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Purification, Renaturation, and Stability Study of FlaA Protein

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ABSTRACT

Objective: To establish an efficient purification and renaturation process for Helicobacter pylori flagellin A (FlaA) and to investigate its storage stability. Methods: Recombinant Escherichia coli cells were disrupted by high-pressure homogenization. Inclusion bodies were purified by washing with Triton X-100 and low-concentration urea. Single-factor experiments were conducted to optimize initial protein concentration, pH, temperature, and the concentrations of glycine, arginine, and glycerol during dialysis renaturation. Furthermore, an ultrafiltration-dialysis combined renaturation process was established. The Bradford method was used to monitor protein concentration changes to evaluate storage stability. Results: High-pressure homogenization effectively disrupted bacterial cells. After purification, the purity of FlaA protein exceeded 90%. The optimal conditions for dialysis renaturation were 0.1 mol/L glycine and 0.3 mol/L arginine, yielding a renaturation recovery of 80.67%. The ultrafiltration-dialysis combined renaturation achieved a recovery of 75.32% while significantly reducing processing time. Storage at a protein concentration of 0.5 mg/mL resulted in the slowest degradation rate at low temperature. Conclusion: This study establishes an efficient and economical process for the purification and renaturation of FlaA inclusion bodies, providing technical support for the large-scale preparation of anti-H. pylori egg yolk antibodies.

KEYWORDS

Helicobacter pylori; FlaA protein; Inclusion body renaturation; Dialysis renaturation; Ultrafiltration renaturation; Protein stability.

1. INTRODUCTION

Helicobacter pylori (H. pylori) is a pathogen responsible for numerous gastrointestinal diseases. Extensive studies have confirmed its close association with the development and progression of severe conditions such as chronic active or superficial gastritis, gastroduodenal ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma. The global infection rate is approximately 43% [1, 2]. Current clinical treatment of H. pylori-related diseases primarily involves combination therapy with proton pump inhibitors and antibiotics. However, with the increasingly serious problem of antibiotic resistance, the development of novel anti- H. pylori agents has become a research hotspot. Egg yolk antibody (immunoglobulin of yolk, IgY) offers advantages such as high specificity and no induction of drug resistance, showing promising application prospects against H. pylori infection [3]. However, its preparation is limited by issues such as high production costs of target antigen proteins and difficulties in inclusion body renaturation. Flagellin A (FlaA), the main structural subunit of the H. pylori flagellum, plays a key role in bacterial colonization and immune evasion [4] and holds significant potential in antigen preparation and vaccine development. Nevertheless, FlaA is predominantly expressed as inactive inclusion bodies in the E. coli expression system, requiring denaturation and renaturation to obtain soluble, biologically active protein [5].

Inclusion body renaturation remains a major challenge in the biopharmaceutical field. Traditional dialysis renaturation is time-consuming and costly, making it difficult to meet large-scale production demands. Ultrafiltration renaturation is efficient, but the high-pressure shear forces may affect protein activity [6]. In this study, using recombinant FlaA protein, we systematically optimized the purification, renaturation, and storage conditions, and innovatively combined ultrafiltration with dialysis technology, aiming to establish an efficient and economical process for FlaA protein preparation, thereby providing technical support for the large-scale production of anti- H. pylori specific IgY.

2. MATERIALS

2.1. Main Reagents

Lysozyme was purchased from Shanghai Yuanye Co., Ltd. The protein marker was purchased from Thermo Fisher Scientific. Triton X-100, urea, glycine, arginine, glycerol, Tris, and other reagents were of analytical grade.

2.2. Main Instruments

High-pressure homogenizer and ultrasonic cell disruptor were purchased from Ningbo Xinzhi Biotechnology Co., Ltd. Electrophoresis system and gel imaging analysis system were purchased from Tanon Corporation. Refrigerated centrifuge was purchased from Sichuan Shuke Instrument Co., Ltd. Micro-spectrophotometer was purchased from Thermo Fisher Scientific. Nanofiltration cross-flow filtration machine was purchased from Zhejiang Haina Membrane Technology Co., Ltd.

2.3. Experimental Strain

The protein expression plasmid pET-28a(+)-FlaA was constructed in our laboratory. After correct identification, it was induced for expression. The bacterial pellet was collected by centrifugation and stored in a -80°C refrigerator for subsequent experiments.

