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Original Article

OPTIMIZATION OF REFOLDING CONDITIONS FOR RECOMBINANT OMP19 PROTEIN FROM BRUCELLA SPP.

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Abstract. The recombinant Omp19 protein of *Brucella* spp. is considered as a promising antigen for the development of diagnostic test systems and immunobiological preparations. However, obtaining its soluble form remains a significant challenge. The aim of this study was to optimize the dialysis conditions for the recombinant Omp19 protein following purification by ion-exchange chromatography.

Dialysis was performed sequentially in several buffer systems with varying compositions of salts and stabilizing components. Dialysis efficiency and protein solubility were assessed using the SDS-PAGE method, with analysis of the supernatant and pellet fractions under various storage temperature conditions. The results showed that when buffers 1-4 were used, the recombinant protein predominantly converted to an insoluble form and was detected in the pellet. To increase the protein's solubility, the concentration of L-arginine in the dialysis buffer was optimized. It was found that using a buffer containing 880 mM L-arginine ensured that the protein remained in the soluble fraction and prevented the formation of a visible pellet.

Based on these results, we recommend using 880 mM L-arginine to stabilize the recombinant Omp19 protein during purification and storage, which is of practical importance for the further use of this protein in diagnostics and biotechnology research.

Keywords: *Brucella* spp.; Omp19; recombinant protein; dialysis; SDS-PAGE.

Introduction

Brucellosis remains one of the most significant zoonotic infections and has a substantial impact on animal and public health in endemic regions. In the Republic of Kazakhstan, the disease continues to be reported in livestock, highlighting the need to improve laboratory diagnosis and develop highly specific serological assays [1,2]. National studies have documented the circulation of *Brucella abortus* and *Brucella melitensis* in different regions of Kazakhstan, underscoring the importance of pathogen surveillance and molecular genetic characterization [3]. In this context, recombinant antigens are particularly valuable because they may improve the sensitivity and specificity of diagnostic methods compared with conventional serological tests.

Outer membrane proteins (Omp) of *Brucella* spp., including Omp19, Omp25, and Omp31, are important immunodominant antigens. Omp19 is an approximately 19kDa lipoprotein that is highly conserved and capable of inducing a humoral immune response [4]. Studies by Kazakh researchers have shown that recombinant Omp antigens can substantially improve the diagnostic performance of enzyme-linked immunosorbent assays compared with conventional methods [5].

Heterologous expression of recombinant proteins in *Escherichia coli* frequently results in the formation of insoluble aggregates and inclusion bodies, necessitating multistep purification that includes solubilization, ion-exchange chromatography, and subsequent renaturation of proteins. In the Laboratory of Molecular Biology and Genetic Engineering at RIBSP, LLP, imidazole-containing buffer systems and detergents are widely used to increase the recovery of the target protein in soluble form [6].

One of the most effective renaturation methods is stepwise dialysis with sequential changes in buffer systems, which allows for the gradual removal of denaturing agents and the stabilization of the

protein's native conformation. Particular attention is paid to the addition of L-arginine, which, according to several studies, reduces the aggregation of intermediate protein forms and increases the yield of the soluble fraction [7].

Despite the existence of research on recombinant *Brucella* spp. antigens, including studies conducted in Kazakhstan (S. Seifullin Kazakh Agrotechnical Research University and the RIBSP), the conditions for effective renaturation of Omp19 remain insufficiently optimized, which limits the production of a stable, functional preparation.

Therefore, this study aimed to evaluate the efficiency of stepwise dialysis of recombinant Omp19 from *Brucella* spp. in various buffer systems and to analyze the distribution of the protein between soluble and insoluble fractions using the SDS-PAGE method.

Materials and methods. The subject of this study is the recombinant Omp19 protein, purified by ion-exchange chromatography, at the "Molecular Biology and Genetic Engineering" laboratory of the RIBSP LLP.

The purification by ion-exchange chromatography of the recombinant Omp19 protein from *Brucella* spp. included the following steps: equilibration, washing, and elution using various concentrations of buffer solutions containing imidazole and 1% Triton X-100. The total protein concentration in the sample, as determined by the Lowry method, was 95 μg/mL.

