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Original Article

Optimized Expression and Highly Efficient Purification of the Anti-inflammatory Drug rIL-1Ra from *E. coli* using Ni/Silica-Coated Magnetic Nanoparticles

Mohammad Sadegh Hashemzadeh [1*], **Gholamhossein Alishiri** [2]

[1] Nanobiotechnology Research Center, New Health Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2] Department of Rheumatology, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran

***Corresponding Author:** Mohammad Sadegh Hashemzadeh, Ph.D., Nanobiotechnology Research Center, New Health Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel: + 989919001032, Email: Dr_Hashemzadeh@bmsu.ac.ir

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Abstract

Background: IL-1β is a key mediator of inflammation in the body. Upon inflammasome activation, the IL-1 receptor antagonist (IL-1Ra) serves as the primary natural inhibitor of IL-1β by competitively binding to its receptor, thereby limiting inflammatory signaling. Due to this mechanism, IL-1Ra has garnered significant interest as a biological anti-inflammatory drug.

Objectives: This study aimed to produce recombinant IL-1Ra (rIL-1Ra) in *E. coli* using optimized expression conditions and to develop a highly efficient purification process utilizing Ni/silica-coated magnetic nanoparticles.

Methods: The *IL-1Ra* gene was cloned into the pET-28a expression vector. The correct construction of the recombinant plasmid was verified by PCR and DNA sequencing. Expression of rIL-1Ra was carried out in *E. coli* BL21 (T7 Express) under optimized conditions (induction with 0.5 mM IPTG at 25 °C for 16-18 h). The expressed protein was analyzed by SDS-PAGE and Western blot. Purification was performed using Ni/silica-coated magnetic nanoparticles, followed by protein concentration via polyethylene glycol (PEG). The protein concentration was determined by Bradford assay, and the product was subsequently stabilized by buffer exchange into PBS (pH 7.4) through dialysis, supplemented with 10% glycerol, and stored at -20 °C.

Results: PCR and sequencing confirmed the successful construction of the expression cassette, showing the expected ~450 bp insert. SDS-PAGE and Western blot analyses detected a protein of approximately 19.8 kDa, confirming the expression and identity of rIL-1Ra. Maximum soluble expression was achieved under the optimized conditions. Purification using Ni/silica-coated magnetic nanoparticles yielded 10 mg of rIL-1Ra per 1000 ml of bacterial culture (10 mg/L).

Conclusion: The *E. coli* BL21 (T7 Express) system proved to be an effective and cost-efficient host for producing soluble rIL-1Ra. Furthermore, the use of Ni/silica-coated magnetic nanoparticles provided an efficient and scalable purification method, yielding a substantial amount of the recombinant protein suitable for further research and potential therapeutic applications.

Keywords: Interleukin 1 Receptor Antagonist Protein, Arthritis, Rheumatoid, Anti-Inflammatory Agents, Magnetite Nanoparticles

1. Background

Inflammation is a non-specific defense mechanism that arises in response to the destructive function of physical, chemical, and biological threatening agents. Prolonged inflammation, especially in atypical conditions, can lead to irreparable damage such as rheumatoid arthritis, a chronic autoimmune disease.[1-4] Interleukin-1 beta (IL-1β) is known as one of the crucial cytokines involved in inflammation in mammalian tissues. In contrast, interleukin-1 receptor antagonist (IL-1Ra) has been identified as an anti-inflammatory cytokine that inhibits the inflammation triggered by IL-1. This antagonist is secreted by immune cells such as monocytes, macrophages, and other non-immune cells like fibroblasts and hepatoblasts. This 153-amino acid polypeptide has a molecular weight of 19.8 kDa.[5] Since IL-1β plays a key role in causing inflammation in the body following the formation of the inflammasome complex, the presence of adequate levels of IL-1Ra in the blood limits the inflammatory effects of IL-1β, making it the most important inhibitor. Therefore, IL-1Ra is of interest as a biological anti-inflammatory drug. IL-1Ra is a member of the IL-1 family, existing in both secretory and intracellular forms resulting from the transcription of the same gene.[6]

