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ORIGINAL ARTICLE

Optimized isolation and purification of native glycoprotein B from herpes simplex virus 1: a streamlined approach

Mohammad Yasaghi[1,2], Ahad Yamchi[3*], Alijan Tabarraei[2*], Sara Salari[4], Abdolvahab Moradi[1], Seyedeh Delafruz Hosseini[1]

[1]Department of Microbiology, School of Medicine, Golestan University of Medical Sciences, Gorgan, Iran
[2]Infectious Diseases Research Center, Golestan University of Medical Sciences, Gorgan, Iran
[3]Department of Plant Breeding and Biotechnology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[4]Department of Veterinary Medicine, Azad University, Garmsar Branch, Iran

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ABSTRACT

Background and Objectives: Viral membrane glycoproteins are essential for host cell recognition, membrane fusion and immune evasion, making them critical targets for antiviral therapies and vaccine development. However, their isolation in native conformation is challenging due to structural complexity and limitations of conventional purification methods. The aim of current study was to develop a cost-effective, reproducible method for the isolation and purification of glycoprotein B (gB) from Herpes Simplex Virus type 1 (HSV-1) while maintaining its native conformation for functional and interaction studies.
Materials and Methods: HSV-1 particles were concentrated via ultracentrifugation and membrane proteins were extracted using a modified protocol of the Mem-PER™ Plus Membrane Protein Extraction Kit. Native PAGE with a 4-8% gradient gel was employed to isolate multimeric gB (~300 kDa), followed by electroelution to extract the protein from the gel. The purity and integrity of gB were validated using SDS-PAGE and Western blot analysis.
Results: The method successfully isolated glycoprotein B in its native multimeric form with high purity and adequate concentration (0.157 mg/mL). The pH of the native gel (8.3) and the high molecular weight of gB facilitated separation from other viral surface proteins. SDS-PAGE and Western blot confirmed the specificity and structural integrity of the purified protein.
Conclusion: This study introduces a cost-effective and reliable method for isolating viral glycoproteins in their native conformation. The approach offers significant advantages over traditional chromatography-based techniques, making it ideal for research-scale applications, including functional and interaction studies.

Keywords: Herpes simplex virus 1; Electroelution; Native polyacrylamide gel electrophoresis; Viral envelope proteins; Protein purification

*Corresponding authors: Ahad Yamchi, Ph.D, Department of Plant Breeding and Biotechnology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. Tel: +98-9133062687 Email: Yamchi@gau.ac.ir

Alijan Tabarraei, Ph.D, Infectious Diseases Research Center, Golestan University of Medical Sciences, Gorgan, Iran. Tel: +98-9112733321 Email: alijant@gmail.com

INTRODUCTION

The proteins of viral membranes are essential components of viruses, primarily involved in host cell recognition, membrane fusion and immune evasion (1, 2). These proteins are critical in virological research, therapeutic targeting and vaccine development. However, studying native viral membrane proteins poses challenges due to difficulties in maintaining structural integrity during isolation and purification (3, 4).

Conventional approaches for isolating membrane proteins have employed techniques like gel filtration chromatography and high-performance liquid chromatography (5, 6). Although these techniques can frequently achieve high purities, they often entail significant drawbacks. The combination of elevated consumable and reagent costs, alongside the requirements for specialized equipment and expertise renders these techniques less accessible, particularly for smaller research laboratories. Additionally, many of these methods depend on denaturing or harsh conditions that may disrupt the native conformation of viral membrane proteins, particularly multimeric complexes (7).

The preservation of the conformation of native viral membrane proteins is crucial for studying their biological functions and interactions (8, 9). The significance of this is heightened when proteins typically exist in a multimeric format, as higher-order assembly is preserved solely under precisely defined conditions. The reassembly of isolated protein forms is not only time-consuming but also frequently results in incomplete or misfolded structures (10, 11).

This study presents a cost-effective and scalable approach for purifying native viral membrane proteins. Our methodology utilizes glycoprotein B as a model, circumventing costly chromatographic techniques by employing Native PAGE and electroelution. This method maintains the protein's native conformation and is suitable for research-scale applications due to its practicality and accessibility. This protocol offers a valuable tool for investigating viral membrane proteins in their biologically relevant state, providing a reliable and efficient alternative to traditional methods.