3. METHODS

3.1. Cell Disruption and Solubility Assessment

The frozen cell pellet was taken out from -80°C, and 20 volumes (w/v) of sterile PBS solution were added. After natural thawing, the suspension was thoroughly mixed using a magnetic stirrer to prepare a cell suspension, which was stored at 4°C. Cells were disrupted by both sonication and high-pressure homogenization, and the disruption efficiency was compared. The specific methods are as follows.

Ultrasonic disruption method: Lysozyme solution was added to the cell suspension to a final concentration of 0.1 mg/mL, followed by enzymatic digestion at 37°C for 2 h. After digestion, the suspension was frozen at -80°C until completely solidified, then thawed in a 37°C water bath; this freeze-thaw cycle was repeated twice. After freeze-thawing, the cells were disrupted by ultrasonication in an ice-water bath at 200 W, with a cycle of 2 s on and 3 s off for 40 min. After sonication, the lysate was stored at 4°C.

High-pressure homogenization method: The cooling cycle of the high-pressure homogenizer was set to 4°C. The homogenizer was purged of 75% ethanol, rinsed with deionized water, and then rinsed with sterile PBS solution. The cell suspension was added. After the material flowed out steadily, the outlet hose was placed back into the inlet for recirculation. The pressure valve was slowly adjusted to 800 bar for homogenization. After homogenization, the pressure was slowly released, and the disrupted cell suspension was collected in a clean beaker. Before the system was completely emptied,

20 mL of PBS solution was added. When the liquid at the outlet became clear, the outlet was directed to a waste container. The homogenized cell suspension was stored at 4°C.

Samples of the supernatant and pellet from each disruption protocol were collected and analyzed by SDS-PAGE to compare disruption efficiency and the localization of the target protein.

Cells induced at 17°C and 37°C were separately disrupted, and the supernatant and pellet were analyzed by SDS-PAGE to determine the localization of the target protein.

3.2. Purification and Denaturation of FlaA Protein

Inclusion body purification: The cell pellet after disruption was resuspended 1:10 (w/v) in PBS washing buffer containing 0.5% Triton X-100 and 1 mol/L urea. After thorough mixing, the suspension was centrifuged at 7,000 r/min for 10 min at 4°C. The supernatant was discarded, and the pellet was collected, weighed, and washed again under the same conditions (repeated twice). Then, the pellet was resuspended 1:20 (w/v) in PBS containing 2 mol/L urea, washed twice, and the final pellet was collected.

Inclusion body denaturation: The purified inclusion bodies were resuspended 1:40 (w/v) in denaturation solution (50 mmol/L Tris-HCl, pH 8.5) containing 8 mol/L urea. The suspension was stirred at 4°C for 1 h, then centrifuged at 8,000 r/min for 5 min. The supernatant was collected and stored at 4°C. Samples from the purification and denaturation processes were analyzed by SDS-PAGE.

3.3. Optimization of Dialysis Renaturation Conditions

Dialysis tubing (10 kDa molecular weight cutoff) was boiled in 50% ethanol, rinsed with deionized water, and then filled with denatured protein solution. The tubing was sequentially placed in dialysis solutions containing 6 M, 4 M, 2 M, 1 M, and 0 M urea (the dialysis solution contained 0.1 M arginine, 0.1 M glycine, 25 mL glycerol, and Tris buffer to a final volume of 500 mL). Single factors were varied while keeping others constant. Glycine concentrations tested: 0, 0.05, 0.1, 0.15, 0.2 mol/L. Arginine concentrations tested: 0, 0.1, 0.2, 0.3, 0.4, 0.5 mol/L. Dialysis was performed for 3 h at each urea concentration gradient. After renaturation, the protein solution was centrifuged at 4°C and 8,000 r/min for 5 min. The supernatant was collected, and the protein concentration was determined by the Bradford method. Renaturation yield (%) = (mass of protein in supernatant after renaturation / total mass of denatured protein) × 100%.