Preparation of Dialysis Buffers. All solutions were prepared in deionized water using analytical-grade reagents. After the components were added, the solutions were thoroughly mixed until all substances had completely dissolved.

Buffer 1 (20 mM Tris-HCl, 300 mM NaCl, and 1 mM ethylenediaminetetraacetic acid [EDTA]), to prepare 1 L of buffer, 20 mL of 1 M Tris-HCl, 60 mL of 5 M NaCl, and 2 mL of EDTA solution were added to a volumetric flask. The volume was adjusted to 1 L with deionized water, and the solution was thoroughly mixed.

Buffer 2 (1 M L-arginine, 300 mM NaCl, 8 mM Na_2HPO_4, and 2 mM NaH_2PO_4).

A total of 60 mL of 5 M NaCl, 8 mL of 1 M Na_2HPO_4, and 2 mL of 1 M NaH_2PO_4 were added to a volumetric flask. Next, 210.7 g of L-arginine was added and mixed until completely dissolved. The volume was adjusted to 1 L with deionized water.

Buffer 3 (phosphate-salt buffer), to prepare 2 L of buffer, 2.88 g of Na_2HPO_4, 0.61 g of KH_2PO_4, 17.5 g of NaCl, and 0.4 g of KCl were dissolved in deionized water. After all components had dissolved, the volume was adjusted to 2 L.

Buffer 4 (20 mM PBS), to prepare 2 L of solution, 5.68 g of Na_2HPO_4, 0.48 g of KH_2PO_4, 16 g of NaCl, and 0.4 g of KCl were dissolved in deionized water. After all components had dissolved, the volume was adjusted to 2 L with purified water.

Buffer 5 (55 mM Tris-HCl, 20 mM NaCl, 0.88 mM KCl, and 880 mM L-arginine), the required amounts of each component were dissolved in deionized water, and the solution was brought to the calculated final volume. This buffer was used to stabilize the protein after dialysis.

Preparation of 2× buffer for SDS-PAGE samples. To prepare 20 mL of 2× sample buffer, 2.5 mL of 4× Tris-HCl/SDS buffer (pH 6.8), 5 mL of glycerol, 0.32 g of SDS, and 2 mg of bromophenol blue were combined. The volume was adjusted to 20 mL with deionized water, and the solution was mixed until all components had completely dissolved. Immediately before use, 50 μL of β-mercaptoethanol was added to 950 μL of the prepared buffer. The buffer was stored at room temperature for no longer than one year.

Dialysis Procedure. For dialysis, 3-mL aliquots of purified recombinant Omp19 from *Brucella* spp. were loaded into dialysis cassettes. The cassettes were sequentially placed in five buffer solutions (buffers 1, 2, 3, 4, and 5), each with a volume of 500 mL. The flasks containing the buffers were placed on magnetic stirrers and incubated at 4 °C. After 2 h, each buffer was replaced with the next freshly prepared solution, and dialysis was continued for 16 h at 4 °C with constant stirring. Sequential buffer exchange was performed to gradually remove denaturing components and promote refolding of the recombinant protein [8].

After dialysis, the protein samples were removed from the cassettes and transferred to sterile microcentrifuge tubes. The samples were centrifuged at the maximum available speed for 30 min. The supernatants were carefully transferred to new sterile tubes. Each pellet was resuspended in 1.5 mL of

deionized water and then mixed with 1.5 mL of 2× sample buffer. A 20-μL aliquot of each sample was collected for subsequent SDS-PAGE analysis [9].

Polyacrylamide Gel Casting. For electrophoretic analysis, two glass or plastic cassettes for casting 10 × 10 × 1 mm mini-gels were prepared. Freshly prepared resolving-gel solution was immediately transferred into the cassettes with a Pasteur pipette to a height of approximately 8 cm. The gel surface was carefully overlaid with approximately 1 cm of deionized water to prevent contact with atmospheric oxygen and ensure uniform polymerization. The resolving gel was polymerized at room temperature for 30–60 min. Completion of polymerization was indicated by the appearance of a distinct boundary between the water layer and the gel surface. After polymerization, the water was removed and the gel surface was rinsed with 1× Tris-HCl/SDS buffer (pH 8.8). The concentrating gel solution was then prepared and added until the space above the resolving gel was completely filled. Combs were inserted to form wells, and the stacking gel was allowed to polymerize at room temperature for 30–45 min until completely solidified [10].

