storage further supports successful refolding.

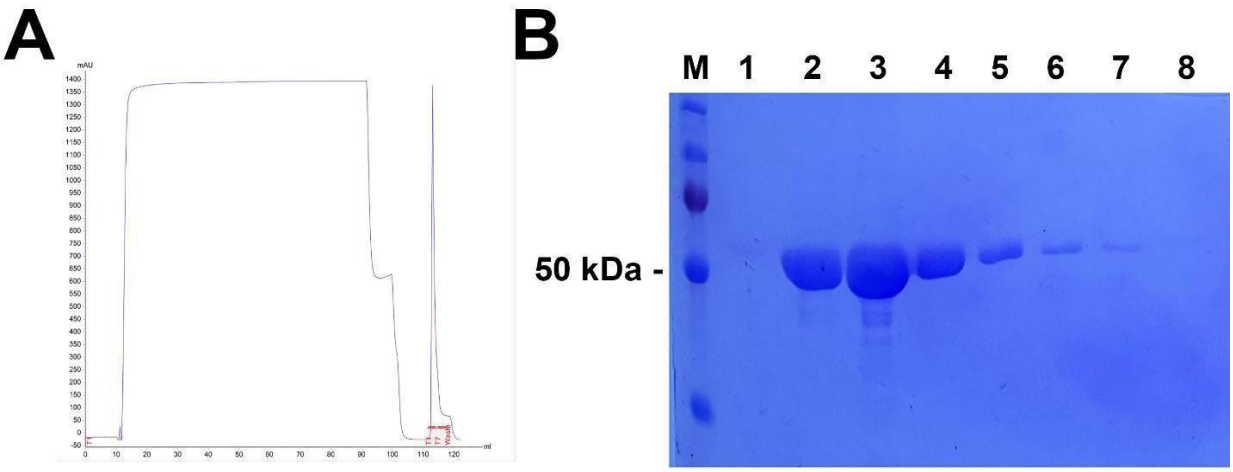

Figure 4 – Chromatographic purification of the G protein ectodomain.
A) Chromatographic purification histogram; B) SDS-PAGE analysis of purified protein fractions.
M – PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa;
1–8 – purified protein fractions corresponding to tubes 4 through 11

Western blot analysis using serum from rabbits immunized with inactivated rabies virus provided critical insight into the antigenic integrity of the recombinant rRABV-GE protein (Figure 5). The observed specific recognition of rRABV-GE by anti-rabies virus antibodies indicates that key antigenic determinants present in native viral glycoprotein G are preserved in the recombinant protein, despite its bacterial origin and the substitution of hydrophobic residues within the fusion loop regions.

Importantly, this finding demonstrates that the Gly-Gly-Ser-Gly-Gly linker substitutions at positions 73–79 and 117–125 do not disrupt major linear or conformational epitopes recognized by polyclonal immune sera. This observation is consistent with previous reports showing that the antigenic sites of RABV-G are distributed across multiple regions of the ectodomain and are relatively tolerant to localized sequence modifications, particularly within fusion-associated regions [14,15]. The ability of polyclonal serum to recognize rRABV-GE also suggests that at least a subset of conformational epitopes is retained following denaturation and refolding, highlighting the effectiveness of the applied refolding protocol. Given that Western blot analysis involves protein denaturation, the observed immunoreactivity further implies preservation of linear epitopes, which are crucial for diagnostic applications such as ELISA-based assays [25].

Taken together, the Western blot results provide strong evidence that rRABV-GE maintains immunological relevance and supports its suitability as a recombinant antigen for serological detection of rabies virus–specific antibodies. These findings validate the design strategy and justify further development of rRABV-GE for diagnostic and potentially immunotechnological applications.

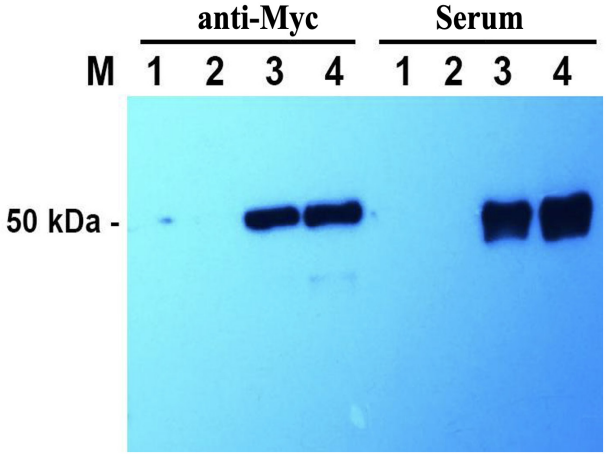

Figure 5 – Western blot analysis of the rRABV-GE protein. 1–2 – Cell lysate: proteins induced with 0.5 mM IPTG at 30 °C, overnight (ON); 3–4 – Pellet: proteins induced with 0.5 mM IPTG at 30 °C, overnight (ON)