3.4. Ultrafiltration Combined with Dialysis Renaturation

Ultrafiltration renaturation: 1.5 L of denatured protein solution (0.5 mg/mL, 8 M urea) was subjected to ultrafiltration using a 10 kDa spiral-wound ultrafiltration membrane. Operating pressure was 2.0–2.5 MPa, temperature 4°C. Dialysis buffer was added in batches to maintain constant solution volume inside the system. The dilution formula $C_1V_1 = C_2V_2$ was used to calculate the required buffer volume. Initial denatured protein solution volume was 1.5 L, urea concentration 8 M. To reduce the concentration to 6 M, total volume needed to be expanded to V_2 = (8 M × 1.5 L) / 6 M = 2 L, thus requiring addition of 0.5 L dialysis buffer. During operation, when the ultrafiltration permeate volume reached 0.5 L, an equal volume of dialysis buffer was added to maintain constant liquid volume. Subsequent additions were calculated similarly to reduce urea concentration stepwise to 6 M, 4 M, 2 M, and 0 M. Samples were taken before each addition. After renaturation, the solution was centrifuged at 4°C and 8,000 r/min for 5 min. The supernatant was collected, protein concentration measured, and yield calculated. Protein purity was assessed by SDS-PAGE.

Ultrafiltration combined with dialysis renaturation: Ultrafiltration renaturation was used to reduce the urea concentration as described above. When the urea concentration reached 2 M, the protein solution was collected. Dialysis renaturation was then performed by sequentially placing the dialysis bag in

dialysis solutions containing 1 M and 0 M urea. After renaturation, the solution was centrifuged at 4°C and 8,000 r/min for 5 min. The supernatant was collected, protein concentration measured, yield calculated, and purity assessed by SDS-PAGE.

3.5. Protein Stability Study

Renatured FlaA protein was diluted with Tris buffer to final concentrations of 0.5, 1.0, and 1.5 mg/mL, sealed, and stored at -80°C. Take 3 samples randomly every two weeks, and protein concentration was determined by the Bradford method. Monitoring continued for 18 weeks.

3.6. Data Analysis

All experiments were performed in three independent replicates. Results are presented as mean ± standard deviation ($\bar{x} \pm s$). One-way ANOVA was performed using GraphPad Prism 9. A P-value < 0.05 was considered statistically significant.

4. RESULTS

4.1. Results of Cell Disruption

E.coli cells were disrupted by both sonication and high-pressure homogenization. After centrifugation, supernatant and pellet were analyzed by SDS-PAGE. As shown in Figure 1A, for the sonication group, the supernatant after lysozyme treatment showed some impurity proteins. After freeze-thaw treatment, impurity proteins in the supernatant increased slightly, while those in the pellet decreased slightly. After sonication, the impurity protein bands in the supernatant increased significantly, while impurity proteins in the target protein fraction decreased significantly. The target protein band was consistently present in the pellet. For the high-pressure homogenization group, the supernatant after treatment contained significantly more impurity proteins compared to the sonication group, while the pellet after homogenization contained significantly fewer impurity proteins compared to the sonication group. The target protein was present in the pellet. Therefore, both methods could disrupt E. coli cells, but high-pressure homogenization resulted in more thorough disruption, and the target protein existed as inclusion bodies in the pellet.

Cells induced at 17°C and 37°C were disrupted, and the supernatant and pellet were analyzed by SDS-PAGE. As shown in Figure 1B, no specific band was detected in the supernatant of the 37°C induction group; the specific band was present in the pellet. In the 17°C induction group, a specific band was detected in the supernatant, and a small amount of specific band was present in the pellet. Therefore, regardless of low or high temperature, FlaA protein was expressed as inclusion bodies.

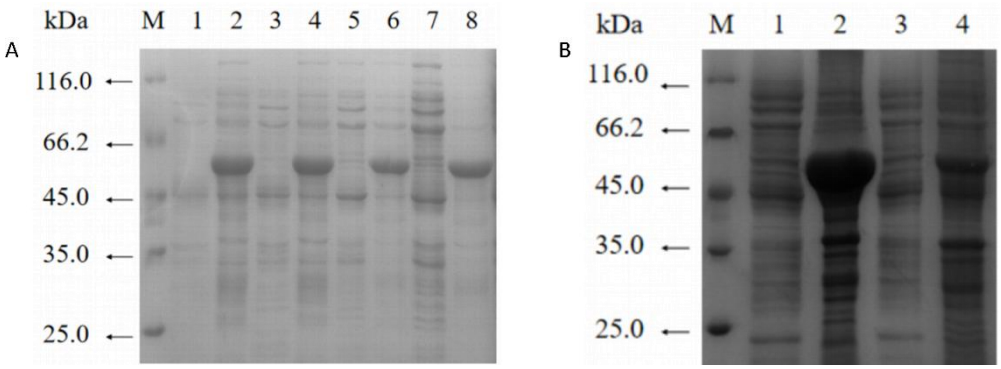

Figure 1. SDS-PAGE detection results of two lysis methods

Figure legend: A) SDS-PAGE results of two lysis methods. M) protein marker; 1) supernatant after lysozyme digestion; 2) pellet after lysozyme digestion; 3) supernatant after freeze-thaw; 4) pellet after