Although the intracellular type has no apparent function, its secretory form tightly and competitively binds to the IL-1 receptor. The three-dimensional structure of IL-1Ra is totally similar to IL-1, with no apparent biological activity or side effects on the cell. Thus, through the occupation of the active site of the cell surface receptor by IL-1Ra, the anti-inflammatory effects are apparent.[7] Restoration of the balance between IL-1

and IL-1Ra with the administration of recombinant exogenous IL-1Ra is a therapeutic approach that has been considered in recent years.[8] Generation of recombinant products is a precise approach to supply a large amount of protein with medical applications.[9]

Another significant advantage of producing recombinant drugs is the safety for human health compared to protein extraction from human fluids. There is compelling evidence that IL-1Ra plays a vital role in improving the signs and symptoms of RA and joint damage.[10] Also, the IL-1Ra can be used to treat a wide range of inflammatory diseases. Various recombinant drugs with inhibitory effects on IL-1, such as Rilonacept (Arcalyst) and Anakinra (Kineret), have fewer side effects compared to other biologic drugs and lower production costs in laboratory or industrial settings than monoclonal antibodies. *E. coli* is the common bacterium used for cloning and expressing recombinant drugs. This expression system has many advantages, such as simple control of gene expression, high efficiency of recombinant protein production (up to 50% of total cell proteins), different cloning vectors, and simple culturing.[11]

2. Objectives

Based on the importance of IL-1β in inflammatory processes, this study aimed to produce a laboratory-scale sample of recombinant mouse IL-1Ra (rIL-1Ra) in *E. coli* BL21 (T7 Express). The objectives were to optimize the recombinant expression of this biological anti-inflammatory drug to obtain the protein in its soluble, native-like form and to establish a highly efficient purification process using Ni/silica-coated magnetic nanoparticles. The final product is intended for future pre-clinical studies.

3. Methods

3.1. Preparation of Reagents, Genetic Constructs, and Bacterial Strains

The pET-28 expression vector harboring the IL-1Ra gene was obtained from GenScript (USA). Horseradish Peroxidase (HRP)-conjugated antibody, anti-6×His tag mouse monoclonal antibody, and kanamycin were purchased from Sigma (USA) and Roche (Germany), respectively. Restriction enzymes (*Nde I*, *BamH I*) and T4 DNA ligase were acquired from New England Biolabs (UK). *Taq* DNA polymerase was procured from Sinaclon (Iran).

The *E. coli* strains DH5α and BL21 (T7 Express) were sourced from Novagen and New England Biolabs, respectively. For bacterial growth, liquid and solid LB media were used. Plasmid purification was performed using the DNA-spin™ Plasmid DNA Purification Kit (Intron, Korea). Ni/Silica-coated magnetic nanoparticles were provided by the Nanobiotechnology Research Center of Baqiyatallah University of Medical Sciences (Iran).

3.2. In Silico Gene Design and Synthesis

To synchronize the codon usage pattern of the target gene with that of the expression host, the *IL-1Ra* sequence was optimized using GenScript software. Following optimization, a recombinant expression cassette containing the *IL-1Ra* gene was designed and synthesized within the pET-28a vector.

3.3. Preparation of Competent Cells and Transformation

E. coli BL21 competent cells were transformed with the pET-28a-rIL-1Ra plasmid for protein expression. Before transformation, the plasmid was amplified in *E. coli* DH5α. The transformed cells were then plated on Luria-Bertani (LB) agar plates containing kanamycin (50 µg/ml) and incubated at 37 °C for 16 h. Following colony formation, white colonies were selected, inoculated into 100 ml of LB liquid medium supplemented with kanamycin (50 µg/ml), and cultured in a shaker incubator (12 h, 37 °C, 200 rpm).

The cells were harvested by centrifugation, and the plasmid DNA was extracted using a plasmid purification kit (Qiagen, Germany). The extracted plasmid was verified by 1% agarose gel electrophoresis. Plasmid concentration was determined by measuring the optical density at 260 nm (OD260) and was calculated to be 35 ng/µl, with the bacterial culture density confirmed at an OD600 of 0.8-1.0 before harvesting.