Functionally, the refolded rRABV-GE protein demonstrated strong immunoreactivity in an indirect ELISA format. When used as a capture antigen, it reliably detected rabies virus–specific antibodies in sera from rabbits immunized with a commercial Vero cell–derived vaccine, while sera from non-immunized animals showed minimal reactivity (Table 1). The clear correlation between optical density values and serum antibody titers indicates that the recombinant protein retains conformational epitopes relevant for antibody recognition.

Overall, these results demonstrate that although rRABV-GE is expressed in *E. coli* predominantly as an insoluble protein, it can be efficiently purified, refolded, and retain strong antigenic properties. This highlights its potential utility as a cost-effective recombinant antigen for diagnostic applications and supports further development toward improved expression strategies or fusion-based designs aimed at enhancing solubility and structural stability.

Table 1 – List of anti-Rabies virus antibodies OD450 values measured by ELISA in rabbits' serum samples

Sample	OD450 value*	Rabies vaccine inoculation
^1:500	0.605 ± 0.032	+
^1:1000	0.347 ± 0.019	+
^1:2000	0.207 ± 0.015	+
^1:4000	0.131 ± 0.009	+
^1:8000	0.095 ± 0.007	+
^1:16000	0.088 ± 0.009	+
^1:32000	0.075 ± 0.008	+
^1:64000	0.074 ± 0.008	+
^1:128000	0.069 ± 0.010	+
#1	0.055 ± 0.008	-
#2	0.063 ± 0.009	-
#3	0.049 ± 0.006	-

^ Samples titer: serum sample from rabbits immunized with rRABV-GE.

Sample 1-3: serum sample from rabbits who did not undergo rRABV-GE inoculation.

* The antibody titer is determined as the maximum dilution of the serum at which the ratio of A450 (A450 of the serum after immunization / A450 of the serum before immunization) exceeds 2:1. Data are presented as the mean ± standard deviation (SD).

Conclusion

The production of a soluble form of the rabies virus glycoprotein ectodomain (rRABV-GE) has long been challenged by the intrinsic structural complexity, hydrophobic regions, and conformational instability of this viral protein, which collectively complicate its heterologous expression and purification. In the present study, these limitations were successfully addressed through the development of a robust and reproducible strategy for the expression, purification, and refolding of rRABV-GE in a bacterial expression system. Codon optimization, targeted substitution of hydrophobic fu-

sion loop residues with flexible linker sequences, and carefully controlled purification and refolding conditions enabled the production of a highly pure and stable recombinant protein. Immunological characterization demonstrated that the purified rRABV-GE retains antigenic determinants relevant to rabies virus–specific immune responses. A strong correlation between serum neutralizing antibody titers and ELISA optical density values confirmed the suitability of rRABV-GE as a sensitive and specific diagnostic antigen. These findings indicate that the truncated ectodomain preserves key neutralizing epitopes, supporting its applicability in immunodiagnostic assays and underscoring its potential relevance for subunit-based vaccine research. Importantly, the optimized production workflow allowed consistent recovery of rRABV-GE at milligram-scale yields with high purity and stability, highlighting the scalability and practicality of the approach for both laboratory and applied use. This overcomes major obstacles previously associated with insolubility and aggregation of RABV-G–derived recombinant proteins in prokaryotic systems.

In summary, this study establishes an efficient and reproducible platform for the production of soluble, immunoreactive rRABV-GE. The availability of such a recombinant antigen provides a valuable foundation for the development of improved ELISA-based diagnostic tools and supports further exploration of rRABV-GE as a component of next-generation rabies vaccines.

Acknowledgments

This project was supported by the Ministry of Science and Higher Education of the Republic of Kazakhstan (grant number AP14870256).

Conflict of interest

All authors are aware of the article's content and declare no conflict of interest.