freeze-thaw; 5) supernatant after sonication; 6) pellet after sonication; 7) supernatant after homogenization; 8) pellet after homogenization. B) SDS-PAGE results after disruption of cells induced at 17 and 37°C. M) protein marker; 1) supernatant after disruption of 37°C-induced cells; 2) pellet after disruption of 37°C-induced cells; 3) supernatant after disruption of 17°C-induced cells; 4) pellet after disruption of 17°C-induced cells.

4.2. Protein Purification Results

The disrupted recombinant *E. coli* was washed with Triton X-100 and finally dissolved with 8 M urea. Samples were collected after centrifugation and analyzed by SDS-PAGE. As shown in Figure 2, after Triton X-100 washing, high-molecular-weight impurity proteins were significantly reduced. After further washing with 2 M urea, low-molecular-weight impurity proteins were also reduced. The inclusion body protein was denatured with 8 M urea, and the SDS-PAGE result (lane 4) showed the molecular weight is consistent with expectations. Therefore, the use of washing buffers containing 0.5% Triton X-100 with 1 M urea and 2 M urea effectively removed impurity proteins.

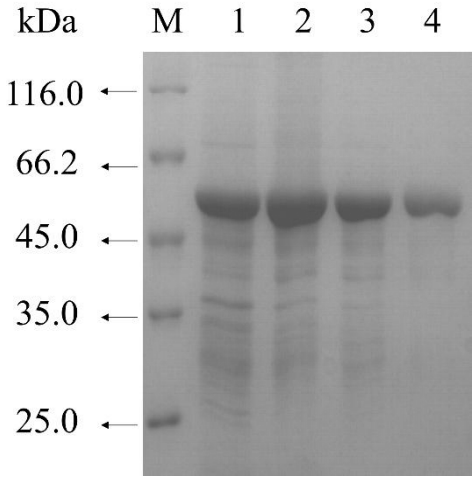

Figure 2. SDS-PAGE detection results during the washing process

Figure legend: M) protein marker; 1) before purification; 2) after Triton X-100 washing; 3) after 2 M urea washing; 4) after denaturation with 8 M urea.

4.3. Dialysis Renaturation Results

By varying single factors, the concentrations of arginine and glycine were adjusted separately. Denatured protein was renatured by dialysis. After renaturation, the supernatant was collected by centrifugation, and protein concentration was measured. As shown in Figure 3, as glycine concentration increased, the protein yield first increased and then decreased. The highest protein yield (69.07%) was achieved at 0.1 M glycine. The yield difference was small between 0.1 and 0.2 M glycine (Figure 3A). As arginine concentration increased, the protein yield also first increased and then decreased. The highest protein yield (80.67%) was achieved at 0.3 M arginine. The yield difference for FlaA protein was small between 0.3 and 0.5 M arginine (Figure 3B). Therefore, 0.1 mol/L glycine and 0.3 mol/L arginine were more favorable for FlaA protein dialysis renaturation.