3.4. Confirmation of the pET-28a–rIL-1Ra Plasmid

The correctness of the r*IL-1Ra* gene insert within the pET-28a vector was confirmed by colony PCR and DNA sequencing. For colony PCR, a single colony (>1 mm diameter) was resuspended in 50 µl of sterile distilled water. Cell lysis was performed by incubation at 99 °C for 5 min, followed by centrifugation at 12,000 × g for 1 min to pellet cell debris. A 10 µl aliquot of the supernatant was used as the PCR template.

The PCR reaction mixture (total volume 25 µl) consisted of 1 µl of template, 1 µl of 10 mM dNTP mix, 1 µl of each forward and reverse primer (5 pmol/µl), 2.5 µl of 10× PCR buffer, 1.5 µl of 25 mM $MgCl_2$ (final concentration 1.5 mM), and 0.2 µl of *Taq* DNA polymerase. The pET-28a-specific primers used were:

Forward: 5′-ATTGTGAGCGGATAACAATTCC-3′

Reverse: 5′-TTCCTTTCGGGCTTTGTTAG-3′

The PCR product was analyzed by 1% agarose gel electrophoresis, which revealed an amplification band of the expected size. For definitive confirmation, the plasmid construct was subsequently verified by DNA sequencing.

3.5. Optimized Expression of rIL-1Ra in E. coli BL21 (T7 Express)

Recombinant IL-1Ra was expressed in *E. coli* BL21 (T7 Express) via IPTG induction. A single colony harboring the pET-28a–*rIL-1Ra* plasmid was inoculated into 100 ml

of LB broth supplemented with kanamycin (50 µg/ml) and cultured overnight at 37 °C with shaking. This starter culture was used to inoculate a fresh medium, which was grown until the OD600 reached approximately 0.6. Protein expression was then carried out under optimized conditions (0.5 mM IPTG, 25 °C, 16-18 h), which were determined based on prior optimization studies that tested various IPTG concentrations (0.1-1 mM), temperatures (18, 25, 37 °C), and induction times (5-7, 16-18, 24 h).

The cells were harvested by centrifugation at 6,000 rpm for 5 min. The cell pellet was resuspended in a suitable lysis buffer and disrupted by sonication on ice (10 cycles of 15 seconds pulse and 45 seconds rest, 75% power). The resulting lysate was clarified by centrifugation at 14,000 rpm for 20 min at 4 °C. The supernatant, containing the soluble recombinant protein, was collected and stored at 4 °C for subsequent purification.

3.6. Analysis of Recombinant Protein Expression

Recombinant IL-1Ra expression was analyzed by SDS-PAGE and Western blotting. Bacterial cells from induced and non-induced cultures were harvested by centrifugation. The cell pellets were lysed, and the total protein content of the lysates was quantified. Equal amounts of protein (e.g., 20 µg) from each sample were separated on a 12% SDS-polyacrylamide gel.

For Western blot analysis, the proteins were electrophoretically transferred from the gel onto a PVDF membrane. The membrane was then blocked with 5% skimmed milk in TBST for 2 h at room temperature to prevent non-specific antibody binding. Subsequently, the membrane was incubated with a primary anti-6×His tag mouse monoclonal antibody (dilution 1:1000) overnight at 4 °C. Following extensive washing with TBST, the membrane was probed with a horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (dilution 1:2000) for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) substrate according to the manufacturer's instructions.

3.7. Purification of His-Tagged rIL-1Ra by Ni/Silica-Coated Magnetic Nanoparticles

Following confirmation of expression, the His-tagged rIL-1Ra was purified under native conditions using Ni/silica-coated magnetic nanoparticles obtained from the Nanobiotechnology Research Center of Baqiyatallah University of Medical Sciences (BUMS).

The nanoparticles were first equilibrated with a binding buffer (20 mM Sodium Phosphate, 300 mM NaCl, pH 8). The clarified bacterial lysate, supplemented with 10 mM imidazole, was incubated with the equilibrated nanoparticles for 60 min at 4 °C with gentle agitation to facilitate binding.