Sample Preparation. The samples were mixed with 2× sample buffer at a 1:1 ratio (v/v). Pellet samples were first resuspended in 1× sample buffer to obtain a homogeneous suspension. The prepared samples were heated in a boiling water bath or heating block at 95 °C for 5–10 min to denature the proteins and reduce disulfide bonds. The samples were then cooled to room temperature and used for SDS-PAGE analysis [10].

Electrophoresis. After polymerization of the stacking gel, the comb was removed and the wells were rinsed with 1× running buffer. The cassettes were installed in a vertical electrophoresis chamber, which was then filled with running buffer. The prepared samples and a molecular-weight marker were loaded into the wells. Electrophoresis was performed at 130 V for 90–100 min, with protein migration monitored using the bromophenol blue dye front [10].

Protein Detection. After electrophoresis, the gel was removed from the cassette and the stacking gel was discarded. Proteins were visualized by staining with Coomassie Brilliant Blue G-250; alternatively, the gel was used for subsequent Western blot analysis. Before staining, the gel was washed three times with distilled water for 10 min per wash and then incubated in staining solution for at least 2 h with constant agitation. Excess stain was removed by washing with distilled water until distinct protein bands became visible [11].

Results. Dialysis efficiency for recombinant Omp19 from *Brucella* spp. was evaluated by SDS-PAGE. Samples obtained at different stages of dialysis against four sequential buffers were separated into supernatant (S) and pellet (P) fractions and analyzed after exposure to different temperature conditions (37 °C, room temperature [RT], −20 °C, and −70 °C). A standard protein ladder was used as the molecular-weight marker (Figure 1A–D).