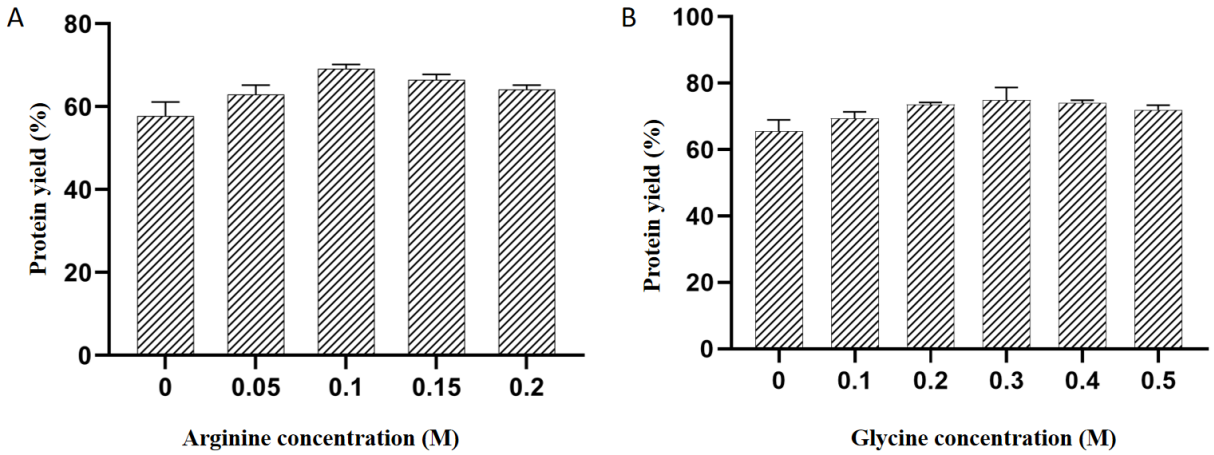

Figure 3. Renaturation yield of FlaA protein

Figure legend: A): Yield of FlaA protein under different glycine concentrations; B): Yield of FlaA protein under different arginine concentrations.

4.4. Ultrafiltration-Dialysis Combined Renaturation

When using ultrafiltration renaturation alone, as the urea concentration decreased, the FlaA protein concentration showed a decreasing trend. When the urea concentration dropped below 2 M, the protein concentration decreased sharply, indicating renaturation failure (Figure 3A – this refers to a figure within the original; likely Figure 4A). As the urea concentration decreased, the FlaA protein band gradually became lighter; at 1 M urea, almost no target band was visible (Figure 3B – likely Figure 4B). When using ultrafiltration combined with dialysis for FlaA protein renaturation, as the urea concentration decreased, the FlaA protein concentration also showed a decreasing trend, but the decrease was slower compared to ultrafiltration alone. After renaturation, the FlaA protein yield was approximately 75.32% (Figure 3C – likely Figure 4C). SDS-PAGE analysis of the renatured protein showed a target band at the expected size, and ImageJ analysis indicated a purity greater than 90%. Therefore, ultrafiltration alone could not effectively renature FlaA protein, but ultrafiltration combined with dialysis successfully restored the inclusion body FlaA protein to soluble, active protein.