The nanoparticles were then separated using a magnetic stand, and the flow-through was collected. To remove non-specifically bound proteins, the nanoparticles were washed twice with a wash buffer (binding buffer containing 40 mM imidazole). The bound His-tagged rIL-1Ra was subsequently eluted by incubating the nanoparticles with an elution buffer (binding buffer containing 250 mM imidazole) for 10 min. The elution step was performed twice to maximize yield.

Fractions from each step - flow-through, wash, and elution - were analyzed by SDS-PAGE to confirm the purity and efficiency of the purification.

3.8. Quantification and Stabilization of Recombinant Protein

The concentration of the purified rIL-1Ra was determined using the Bradford assay. A standard curve was prepared using known concentrations of Bovine Serum Albumin (BSA). For the assay, 10 µl of each standard or sample was mixed with 1 ml of Bradford reagent in a cuvette. The mixture was incubated at room temperature for 5-10 min, and the absorbance was measured at 595 nm. A blank consisting of 10 µl of phosphate-buffered saline (PBS) mixed with 1 ml of Bradford reagent was used to zero the spectrophotometer.

Following quantification, the purified protein was stabilized via buffer exchange into PBS (pH 7.4) using dialysis. The final product was supplemented with stabilizers (10% glycerol and 0.05% BSA) and stored at -20 °C for subsequent use.

4. Results

4.1. Verification of pET-28a-rIL-1Ra Plasmid

The successful construction of the recombinant pET-28a/IL-1Ra plasmid was confirmed through PCR and restriction enzyme analysis. As shown in Figure 1, colony PCR of transformed *E. coli* DH5α cells demonstrated the presence of the rIL-1Ra insert. Definitive verification was

Figure 1. Verification of the recombinant pET-28a/*IL-1Ra* plasmid. The figure shows the confirmation of the *IL-1Ra* gene insert via colony PCR and plasmid analysis. Lane M: 1 kb DNA ladder; Lane 1: PCR products showing the expected ~700 bp fragment; Lane 2: Negative control (no template); Lane 3: Undigested plasmid DNA extracted from a colony.

achieved by double digestion of the purified plasmid with NdeI and BamHI. Agarose gel electrophoresis (1%) of the digest revealed a fragment of approximately 700 bp, corresponding to the released rIL-1Ra gene, confirming its correct insertion into the vector backbone.

4.2. High-Yield Expression of rIL-1Ra in E. coli BL21 (T7 Express) and Western Blot Analysis

Following confirmation, recombinant protein expression was carried out under optimized conditions. As shown in Figure 2, a band of approximately 19.8 kDa was observed, confirming successful expression. The highest level of expression was achieved using the *E. coli* BL21 (T7 Express) system. Furthermore, Western blot analysis (Figure 3) confirmed the identity of the expressed protein.