MATERIALS AND METHODS

Cell culture and virus propagation. A549 cell lines (ATCC number: CCL-185) were cultured in T175 flasks to support large-scale virus production. A total of 34 flasks were prepared, each containing 40 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and monitored daily for confluence. Once the cells reached approximately 90% confluence, they were prepared for HSV-1 infection.

HSV-1 KOS strain stock solution was diluted in serum-free DMEM to achieve a multiplicity of infection (MOI) of 0.1 for each T175 flask. For infection, 20 mL of the virus-containing DMEM was added to each flask. The flasks were incubated at 37°C for 1 hour to facilitate viral adsorption, with gentle rocking every 15 minutes to ensure even distribution of the virus. Following the adsorption period, the virus-containing medium was carefully removed, and 30 mL of serum-free DMEM was added to each flask to support viral replication.

Cytopathic effects (CPE), including cell rounding, detachment and syncytium formation were monitored daily under a light microscope. The culture was maintained for 5 days post-infection until most of the cells had detached from the flask surface, indicating extensive viral replication. At this stage, the supernatant containing viral particles and cell debris was collected from all 34 flasks, resulting in approximately 1 liter of virus-containing medium. To remove the cell debris, the collected medium was centrifuged at 10,000 g for 15 minutes at 4°C, and the clarified supernatant was carefully separated.

Plaque titration. The plaque assay was performed using A549 cell monolayers cultured in 6-well plates to determine the viral titer. Serial tenfold dilutions of the HSV-1 stock were prepared in serum-free DMEM, and 500 µL of each dilution was added to the cells. Following an adsorption period of one hour at 37°C with gentle rocking every 15 minutes, the inoculum was removed, and the cells were overlaid with 1% CMC in DMEM supplemented with 2% FBS. Plates were incubated for 48-72 hours, allowing plaques to develop. The cells were then fixed with 10% formaldehyde and stained with 0.1% crystal violet to visualize the plaques. Plaques were manually counted, and the viral titer was calculated in plaque-forming units (PFU) per milliliter (12).

Virus concentration via centrifugation. To concentrate the viral particles and prepare them for membrane protein extraction, the harvested supernatant (~1 liter) underwent Ultracentrifugation process. The concentrated medium was processed using ultracentrifugation at 70,000 × g for 3 hours at 4°C. This step utilized high-speed centrifugal force to separate the viral particles from the residual medium. The extended centrifugation time and high g-force ensured optimal sedimentation of the viral particles, forming a dense pellet at the bottom of the tube (12).

Extraction of the membrane proteins. The viral pellet was processed using the Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, Catalog No. 89842) to isolate the cytosolic and membrane-associated proteins. Initially, 0.75 mL of Permeabilization Buffer was added to the pellet, vortexed briefly for homogeneity, and incubated at 4°C for 10 minutes with constant mixing. The permeabilized particles were centrifuged at 16,000 × g for 15 minutes at 4°C, and the supernatant containing cytosolic proteins was collected. For membrane protein solubilization, the remaining pellet was resuspended in 0.5 mL of Solubilization Buffer, incubated at 4°C for 30 minutes and centrifuged again at 16,000 × g for 15 minutes. The resulting supernatant, containing solubilized membrane proteins, was transferred to a new tube for immediate use or stored for later downstream applications (13).

Validation of the surface protein extraction. To confirm the successful extraction of surface and membrane-associated proteins, the protein fractions obtained in previous step were analyzed using SDS-PAGE (14). This method enabled the separation of proteins under denaturing conditions, providing a clear profile of the extracted proteins based on their molecular weight. Briefly, protein samples of membrane fractions were mixed with SDS sample buffer containing a reducing agent and boiled at 95°C for 5 minutes to denature the proteins completely. The samples, along with a molecular weight marker, were loaded onto a 12% polyacrylamide gel, and electrophoresis was performed at a constant voltage (15).

Predicting the physicochemical properties of the target proteins. To predict the physicochemical properties of the target proteins, the ProtParam tool was utilized (16). ProtParam is a reliable and widely used tool for calculating various parameters of proteins, based on sequences. In this study, the molecular weight and theoretical isoelectric point (pI) of each target protein were computed by inputting their sequences into the tool.

