ISSN (O): 2581-8740 | ISSN (P): 2581-8732
Thio-Ngo *et al.*, 2020
DOI: 10.21276/SSR-IIJLS.2020.6.3.1

Research Article

open access

# Cloning, Expression and Purification of Recombinant Interleukin 1 Receptor Antagonist (IL-1RA) in *Escherichia coli*

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**Received: 21 Jan 2020/ Revised: 24 Mar 2020/ Accepted: 29 Apr 2020**

**ABSTRACT**

**Background:** Interleukin 1 (IL-1) is a cytokine that plays an important role in the immune system. However, the excessive IL-1 secretion is responsible for several diseases such as septic shock, cancer, Alzheimer's, etc. Interleukin 1 Receptor Antagonist (IL-1Ra) has been studied and demonstrated the ability to prevent the effects of IL-1 on rheumatoid arthritis. In this study, *E. coli* BL21(DE3) strain carrying human IL-1Ragene was structured to produce recombinant IL-1Ra as an initial source for rheumatoid arthritis and some other autoimmune diseases application testing.

**Methods:** The IL-1Ra gene encodes for IL-1Ra was codon optimized, chemically synthesized and cloned into the pET-His vector, creating recombinant plasmid pETHis-il1ra to express IL-1Ra under control of T7 promoter. *E. coli* BL21(DE3)/ pETHis- IL-1Ra strain was formed by the transformation of pETHis-il1ra into *E. coli* BL21(DE3), cultured in LB medium containing Ampicillin, supplemented with IPTG for the induction T7 promoter.

**Results:** IL-1Ra was excessively expressed in the cytoplasmic in soluble form. *E. coli* BL21(DE3)/ pETHis-il1ra strain was fermented in an one-liter bioreactor. IL-1Ra began to be expressed after induction of IPTG and reached a large amount after 8 hours of induction. IL-1Ra was purified by cation exchange chromatography with the amount of IL-1Ra protein obtained 43.11 mg with a purity of 95.8%.

**Conclusion:** We successfully cloned IL-1Ra gene into *E. coli* BL21(DE3) strain as a source of material for production of IL-1Ra. Furthermore, our one-step purification of recombinant IL-1Ra using cation exchange chromatography with Tris-HCl solution was applicable for large scale production. This result laid the groundwork for the study of applying IL-1Ra in the treatment of IL-1.

**Key-words:** Cation exchange chromatography, Fermentation, IL-1, IL-1Ra, Rheumatoid arthritis

## INTRODUCTION

Interleukin 1 (IL-1), a cytokine produced by macrophage, neutrophil and monocyte, plays an important part in pro-inflammatory responses, attracting other immune cells to the infection site, dilating blood vessels and inflammation [1]. However, when there is severe inflammation or any abnormal activities in the immune system, in which the production of IL-1 was uncontrollable, IL-1 was secreted into the circulation system and acted as a hormone. IL-1 might enhance the blood coagulation, lower blood pressure through lowering the heartbeat and widening blood vessels, causing fever, stimulating the liver to produce proteins such as fibrinogen, C response protein and haptoglobin thus leads to body failure. Effects of IL-1 were observed and have scientifically linked to autoimmune such as septic shock, rheumatoid arthritis, Alzheimer, and some cancers, etc [2].

Till now, researchers have found several methods to prevent IL-1 related diseases, including corticosteroid, interferon, IL-1 monoclonal antibodies, etc. Nevertheless, disadvantages of these methods were still remained, which could be stated such as unwanted effects onto other cells and high cost. Therefore, more findings for the prevention of IL-1 related diseases with lower cost and higher effective was essential. Recent

**How to cite this article**
Thi-Ngo KH, Nguyen-Le D, Nguyen-Phuoc T, Tran TL, Tran-Van H. Hieu Tran-Van, Associate Professor, Department of Molecular and Environmental Biotechnology, University of Science, Ho Chi Minh City, Vietnam. SSR Inst. Int. J. Life Sci., 2020; 6(3): 2528-2535.

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ISSN (O): 2581-8740 | ISSN (P): 2581-8732
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studies have shown that by using IL-1 Receptor Antagonist (IL-1Ra), rheumatoid arthritis was completely prevented [3-5]. Besides, IL-1Ra also possessed the ability to inhibit the effects of IL-1 on animal models such as septic shock, asthma, diabetes and some cancers, etc promising the use of IL-1Ra as a novel therapy in preventing diseases [6-8].