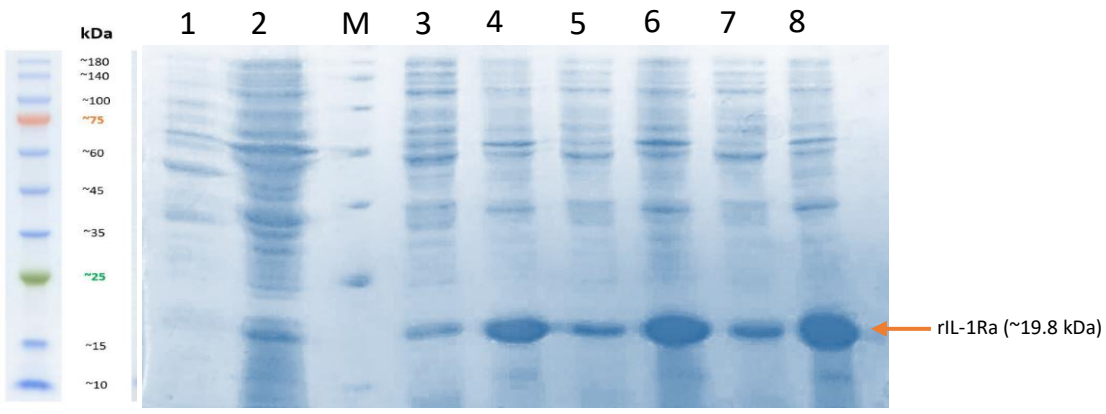

Figure 2. SDS-PAGE analysis of recombinant protein expression under optimized conditions. Lane M: Protein molecular weight marker. Lane 1: Supernatant of non-induced control; Lane 2: Pellet of non-induced control; Lanes 3, 5, and 7: Supernatant fractions from three induced colonies (containing soluble proteins); Lanes 4, 6, and 8: Pellet fractions from the same three induced colonies, respectively (containing insoluble proteins). The arrow indicates the expressed recombinant protein at approximately 19.8 kDa.

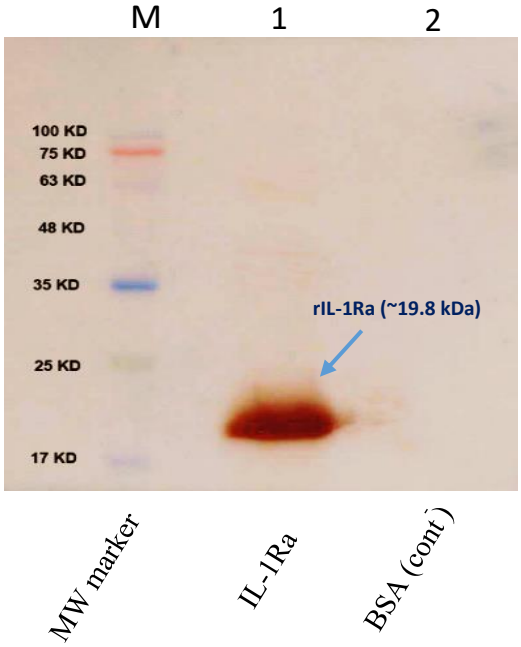

Figure 3. Western blot assay of recombinant IL-1Ra protein with amino-terminal anti-hexa-histidine-specific monoclonal the antibody. Column 1: Protein marker (SinaClon); Column 2: Reaction between antibody and the target protein (19.8 KDa) indicating the accurate expression of IL-1Ra; Column 3: No reaction between the antibody and BSA protein (Negative control).

4.3. Magnetic Nanoparticle-Based Purification, PEG-Mediated Concentration, and Stabilization of rIL-1Ra

Following expression in *E. coli* BL21 (T7 Express) system, the rIL-1Ra was purified using Ni/silica-coated magnetic nanoparticles. SDS-PAGE analysis confirmed the successful purification, showing a distinct band at the expected molecular weight (Figure 4). The purified protein was concentrated 3-fold, from 0.5 mg/ml to 1.5 mg/ml, using polyethylene glycol (PEG)-mediated dialysis.[11] Finally, to stabilize the protein product and prevent aggregation and precipitation, buffer exchange was performed by dialysis against PBS (pH 7.4). The final protein product was stored at -20 °C until further use (Figure 5). The purification process yielded 10 mg of

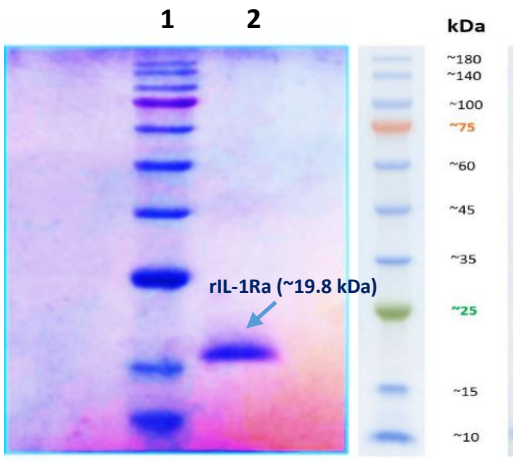

Figure 4. SDS-PAGE analysis of purified rIL-1Ra using Ni/silica-coated magnetic nanoparticles. Lane 1: Protein molecular weight marker (SinaClon); Lane 2: 10 µl of purified rIL-1Ra (0.5 mg/ml), showing a single band at approximately 19.8 kDa.