Native PAGE. To ensure the preservation of the structural and functional integrity of membrane-associated proteins including glycoprotein B, the extracted membrane protein fractions were analyzed using Native Polyacrylamide Gel Electrophoresis (Native PAGE) (17). Unlike SDS-PAGE, which denatures proteins, Native PAGE separates proteins under non-denaturing conditions, maintaining their native conformation. A 4-8% gradient polyacrylamide gel was prepared to allow the separation of proteins across a wide molecular weight range. Protein samples were mixed with a non-denaturing sample buffer and loaded onto the gel alongside a molecular weight marker for size estimation. Electrophoresis was performed at 4°C using a constant voltage to prevent protein denaturation during migration. Upon completion of the run, the gel was stained with Coomassie Brilliant Blue to visualize the separated protein bands. Furthermore, to isolate glycoprotein B (gB) in its native conformation, the entire protein content extracted from the membrane fraction was run on another native gel as describe in aforementioned paragraph. Finally, to isolate glycoprotein B for further analysis, the specific band identified on the gel was carefully excised using a sterile scalpel (18).

Electroelution. The glycoprotein was recovered from native PAGE using the Bio-Rad Model 422 Electro-Eluter, following the manufacturer's guidelines. In brief, protein bands were carefully excised from four native gels and placed into electroelution tubes equipped with a frit at the bottom. The tubes were then filled with an elution buffer (25 mM Tris / 199 mM glycine), and silicone adaptors with membrane caps (MWCO 12,000–15,000 Daltons) were attached to the ends of the tubes. The membrane caps were also filled with the same elution buffer to ensure proper conductivity (15).

Electroelution was performed at a constant current of 10 mA per tube for five hours, allowing the proteins to migrate through the gel into the elution buffer. After completion, the eluted proteins were carefully pipetted from the membranes. Dialysis was not required, as the elution buffer did not contain SDS,

ensuring that the proteins remained in their native state. Afterwards, the protein was concentrated by lyophilization (19). The protein concentration was determined by UV-Vis spectroscopy at 280 nm using a known extinction coefficient for gB according to Gill and von Hippel (1989) (20).

Validation of glycoprotein B isolation. The protein solution obtained after electroelution was validated for the presence and purity of glycoprotein B using SDS-PAGE, as previously described. Subsequently, Western blot analysis was conducted employing a mouse monoclonal anti-glycoprotein B antibody (Abcam, Catalog No. ab6506), with detection achieved through DAB (diaminobenzidine) staining (21).

RESULTS

The procedures of cell culture, virus propagation and protein extraction were executed successfully. The viral titer was determined to be 3.4×10^7 PFU/mL, indicating effective virus propagation. Ultracentrifugation facilitated the concentration of viral particles from 1 liter of viral culture supernatant, resulting in a dense viral pellet. The Mem-PER™ Plus Membrane Protein Extraction Kit effectively isolated the virus's surface proteins for subsequent analysis.

Additionally, the extraction of viral surface proteins was validated through analysis of the extracted fractions using SDS-PAGE, as illustrated in Fig. 1. The observed protein bands correspond with the anticipated molecular weights established from literature (22-24) and computational predictions utilizing the ProtParam tool. Table 1 presents a summary of the predicted and in-vitro molecular weights, along with the theoretical pI values for the identified proteins.
Among the identified proteins, gB, gD and gH/gL are recognized for their ability to form multimers in their native state. The analysis of both monomeric and multimeric forms confirmed that the molecular weights closely aligned with theoretical predictions. The study concentrated on glycoprotein B (gB), which displayed specific bands at approximately 100–110 kDa for the monomeric form and ~300 kDa for the multimeric form, consistent with the anticipated values.

Furthermore, the extracted proteins were analyzed using Native PAGE to preserve their native conformation and evaluate the potential multimeric states. The results presented in Fig. 1B indicate a dense band at approximately 300 kDa, which aligns with predictions from ProtParam and corroborating literature, suggesting the multimeric form of the glycoprotein B (gB). The absence of other prominent bands on the gel indicates the high specificity of the extraction process for gB in its native state. This outcome confirms the effective isolation and maintenance of the structural integrity of gB, preparing it for subsequent analyses. Then the entire extracted protein fraction was subjected to Native PAGE gel electrophoresis, and the band was observed at approximately 300 kDa as previously identified and it was excised from the gel for electroelution. The electroeluted protein solution underwent SDS-PAGE and Western blot analysis to validate the extraction and confirm the presence of the 300 kDa band.