Based on that idea and the essential role of IL-1Ra in blocking IL-1 over effects, we cloned, expressed and purified recombinant IL-1Ra in *E. coli* to generate an IL-1Ra supplement source with low cost for further pharmaceutical applications.

## MATERIALS AND METHODS

This study performed in March, 2019 at the Department of Molecular and Environmental Biotechnology, Faculty of Biology and Biotechnology, University of Science, VNU-HCM, Hochiminh City, Vietnam.

**Host strain and plasmid-** Bacterial strain *E. coli* DH5$\alpha$ [F- endA1 hsdR17 (rk-/mk-) supE44 thi$\lambda$- recA1 gyrA96 $\Delta$lacU169 ($\phi$80 lacZ $\Delta$M15)] (Takara, Japan) was used for plasmid replication. Strain *E. coli* BL21(DE3) [F-, ompT, hsdS (rB- mB), gal (DE3)] was used for high yield expressing recombinant human IL-1Ra. Plasmid pSMART contained codon optimized, chemically synthesized *il1ra* gene encoded for IL-1Ra protein. This plasmid has the size of 1980 bp, including Kanamycine antibiotic encoded gene. Plasmid pET-His (Novagen, Germany) size of 4636 bp, including T7 promoter, which induced by using IPTG (isopropyl $\beta$-D thiogalactoside), ampicillin encoded gene (Amp) was used for constructing pETHis-il1ra plasmid for expression of IL-1Ra.

**Constructing recombinant pETHis-il1ra vector-** The process of constructing recombinant vector pETHis-il1ra was performed according to Ausubel *et al.* [9] with slight changes. In brief, *il1ra* gene encoded for IL-1Ra was obtained by PCR using plasmid pSMART as a template with specific primers IL1RA-F and IL1RA-R (Primers were not listed). PCR products and plasmid pETH was digested with *Bam*HI and *Nde*I. Ligation of the two products was carried out with enzyme T4 ligase. Plasmid pETHis-il1ra was transformed into *E. coli* DH5α strain competent cells through chemical transformation usingthe cold calcium solution. The strains, which carried recombinant pETHis-IL-1Ra were screened on Luria-Bertani (LB) agar plate (containing Ampicillin at 100 µg/ml final concentration) and colony PCR with T7 promoter specific primers for positive colonies. The PCR product of recombinant plasmid pETHis-il1ra had the size of 637 bp and was checked through gel electrophoresis on 1% agarose gel. Recombinant plasmid pETHis-il1ra was obtained from positive colonies and performed sequencing by BigDyeTM Terminator (Macrogene Inc., Korea). Sequences were compared with published sequences by Jellyfish software.

**Expression of recombinant IL-1Ra-** *E. coli* BL21(DE3) strain contained recombinant plasmid pETHis-il1ra was induced for expression of IL-1Ra following the instruction of Novagen [10]. In brief, *E. coli* BL21(DE3)/pETHis-IL-1Ra was cultured in LB medium with Amp at 100 µg/ml final concentration, shook 250 rpm, at 37$^{o}$C until $OD_{600}$ reached 0.8–1.0, then 0.5 mM concentration of IPGT was added, and continued culturing overnight, at 25$^{o}$C. The biomass was obtained, washed and suspended with Tris HCl10 mM, pH7 and EDTA 1 mM solution. Cells were broken using ultrasonic by Ultrasonic Cell Disruptor (USA), centrifuged at 13000 rpm, 4$^{o}$C in 10 minutes to obtain soluble and insoluble phases (debris).

**SDS-PAGE and Western blot-** The expression of IL-1Ra was analyzed by running SDS-PAGE with 15% gel. Proteins were stained with Coomassie Brilliant Blue. Proteins were transferred onto nitrocellulose after performing SDS-PAGE, and the presence of IL-1Ra was detected using IL-1Ra specific antibody (R&D Bioscience) [9].