References

1. Yousaf M., Qasim M., Zia S., Rehman Khan M., Ur Ashfaq U., Khan S. (2012) Rabies molecular virology, diagnosis, prevention and treatment. *Virology Journal*, vol. 9, no. 1, pp. 1–5. https://doi.org/10.1186/1743-422X-9-50

2. Fooks A.R., Banyard A.C., Ertl H.J. (2019) New human rabies vaccines in the pipeline. *Vaccine*, vol. 37, pp. 140–145. https://doi.org/10.1016/j.vaccine.2018.08.039

3. Fisher C.R., Streicker D.G., Schnell M.J. (2018) The spread and evolution of rabies virus: Conquering new frontiers. *Nature Reviews Microbiology*, vol. 16, no. 4, pp. 241–255. https://doi.org/10.1038/nrmicro.2018.11

4. Dhulipala S., Uversky V. (2022) Looking at the pathogenesis of the rabies lyssavirus strain Pasteur Vaccins through a prism of the disorder-based bioinformatics. *Biomolecules*, vol. 12, no. 10, p. 1436. https://doi.org/10.3390/biom12101436

5. Charlton K.M., Casey G.A. (1981) Experimental rabies in skunks: Persistence of virus in denervated muscle at the inoculation site. *Canadian Journal of Comparative Medicine*, vol. 45, pp. 357. PMID: 7337867; PMCID: PMC1320163

6. Dietzschold B., Wunner W., Smith C. (1988) The molecular biology of rabies virus. *Clinical Infectious Diseases*, vol. 10, no. 4, pp. 771–784. https://doi.org/10.1093/clinids/10.supplement _4.s771

7. Targovnik A.M., Ferrari A., Mc Callum G.J., Arregui M.B., Smith I., Bracco L.F., Alfonso V., López M.G., Martínez-Solís M., Herrero S., Miranda M.V. (2019) Highly efficient production of rabies virus glycoprotein G ectodomain in Sf9 insect cells. *Biotech.*, vol 9, article number 385. https://doi.org/10.1007/s13205-019-1920-4

8. Li X., Luo J., Wang S., Shen Y., Qiu Y., Wang X., Deng X., Liu X., Bao W., Liu P., Zhou J., Ding C., Ma Z. (2010) Engineering, expression, and immuno-characterization of recombinant protein comprising multi-neutralization sites of rabies virus glycoprotein. *Protein Expression and Purification*, vol. 70, no. 2, pp. pp. 179-183. https://doi.org/10.1016/j.pep.2009.09.005

9. Das A., Bysack A., Raghuraman H. (2021) Effectiveness of dual-detergent strategy using Triton X-100 in membrane protein purification. *Biochemical and Biophysical Research Communications*, vol. 578, pp. 122–128. https://doi.org/10.1016/j.bbrc.2021.09.031

10. Geiger T., Clarke S. (1987) Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. *Journal of Biological Chemistry,* vol. 262, no. 2, pp. 785–794. https://doi.org/10.1016/S0021-9258(19)75855-4

11. Gaudin Y. (2000) Rabies virus-induced membrane fusion pathway. *Journal of Cell Biology*, vol. 150, no. 3, pp. 601–612. https://doi.org/10.1083/jcb.150.3.601

12. Pan L.Q., Xie Z.M., Tang X.J., M. Wu, Wang F.R., Naranmandura H., Chen S.Q. (2013) Engineering and refolding of a novel trimeric fusion protein TRAIL-collagen XVIII NC1. *Appl Microbiol Biotechnol.*, vol. 97, no 16, pp. 7253-7264. https://doi.org/10.1007/s00253-012-4604-0

13. Singh S.M., Panda A.K. (2005) Solubilization and refolding of bacterial inclusion body proteins. *Journal of Bioscience and Bioengineering*, vol. 99, no. 4, pp. 303–310. https://doi.org/10.1263/jbb.99.303

14. Yang F., Lin S., Ye F., Yang J., Qi J., Chen Z., et al. (2020) Structural analysis of rabies virus glycoprotein reveals pH-dependent conformational changes and interactions with a neutralizing antibody. *Cell Host & Microbe*, vol. 27, no. 3, pp. 441–453. https://doi.org/10.1016/j.chom. 2019.12.012

15. Berkmen M. (2012) Production of disulfide-bonded proteins in Escherichia coli. *Protein Expression and Purification*, vol. 82, no. 1, pp. 240–251. https://doi.org/10.1016/j.pep.2011.10.009