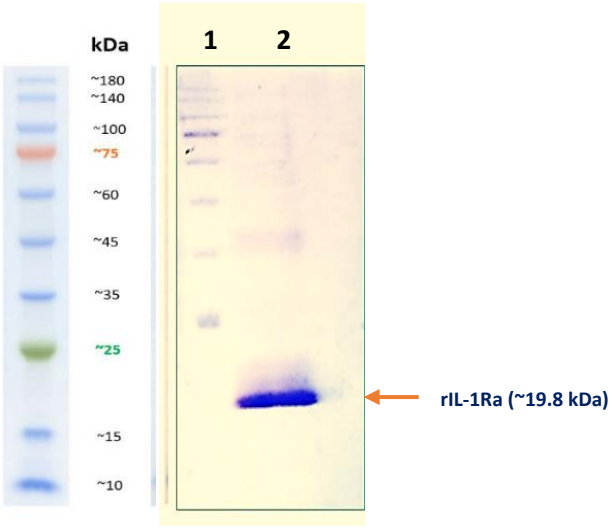

Figure 5. Analysis of PEG-mediated concentration of purified rIL-1Ra. SDS-PAGE analysis of the protein following purification with Ni/silica-coated magnetic nanoparticles and subsequent concentration using polyethylene glycol (PEG). Lane 1: Protein molecular weight marker (SinaClon); Lane 2: 10 µl of purified and 3-fold concentrated rIL-1Ra (1.5 mg/ml), showing a single band at approximately 19.8 kDa.

purified rIL-1Ra per liter of bacterial culture (10 mg/L).

5. Discussion

According to the findings, by examination of the common strain of *E. coli* BL21 (T7 Express) for protein expression, a considerable amount of rIL-1Ra was obtained. The results of this study can be effective and useful in increasing laboratory capacity to establish the process of producing recombinant drugs. Due to the high rate of bacterial growth and the powerful bacterial system for protein production in the form of inclusions, these cells can be used to produce different classes of proteins. In this study, *E. coli* BL21 (T7 Express) was considered a suitable bacterial strain for high recombinant protein expression. This is attributed to the presence of mutations in thioredoxin reductase and glutathione reductase enzymes, which facilitate disulfide bond formation.[12] The study concluded that the native-like form of this protein (soluble form) can be expressed in *E. coli* BL21 (T7 Express) systems. This system offers several advantages, including simple gene expression control, high recombinant protein yield (up to 50% of total cell protein), and easy culture.[13]

The most powerful system ever designed to clone and express recombinant proteins in *E. coli* is the pET system, developed by Novagen.[14] *E. coli* BL21 has the *T7* gene and defects in Lon and OmpT proteases.[15] The expression plasmid used in this study was pET28a, which has important properties such as a potent T7 promoter for further protein production.[16]

Since the recombinant form of IL-1Ra is non-glycosylated, it does not require a yeast expression system or a mammalian cell. According to the results obtained from the expression of this protein in bacteria, a significant difference was observed in the expression of this protein in *E. coli* BL21 (T7 Express). In one study, protein expression was increased, and the researchers determined the amount of expression under different environmental conditions, including varying concentrations of IPTG and specific time intervals. They found that the highest expression level is likely achieved when the induction of bacteria with 0.5 mM IPTG is incubated overnight at 25 °C. This study was conducted to optimize protein expression in a specific bacterial strain, and the factors influencing protein expression were investigated.

Birikh et al. published a paper on IL-1Ra expression. In that study, the PGMCE expression vector and genetic manipulation of the translation initiation region were used to increase gene expression. They found that protein expression could be increased by manipulating the expression vector, but no study was performed on the expression bacterium.[18] In a high-value protein expression study using an *E. coli* system, a low yield of 0.43 g/L was obtained, likely due to suboptimal fermentation conditions such as pH, temperature, dissolved oxygen, or induction time.[19]