**Fermentation of IL-1Ra using Jar FermentorBioTron-LiFlusone-liter system-** The fermentation of *E.* coli BL21(DE3) strain was conducted following Chen *et al.* [11] with modifications. *E. coli* BL21(DE3) strain containing plasmid pETHis-il1ra was cultured in 100 mL LB-Amp medium (final concentration of 100 µg/ml of Ampicillin) at 37$^{o}$C, 250 rpm in 18 hours. The whole cultured medium was transferred to Jar fermentor with 1 liter of LB-Amp medium. The fermentation parameters were set as the following: pH=7 (adjusted with $NH_4OH$ 15% and HCl 0.5N), relative humidity= 30%, 37$^{o}$C, stirrer speed of 350 rpm, aeration speed of 1 vvm. When $OD_{600}$ reached 0.8-1.0, IPTG was added to the final concentration of 0.5 mM for inducing the expression of IL-1Ra. Meanwhile, the stirrer speed and temperature were adjusted to 200 rpm and 25$^{o}$C, respectively. The total fermentation time

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in the Jar Fermentor system was 22 hours. The biomass and yield of expressing IL-1Ra were collected and measured in every 2 hours. After 22 hours of fermentation, cultured medium was centrifuged to collect the biomass and proceed to French pressure to obtain recombinant IL-1Ra in the soluble phase. Protein concentration was measured via Bradford method.

**Purification of IL-1Ra using cation chromatography-** IL-1Ra protein possesses a pI of 5.4. Therefore, using cation chromatography on the AKTA Explorer chromatography system (GE Healthcare, UK) was carried out with Hitrap SP FF 5 mL column. The process included[9]: (1) the column was equilibrated with Sodium acetate 20 mM pH=5 solution (solution A); (2) sample was loaded into the column; (3) unbound proteins were washed away with solution A; (4) bound proteins were eluted with Tris-HCl, 20 mM, pH=8 solution. Protein fractions were analyzed through SDS-PAGE and the presence of IL-1Ra was confirmed by Western blot probed with IL-1Ra specific antibody. The yield of purification was determined by using the Bradford method combined with gel density analyzed using Quatity One software (Biorad).

## RESULTS

**Obtaining *il1ra* gene and constructing recombinant plasmid pETHis-il1ra-** *il1ra* gene encoded for protein IL-1Ra was obtained using PCR with plasmid pSMART used as a template with two specific primers IL1RA-F and IL1RA-R. The results on gel agarose (Fig. 1) showed a DNA band with the size of approximately 474 bp (Fig. 1, lane 2), equal to the size of designed IL-1Ra encoded gene.

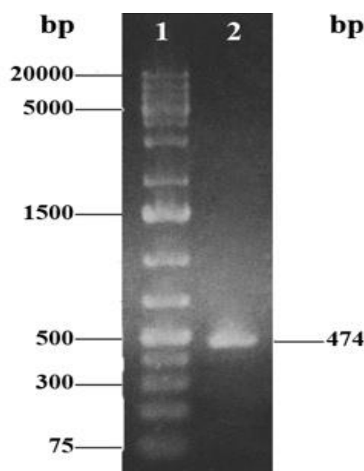


**Fig. 1**: PCR products of IL1Ra gene amplification. 1: DNA marker; 2: IL1Ra gene

After that *il1ra* gene was digested using restriction enzymes *Bam*HI and *Nde*I, then ligated to plasmid pET-His. Ligated product was transformed into *E. coli* DH5α strain, spread on LB-Amp agar plate and incubated at 37°C for 16 hours. Colonies on the plate were screened for positive strains containing the recombinant plasmid.

**Screening for positive *E. coli* DH5α strain containing recombinant plasmid pETHis-il1ra-** Colonies on LB-Amp agar plate were screened for the positive *E. coli* DH5α strain containing recombinant plasmid by PCR colonies with T7 promoter and terminator primers, digested with restriction enzymes and sequenced. Results on gel agarose (Fig. 2A) indicated that IL-1Ra gene was successfully inserted between *Bam*HI and *Nde*I site, with the size of 637 bp (Fig. 2A, lane 3-6). Otherwise, *il1ra* gene failed to insert into plasmid pET-His resulted in a 163 bp length DNA band on gel agarose (Fig. 2A, lane 2). Plasmid from positive colonies were extracted and screened with digestion reaction with *Bam*HI and *Nde*I and PCR plasmid (Fig. 2B). Plasmid pETHis-il1ra after digested by both enzymes had two separated DNA bands with the size of 4601 and 474 bp, respectively (Fig. 2B, lane 7). These two bands were the same size compared with plasmid pET-His and IL1Ra gene. Besides that digesting plasmid pETHis-il1ra and pET-His with *Eco*RI, resulted in 5110 and 4636 bp bands on gel agarose, respectively (Fig. 2B, lane 5 and 6). IL-1Ra gene in recombinant plasmid pETHis-il1ra was sequenced and compared with IL1Ra gene obtained from plasmid pSMART. These two genes showed 100% homology and the same translation frame. Taken together, we successfully screened the *E. coli* DH5α strain containing recombinant plasmid pETHis-IL-1Ra. This plasmid was then transformed into *E. coli* BL21(DE3) strain to construct an expression strain for human recombinant IL-1Ra.