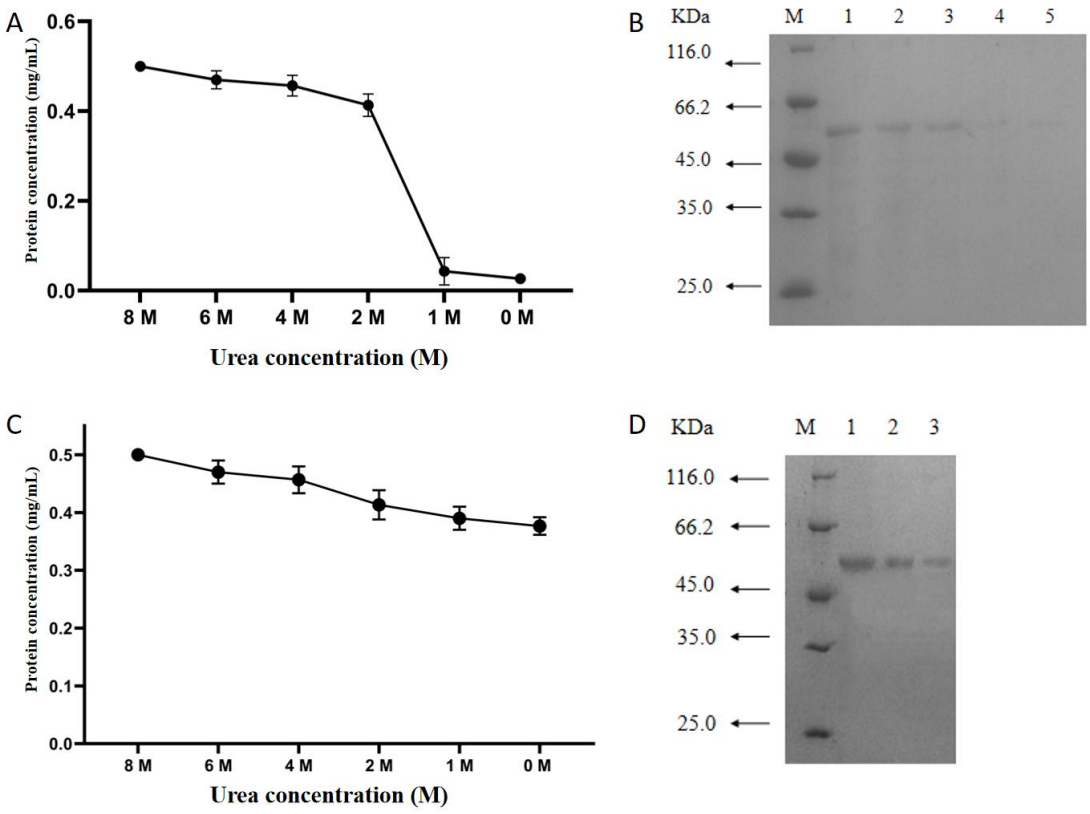

Figure 4. Concentration and SDS-PAGE detection results of two renaturation methods

Figure legend: A, B: Detection results of FlaA protein renaturation using ultrafiltration alone; C, D: Detection results of FlaA protein renaturation using ultrafiltration combined with dialysis.

4.5. Protein Stability Test Results4

Different storage concentrations significantly affected the degradation rate of FlaA protein (Figure 4 – should be Figure 5). Over 20 weeks, the protein concentration in the 0.5 mg/mL group decreased from 0.50 mg/mL to 0.40 mg/mL (retention rate 80.2%), while the retention rates for the 1.0 mg/mL and 1.5 mg/mL groups were 58.6% and 51.3%, respectively. The degradation rate of the 0.5 mg/mL group was significantly lower than that of the higher concentration groups ($P < 0.01$).

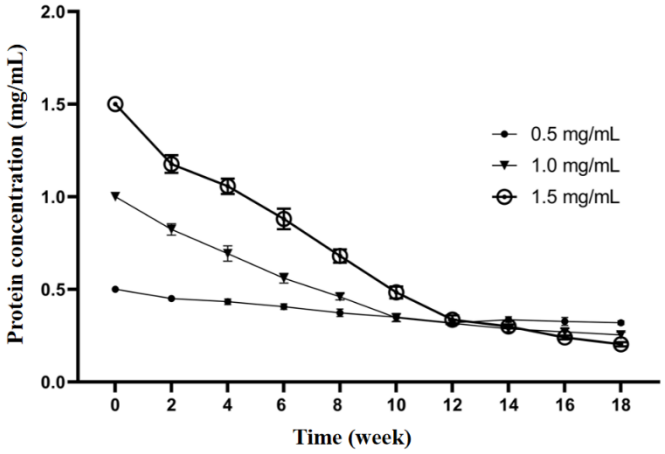

Figure 5. Effect of initial protein concentration on the titer of FlaA protein

5. DISCUSSION

Based on protein structure prediction, the FlaA protein lacks a signal peptide, transit peptide, and transmembrane domain, and is hydrophilic. Theoretically, FlaA should be expressed in a soluble form. However, in practice, we attempted induction at both low temperature (17°C, slow) and high temperature (37°C, fast), and after disrupting the induced cells, we found that the protein was present as inclusion bodies in the pellet regardless of temperature. This indicates that our recombinant E. coli expresses FlaA as inclusion bodies under both conditions. We hypothesize that this may be due to the relatively large molecular weight and complex structure of FlaA. Additionally, FlaA contains four cysteine residues, which may make it difficult to form correct disulfide bonds in the reducing cytoplasmic environment of E. coli, leading to misfolding and aggregation. Therefore, subsequent purification and renaturation are required to restore the active structure of the protein.

Given the high expression level but inclusion body formation of FlaA in E. coli, we first used high-pressure homogenization to disrupt the cells. This method is more efficient and thorough than sonication and is suitable for large-scale production. Washing with Triton X-100 and low-concentration urea effectively removed impurities such as lipopolysaccharides and membrane proteins, resulting in FlaA purity exceeding 90%.