Some research showed that injection of rIL-1Ra could reduce the severity of inflammation and improve the preclinical signs in the model of RA by 67%, which ultimately led to approval from the FDA for the treatment of RA.[20] In 2015, a study on human IL-1Ra in a *E. coli* host was conducted by Barati et al. The results showed that the expression of this protein in a *E. coli* hosts using pET28a expression vector was high. The study also showed a direct relationship between expression and cell harvest time after induction of protein expression using 1 mM IPTG.[21] In another study on *E. coli* BL21, the relationship between induction time and protein expression was investigated. It was concluded that after a day, the expression of this protein was higher.[12] Expression of this

protein in *E. coli* systems, due to its inability to exert post-translational modifications, faces limitations such as disulfide bond formation. In these cases, organisms such as yeast or Chinese Hamster Ovaries (CHO) can be used. However, due to the high cost and low level of protein expression associated with working with these two types of organisms, it was decided to use the *E. coli* expression system to achieve a high amount of protein. Because this protein contains disulfide bonds and *E. coli* BL21 has the ability to form disulfide bonds within the bacterium, a high amount of protein expression is probable.

In the present study, Ni/silica-coated magnetic nanoparticles were employed for the purification of recombinant IL-1Ra, demonstrating several notable advantages over conventional methods such as Ni-NTA column chromatography. The magnetic nanoparticle-based approach proved to be significantly faster, with binding and separation processes completed within minutes rather than hours. This method also exhibits superior efficiency in handling viscous lysates and small sample volumes, which often pose challenges in traditional column-based systems. Furthermore, the scalability of this technique allows for easy adaptation to larger production scales simply by increasing the quantity of nanoparticles and reaction vessels. The compatibility of this system with automated platforms also makes it particularly suitable for high-throughput applications, presenting a robust and versatile alternative for recombinant protein purification in both research and industrial settings. The successful implementation of this purification strategy underscores its potential for broader application in biopharmaceutical production, particularly for therapeutic proteins like IL-1Ra, where purity and efficiency are critical considerations.[22]

6. Conclusion

In this study, optimal expression of soluble recombinant IL-1Ra was achieved in the *E. coli* BL21 (T7 Express) system using 0.5 mM IPTG at 25 °C. The implemented purification strategy employing Ni/silica-coated magnetic nanoparticles resulted in a final yield of 7.5 mg from 750 ml of broth culture. Our findings demonstrate that the *E. coli* BL21 system serves as a cost-effective and efficient host for the large-scale production of soluble rIL-1Ra. Furthermore, the magnetic nanoparticle-based purification method proved to be a highly efficient, scalable, and robust alternative to traditional chromatography, highlighting its significant potential for both research and industrial-scale production of this therapeutic protein.

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Research Highlights

What Is Already Known?

IL-1Ra, as a biological anti-inflammatory drug, is the most important IL-1β inhibitor that limits the inflammatory effects caused by it. Therefore, cost-effective in vitro production of rIL-1Ra similar to its native form in appropriate values is of particular importance. The use of *E. coli* expression systems frequently results in the formation of insoluble protein aggregates, known as inclusion bodies. Consequently, the implementation of optimized expression and purification strategies is essential for obtaining sufficient yields of soluble, functional rIL-1Ra.

What Does This Study Add?

This study establishes an efficient method for producing and purifying the therapeutic protein rIL-1Ra. The study demonstrated that the *E. coli* BL21 (T7 Express) system is ideal for cost-effective, large-scale production of the soluble protein. Furthermore, the study showed that purification using Ni/silica-coated magnetic nanoparticles is a highly efficient and scalable alternative to traditional methods, offering significant potential for both research and industrial applications.

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Author Contributions

MSH: Obtained a grant from the Iran National Science Foundation (INSF), conceptualized the study, designed the methodology, conducted data analysis, and drafted the manuscript. GA: Assisted with study conceptualization and reviewed the manuscript. Both authors approved the final manuscript.

Conflict of Interest Disclosures

All authors declared that they have no conflict of interest.

Ethical Approval

All assessments were conducted in accordance with ethical principles and under the supervision of the University's Ethics Committee (Ethic No. IR.BMSU.REC.1398.301).

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Declaration of Generative AI and AI-assisted Technologies

The authors used the DeepSeek tool to check for grammatical errors, spelling mistakes, and obvious problems. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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