The purified protein exhibited a concentration of 0.157 mg/mL as determined by the Gill and von Hippel method. SDS-PAGE results (Fig. 1A) displayed a singular protein band at approximately 110 kDa, which aligns with prior analyses identifying it as the monomeric form of glycoprotein B (gB). This observation provides substantial evidence that the 300 kDa band in the Native PAGE gel corresponds to the multimeric form of gB.

Furthermore, Western blot analysis (Fig. 1C) confirmed that the extracted protein band corresponds to glycoprotein B, as indicated by the specific reaction with the anti-gB antibody. The results demonstrate the successful extraction of gB from the viral surface, validating the process for isolating and characterizing this key viral surface protein.

DISCUSSION

Viral glycoproteins are vital parts of the viral membrane and are involved in the pathophysiology of virion infections. These elements play a crucial role in the infection process by allowing the virus to bind to host cell receptors, promote membrane fusion and allow viral entrance. Since neutralizing antibodies mostly target these glycoproteins, these proteins are crucial for inducing immunological responses (25). Since viral membrane glycoproteins are crucial during the early stages of infection, and antiviral therapies should target them. The viral replication cycle is successfully disrupted, and infection is avoided by inhibiting functions during receptor binding, fusion, or the entrance (26).

The expression and purification methods of these

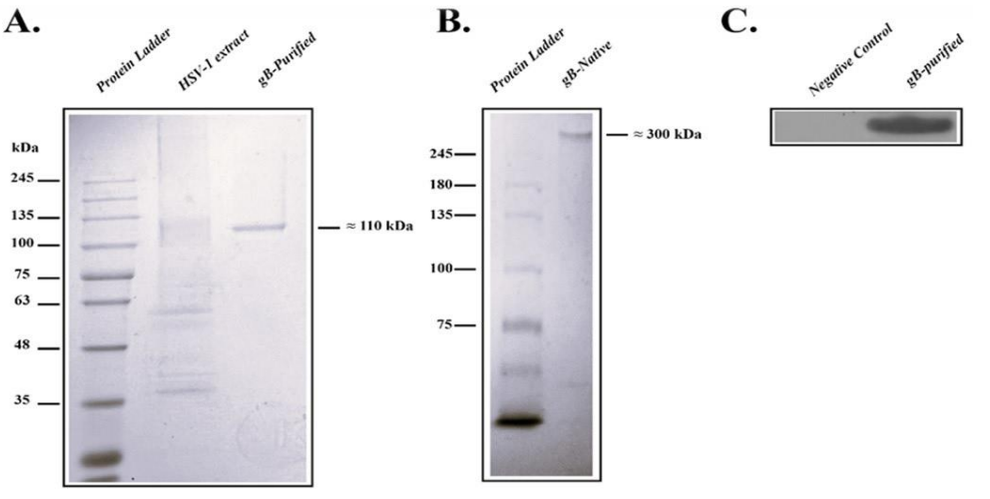

Fig. 1. Analysis of glycoprotein B (gB) extracted and purified from HSV-1.
(A) SDS-PAGE showing the protein ladder, HSV-1 extract, and the purified gB monomer (~110 kDa).
(B) Native PAGE confirming the multimeric form of gB (~300 kDa) in its native state.
(C) Western blot analysis using anti-gB antibody, validating the identity and specificity of the purified gB. Negative control shows no cross-reactivity.

Table 1. Comparative analysis of the predicted and in-vitro molecular weights and the isoelectric points (pI) of HSV-1 glycoproteins