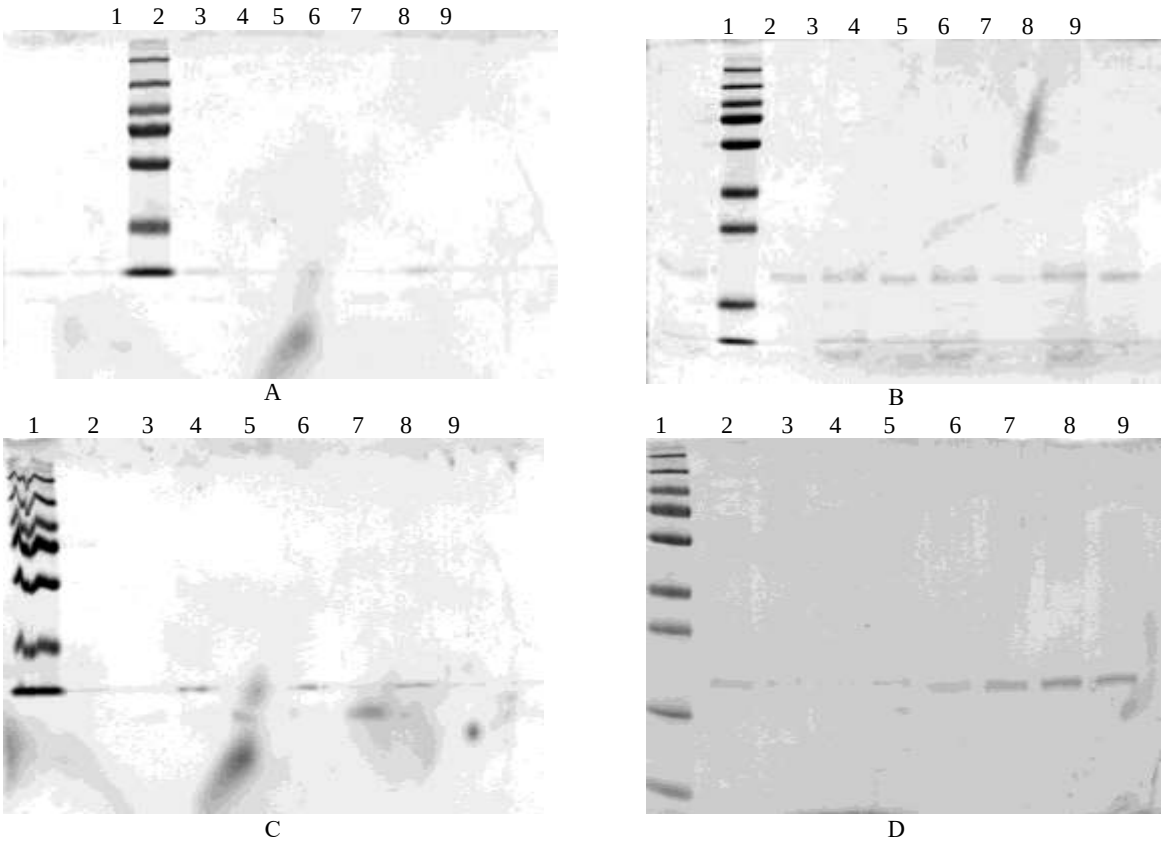

Figure 1. Dialysis efficiency of the recombinant Omp19 protein from *Brucella* spp. in various buffer systems.

A – Dialysis in buffer 1: 1 – pellet (+37), 2 – n.d. (+37), 3 – marker, 4 – pellet (RT), 5 – supernatant (RT), 6 – pellet (-20), 7 – supernatant (-20), 8 – pellet (-70), 9 – supernatant (-70);
B – Dialysis in Buffer 2: 1 – supernatant (+37), 2 – marker, 3 – pellet (+37), 4 – supernatant (RT), 5 – pellet (RT), 6 – supernatant (-20), 7 – pellet (-20), 8 – supernatant (-70), 9 – pellet (-70);
C – dialysis in buffer 3: 1 – marker, 2 – pellet (+37), 3 – supernatant (+37), 4 – pellet (RT), 5 – supernatant (RT), 6 – pellet (-20), 7 – supernatant (-20), 8 – pellet (-70), 9 – supernatant (-70).
D – dialysis in buffer 4: 1 – marker, 2 – supernatant (RT), 3 – supernatant (+37), 4 – supernatant (-20), 5 – supernatant (-70), 6 – pellet (RT), 7 – pellet (+37), 8 – pellet (-20), 9 – pellet (-70).

In buffer 1 (Figure 1A), the protein was detected predominantly in the pellet fraction under all temperature conditions, whereas little or no protein was detected in the supernatant. A similar pattern was observed in buffer 2 (Figure 1B): most of the protein was present in the pellet, while only small amounts were detected in the supernatant under selected conditions.

In buffer 3 (Figure 1C) the protein was predominantly present in the pellet regardless of the incubation temperature, indicating low solubility under these dialysis conditions. In buffer 4 (Figure 1D), the protein also remained predominantly associated with the pellet under all conditions examined.

Overall, the electrophoretic results (Figure 1A–D) showed that recombinant Omp19 predominantly transitioned to an insoluble form during dialysis against the tested buffer systems and was recovered in the pellet. These findings indicate that the initial refolding conditions were insufficient and that the buffer composition required optimization to improve protein solubility.

To optimize the dialysis conditions for recombinant Omp19 from *Brucella* spp., the effect of L-arginine concentration on protein stability was examined. Dialysis was initially performed using 1 M L-arginine. However, protein precipitation was observed at this concentration, indicating reduced solubility and stability. The L-arginine concentration was therefore reduced to 880 mM (buffer 5). The results are shown in Figure 2.