16. Lipinszki Z., Vernyik V., Farago N., Sari T., Puskas L.G., Blattner F.R., Posfai G., Gyorfy Z. (2018) Enhancing the Translational Capacity of E. coli by Resolving the Codon Bias. *ACS Synthetic Biology*, vol. 7, no 11, pp. 2656-2664. https://doi.org/10.1021/acssynbio.8b00332

17. Bhatwa A., Wang W., Hassan Y.I., Abraham N., Li X.-Z.,Zhou T. (2021) Challenges associated with the formation of recombinant protein inclusion bodies in *Escherichia coli* and strategies to address them for industrial applications. *Front. Bioeng. Biotechnol*., vol 9, article 630551. https://doi.org/10.3389/fbioe.2021.630551

18. Hemamalini N., Ezhilmathi S., Mercy Angela A. (2019). Recombinant protein expression optimization in Escherichia coli: A review. *Indian Journal of Animal Research*, vol 54, no 6, pp. 653-660. doi: 10.18805/ijar.B-3808

19. Baneyx F., Mujacic M. (2004) Recombinant protein folding and misfolding in Escherichia coli. *Nature Biotechnology*, vol. 22, no. 11, pp. 1399–1408. https://doi.org/10.1038/nbt1029

20. Sahdev S., Khattar S.K., Saini K.S. (2008) Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Microbial Cell Factories*, vol. 7, no 1-2, pp. 249-264. https://doi.org/10.1007/s11010-007-9603-6

21. Chen R. (2012) Bacterial expression systems for recombinant protein production: E. coli and beyond. *Protein & Cell*, vol. 3, no. 12, pp. 934–947. https://doi.org/10.1016/j.biotechadv.2011.09.013

22. Arakawa T., Ejima D., Tsumoto K., Obeyama N., Tanaka Y., Kita Y., Timasheff S.N. (2007) Suppression of protein interactions by arginine: A proposed mechanism of the arginine effects. *Biophysical Chemistry*, vol. 127, no. 1–2, pp. 1–8. https://doi.org/10.1016/j.bpc.2006.12.007

23. Golovanov A.P., Hautbergue G.M., Wilson S.A., Lian L.-Y. (2004) A simple method for improving protein solubility and long-term stability. *Journal of the American Chemical Society*, vol. 126, no. 29, pp. 8933–8939. https://doi.org/10.1021/ja049297h

24. Maeda Y., Idehara M., Imoto T. (1995) Effective renaturation of reduced lysozyme by gentle removal of urea. *Protein Engineering, Design and Selection*, vol. 8, no. 2, pp. 201–205. https://doi.org/10.1093/protein/8.2.201

25. De-Simone S.G.; Gomes L.R.; Napoleão-Pêgo P.; LechugaG.C.; de Pina J.S.; da Silva F.R. (2021) epitope mapping of the diphtheria toxin and development of an ELISA-specific diagnostic assay. *Vaccines*, vol 9, no 4, Article 313. https://doi.org/10.3390/vaccines9040313

Information about authors:

Gulshat A. Bayandy – M.Sc., PhD Student, Department of Molecular Biology and Genetics, Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University (Almaty, Kazakhstan, e-mail: bayandy.gulshat92@gmail.com).

Nuraiym B. Baltakhozha – M.Sc., PhD Student, Department of Molecular Biology and Genetics, Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University (Almaty, Kazakhstan, e-mail: baltakhozhanuraiym@mail.ru).

Khamida E. Yerkin – M.Sc., Research Intern, Scientific Research Institute of Biology and Biotechnology Problems, Al-Farabi Kazakh National University (Almaty, Kazakhstan, e-mail: e-mail: khamida.yerkin@gmail.com).

Kuralai B. Nurpeis – Bachelor's Student, Department of Molecular Biology and Genetics, Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University (Almaty, Kazakhstan, e-mail: kukalai2004@gmail.com).

Gulzat N. Sailauova – Bachelor's Student, Department of Molecular Biology and Genetics, Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University (Almaty, Kazakhstan, e-mail: saylauova04@mail.ru).

Izat T. Smekenov – PhD, Senior Lecturer, Department of Molecular Biology and Genetics, Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University (Almaty, Kazakhstan, e-mail: smekenovizat@gmail.com).

Amangeldy A. Bissenbaev – Doctor of Biological Sciences, Professor, Department of Molecular Biology and Genetics, Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University (Almaty, Kazakhstan, e-mail: amangeldy.bisenbaev@kaznu.kz).