Arginine is currently recognized as the most effective protein aggregation inhibitor. Its mechanism is not to promote correct folding but to inhibit hydrophobic interactions, thereby preventing aggregation between misfolded molecules [7]. In this study, 0.3 mol/L arginine increased the yield to 74.87%, confirming its effectiveness in FlaA renaturation. The core role of glycine is to help solubilize proteins, inhibit misfolding and aggregation, thereby significantly improving the renaturation success rate and activity recovery of target proteins. Furthermore, Zhang Yu Nan et al. found that initial protein concentration is a key factor affecting renaturation yield. Too high a protein concentration can enhance intermolecular hydrophobic interactions, leading to irreversible aggregation; too low a concentration reduces production efficiency [8]. Del Giudice et al. found that the effect of pH on renaturation is closely related to the isoelectric point [9]. The isoelectric point of FlaA is 6.03. When the dialysis buffer pH is 8.5, far from the isoelectric point, it helps maintain the protein in a negatively charged state and reduce aggregation. Notably, ultrafiltration renaturation alone failed when the urea concentration dropped below 2 mol/L, presumably because high shear forces caused aggregation of folding intermediates or retention by the membrane. By combining ultrafiltration with dialysis, switching to gentle dialysis renaturation after the urea concentration reached 2 mol/L, we utilized the efficiency of ultrafiltration while avoiding the damaging effects of shear forces on folding proteins. This provides a new approach for large-scale renaturation of inclusion body proteins. Regarding storage stability, FlaA protein at 0.5 mg/mL degraded the slowest at -80°C, with a retention rate of 80.2% after 20 weeks, while higher concentrations degraded faster, possibly due to enhanced intermolecular interactions leading to aggregation and precipitation.

6. CONCLUSION

This study established an efficient purification and renaturation process for FlaA inclusion bodies: high-pressure homogenization for cell disruption, purification by washing with Triton X-100 and low-concentration urea. The optimal dialysis renaturation conditions were 0.1 mol/L glycine and 0.3 mol/L arginine, achieving a renaturation yield of 80.67%. The ultrafiltration-dialysis combined renaturation achieved a yield of 75.32% while significantly reducing processing time. This process provides technical and raw material support for the large-scale preparation of anti-H. pylori specific IgY and diagnostic test strips.

ACKNOWLEDGMENTS

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REFERENCES

[1] MALFERTHEINER P, CAMARGO M C, EL-OMAR E, et al. Helicobacter pylori infection [J]. Nature Reviews Disease Primers, 2023, 9(1): 19.

[2] BANISEFID E, NASIRI E, POUREBRAHIMIAN LEILABADI S, et al. The paradox of Helicobacter pylori: how does H. pylori infection protect against esophageal cancer? [J]. 2024, 86(12): 7221-6.

[3] ZHANG L, XIAO Y, JI L, et al. Potential Therapeutic Effects of Egg Yolk Antibody (IgY) in Helicobacter pylori Infections─A Review [J]. Journal of Agricultural and Food Chemistry, 2021, 69(46): 13691-9.

[4] GU H. Role of Flagella in the Pathogenesis of Helicobacter pylori [J]. Current microbiology, 2017, 74(7): 863-9.

[5] NI H, GUO P C, JIANG W L, et al. Expression of nattokinase in Escherichia coli and renaturation of its inclusion body [J]. Journal of biotechnology, 2016, 231: 65-71.

[6] KARAKAŞ F, ARSLANOĞLU A. Gene cloning, heterologous expression, and partial characterization of a novel cold-adapted subfamily I.3 lipase from Pseudomonas fluorescence KE38 [J]. Sci Rep, 2020, 10(1): 22063.

[7] SOLEYMANI B, BARZEGARI E, MANSOURI K, et al. Heterologous expression, purification, and refolding of SRY protein: role of L-arginine as analyzed by simulation and practical study [J]. Molecular biology reports, 2020, 47(8): 5943-51.

[8] ZHANG Y N, LIU J J, ZHANG W, et al. Expression, purification and refolding of pro-MMP-2 from inclusion bodies of E. coli [J]. Protein Expression and Purification, 2023, 208-209: 106278.

[9] DEL GIUDICE A, DEL GIUDICE D, SPATOLA E, et al. An albumin unfolding and refolding cycle induced by a time-controlled pH jump [J]. Organic & biomolecular chemistry, 2024, 23(1): 118-25.