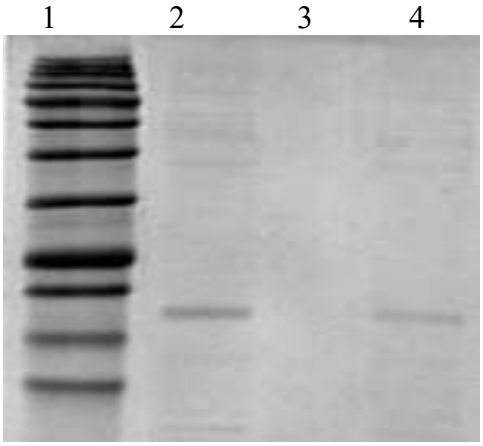

Figure 2. Dialysis results with optimized buffer composition.
Lane 1, marker; lane 2, supernatant; lane 3, pellet; lane 4, supernatant after filtration.

As shown in Figure 2, recombinant Omp19 remained soluble and did not form a visible pellet during dialysis in the presence of 880 mM L-arginine. Therefore, 880 mM L-arginine was selected as the optimal concentration for subsequent purification and storage of recombinant Omp19.

Discussion. The results demonstrated that the efficiency of recombinant Omp19 dialysis depended strongly on the composition of the dialysis buffer. Electrophoretic analysis of samples dialyzed in buffers 1-4 showed that the protein was localized predominantly in the pellet fraction regardless of storage temperature. This distribution indicates protein aggregation and conversion to an insoluble state.

Aggregation is a major challenge in the recovery of recombinant proteins after removal of denaturing agents [12]. Current evidence indicates that the propensity of a protein to aggregate is determined by both its primary structure and environmental conditions, including ionic strength, pH, and buffer composition [13].

The observed behavior may be associated with structural features of Omp19. Omp19 has previously been characterized as an outer membrane lipoprotein of *Brucella* spp. containing hydrophobic regions that mediate interactions with membrane structures [14]. After removal of denaturants, these regions may promote intermolecular interactions and subsequent protein precipitation. Similar difficulties in obtaining soluble Omp19 have been reported in studies involving recombinant expression in *Escherichia coli* [15,16].

To improve recombinant-protein solubility, the concentration of L-arginine was optimized. Arginine is currently regarded as one of the most effective stabilizing additives used during protein refolding. It can suppress protein–protein interactions, reduce hydrophobic aggregation, and help maintain protein solubility during purification and storage [17]. A recent review likewise identified arginine as a widely used additive for recombinant-protein refolding because of its ability to increase solubility and prevent aggregate formation [17].

In the present study, 1 M L-arginine did not fully stabilize Omp19, as a proportion of the protein continued to pellet. When the arginine concentration was reduced to 880 mM, the protein remained in the soluble fraction and no visible pellet formed. Electrophoretic analysis confirmed that the target protein was recovered predominantly in the supernatant after dialysis. This result is consistent with the concentration-dependent effects of arginine, which are governed by the balance of its interactions with the protein surface and solvent molecules [17,18].

The 880 mM concentration likely provided more favorable conditions for stabilizing Omp19 after removal of denaturing agents. Arginine can shield hydrophobic regions of protein molecules and inhibit intermolecular contacts that lead to aggregation [17,18]. This mechanism may explain the greater solubility of Omp19 in the optimized buffer compared with the previously tested dialysis conditions.

Taken together, the results indicate that the standard buffer systems tested did not support efficient refolding of recombinant Omp19 from *Brucella* spp., whereas a buffer containing 880 mM L-arginine

substantially improved protein solubility and stability. These findings are relevant to the further purification, storage, and use of recombinant Omp19 as a diagnostic antigen or vaccine component. Future studies should additionally assess the immunoreactivity and structural integrity of the protein following optimized dialysis.

Conclusions. The result of this research shows that standard buffer systems used in the dialysis of the recombinant Brucella spp. Omp19 protein do not always ensure that it remains in a soluble form, which leads to the formation of a pellet and a decrease in the yield of the target protein. It has been shown that optimizing the composition of the dialysis buffer by using 880 mM L-arginine helps maintain the solubility and stability of the protein.

Based on these results, buffer 5 is recommended for the further purification, storage, and use of the recombinant Omp19 protein as a promising antigen for diagnostic and immunobiological studies.

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Conflict of interest. The authors declare that they have no conflicts of interest related to the conduct of this study or the preparation and publication of this article.