	Molecular weight (kDa)				Theoretical pI	
	Multimer		Monomer		Multimer	Monomer
	Predictive	In-Vitro	Predictive	In-Vitro		
gB	301	330	100	110	7.8	7.63
gC	-	-	55	100	-	7.65
gD	86	90	43	45	8.26	8.11
gH	115	120	90	95	8.16	6.61
gL	115	120	24	35	8.16	9.32
gM	-	-	51	-	-	9
gE	-	-	59	-	-	5.74
gI	-	-	41	-	-	9.05
gG			25	-	-	5.96

glycoproteins differ based on research objectives (27). In vaccine design studies, researchers commonly express these proteins in eukaryotic or prokaryotic expression systems and subsequently purify them using various techniques (28, 29). However, this generally presents particular constraints. Various expression systems may fail to produce the native protein synthesized by the virus in its host, potentially resulting in suboptimal responses. Moreover, if the protein inherently exists in a multimeric form, assembling expressed monomers into a functional multimeric structure in the laboratory becomes quite challenging (30). The purification process is often prolonged, complex and expensive. Although these methodologies have limitations, they are considerably more suitable for large-scale applications including vaccine production.

A number of researches have focused on these glycoproteins is antiviral studies. Investigations generally focus on analyzing the interaction between a target protein and a specific drug or ligand (31, 32). The availability of purified glycoproteins will enable the examination of these interactions in vitro. Recombinant protein production holds promise for addressing

this demand; however, it is accompanied by specific limitations. Research on glycoproteins should be limited to their native form because of their intricate structure and post-translational modifications. This is particularly relevant to glycoproteins that exist as multimers on the surface of a virus. The optimal approach for conducting these studies involves the direct separation and purification of glycoproteins from viral particles. This preserves the protein's native conformation and structural integrity, facilitating advanced analyses and applications (15).

This study presents the successful extraction and purification of gB from the surface of herpesvirus. A Mem-PER™ Plus Membrane Protein Extraction Kit was employed for the extraction of cell surface proteins. Based on the premise that the cellular membrane constitutes the origin of the herpesvirus envelope, we proposed that the kit may be appropriate for the extraction of viral surface proteins. A minor modification in the kit protocol facilitated the successful execution of the extraction and purification process.

A substantial quantity of viral particles was required for this process, as the virus is considerably smaller than a cell. We concentrated approximately 3×1010 viral particles through ultracentrifugation, resulting in a pellet that was subsequently processed according to the steps detailed below for the kit. The method effectively isolated and purified gB for further analyses.

Researchers utilize various protein purification techniques, including gel filtration, ion exchange and HPLC; however, these methods possess notable limitations (33, 34). In addition to the considerable costs associated with these techniques, researchers often face significant limitations imposed by them (35). The intricate configuration of purification procedures in these methods generally requires a significant sample size to guarantee adequate material after the setup. Utilizing expression systems for protein generation may address the issue (7); however, in studies such as ours, where the virus is the source of the target protein, this poses as a challenge. The restricted availability of protein necessitates an efficient, straightforward and cost-effective method to minimize the loss of extracted material during purification (29).

We employed an electroelution technique to extract protein from a native PAGE gel. This method had previously been employed in experiments such as the one for Hashemi et al which proved it to be entirely feasible and accessible (15). Electroelution facilitates the accurate extraction of proteins from the gel, thus reducing losses. This technique is especially effective for the purification of viral proteins when the available source material is restricted (15, 36). The results indicated that the protein eluted from the gel in its native form and exhibited high purity. The concentration of the purified protein was adequate for subsequent applications, such as investigating protein-ligand interactions through surface plasmon resonance and other techniques.

The SDS-PAGE analysis indicated that, despite the kit yielding multiple surface proteins of the virus in the initial extraction, only gB successfully entered the gel. The pH of the native gel was approximately 8.3, which impeded the migration of many proteins into the gel. The native pI ensured that the protein maintained the appropriate charge in the gel environment. A 4-8% gradient gel was employed because of the high molecular weight of gB, since it is the heaviest surface protein of the herpesvirus in its native state. This suggests that smaller proteins, despite penetrating the gel, are clearly distinguished from the gB band, thus aiding in its excision for electroelution. This study employed native PAGE; however, when accessible, alternative methods such as isoelectric focusing may provide enhanced protein separation. IEF enhances the separation of proteins according to their pI value, thereby improving purity and isolation efficiency (37).

CONCLUSION

This technique is a practical procedure that demonstrates excellent reproducibility and significantly reduces costs relative to other purification methods. Although it yields a lower quantity compared to alternative protein purification methods, it is particularly suitable for research applications including studies on protein function and interaction analyses. As a result, it would also draw the interest of explorers, investigators and researchers in general.

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