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BRUCELLA SPP. OMP19 РЕКОМБИНАНТТЫ АҚУЫЗЫНЫҢ РЕФОЛДИНГІ ЖАҒДАЙЛАРЫН ОҢТАЙЛАНДЫРУ

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Аннотация. *Brucella spp.* Omp19 рекомбинантты ақуызы диагностикалық тест-жүйелер мен иммунобиологиялық препараттарды әзірлеуге арналған перспективалы антиген ретінде қарастырылады. Алайда оның ерігіш түрін алу өзекті мәселелердің бірі болып қала береді. Осы зерттеудің мақсаты ион алмастырғыш хроматография әдісімен тазартылғаннан кейінгі Omp19 рекомбинантты ақуызының диализ жағдайларын оңтайландыру болды.

Диализ тұздар мен тұрақтандырғыш компоненттердің құрамы әртүрлі бірнеше буферлік жүйелерде кезең-кезеңімен жүргізілді. Диализдің тиімділігі мен ақуыздың ерігіштігі әртүрлі сақтау температураларында үстіңгі сұйықтық пен тұнба фракцияларын талдау арқылы SDS-PAGE (натрий додецилсульфаты-полиакриламидті гель электрофорезі) әдісімен бағаланды. Зерттеу нәтижелері көрсеткендей, 1–4 буферлер қолданылған кезде рекомбинантты ақуыз негізінен ерімейтін күйге ауысып, тұнба фракциясында анықталды. Ақуыздың ерігіштігін арттыру мақсатында диализдік буфер құрамындағы L-аргининнің концентрациясы оңтайландырылды. Құрамында 880 мМ L-аргинин бар буферді пайдалану ақуыздың ерігіш фракцияда сақталуын қамтамасыз етіп, көзге көрінетін тұнбаның түзілуіне жол бермейтіні анықталды.

Алынған нәтижелер тазарту және сақтау кезеңдерінде Omp19 рекомбинантты ақуызын тұрақтандыру үшін 880 мМ L-аргининді қолдануды ұсынуға мүмкіндік береді. Бұл тәсіл аталған ақуызды диагностикалық және биотехнологиялық зерттеулерде одан әрі қолдану үшін практикалық маңызға ие.

Түйінді сөздер: *Brucella spp.*, Omp19, рекомбинантты ақуыз, диализ, SDS-PAGE.

ОПТИМИЗАЦИЯ УСЛОВИЙ РЕФОЛДИНГА РЕКОМБИНАНТНОГО БЕЛКА OMP19 BRUCELLA SPP.

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Аннотация. Рекомбинантный белок Omp19 *Brucella spp.* рассматривается как перспективный антиген для разработки диагностических тест-систем и иммунобиологических препаратов, однако получение его растворимой формы остаётся актуальной задачей. Целью настоящего исследования

являлась оптимизация условий диализа рекомбинантного белка Omp19 после очистки методом ионно-обменной хроматографии.

Диализ проводили последовательно в нескольких буферных системах с различным составом солей и стабилизирующих компонентов. Эффективность диализа и растворимость белка оценивали методом ДСН-ПААГ (додецил сульфат натрия – полиакриламидный гель) с анализом надосадочных и осадочных фракций при различных температурных режимах хранения. Результаты показали, что при использовании буферов 1-4 рекомбинантный белок преимущественно переходил в нерастворимую форму и обнаруживался в осадке. Для повышения растворимости белка была проведена оптимизация концентрации L-аргинина в составе диализного буфера. Установлено, что применение буфера, содержащего 880 мМ L-аргинина, обеспечивало сохранение белка в растворимой фракции и предотвращало образование видимого осадка.

Полученные результаты позволяют рекомендовать использование 880 мМ L-аргинина для стабилизации рекомбинантного белка Omp19 на этапах очистки и хранения, что имеет практическое значение для дальнейшего применения белка в диагностических и биотехнологических исследованиях.

Ключевые слова: *Brucella spp.,* Omp19, рекомбинантный белок, диализ, ДСН-ПААГ.