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DOI: 10.21276/SSR-IIJLS.2020.6.3.1

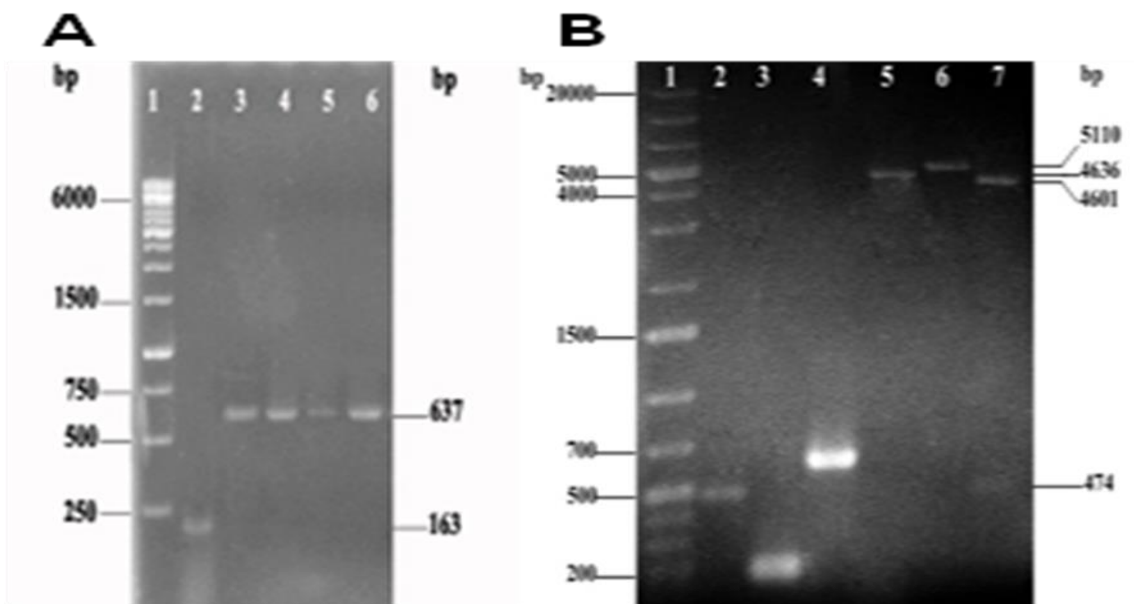


**Fig. 2:** Screening for positive colonies of *E. coli* DH5α strain that contains recombinant plasmid. PCR colonies with T7 promoter/terminator primers; (A) 1: DNA marker; 2: Negative control (T7 promoter sequence); 3-6: Recruited colonies; PCR plasmid and digestion with restriction enzymes *Bam*HI and *Nde*I (B) 1: DNA marker, 2: Il1Ra gene; 3: Negative control (T7 promoter sequence); 4: T7 primers product; 5,6: Plasmid pETHis-il1ra/pET-His *Eco*RI digested; 7: Plasmid pETHis-il1ra *Bam*HI and *Nde*I digested

**Inducing the expression of IL-1Ra in recombinant *E. coli* BL21(DE3)-** In order to assess the expression and the presence of IL-1Ra in inclusion bodies or soluble in bacterial cytoplasm, host strain *E. coli* BL21(DE3)/pETHis-il1ra was cultured in LB-Amp medium (final concentration of Ampicillin was 100 µg/ml) and induced by the presence of IPTG. The biomass was collected after cultured and induced by IPTG, including total, soluble (IL-1Ra in soluble form) and precipitated fraction (IL-1Ra in inclusion bodies). The presence of IL-1Ra in these fractions was analyzed by SDS-PAGE and Western blotprobed with IL-1Ra specific antibody (Fig. 3). Negative control was the *E. coli* BL21(DE3) induced by IPTG without transformed of pETHis-il1ra plasmid. There was an over expression band at about 17 kDa in the total protein fraction (Fig. 3A, lane 4) after inducing with IPTG but not before (Fig. 3A, lane 3). In addition, confirmation using Western blot with anti-IL-1Ra antibody resulted in a band in the film at the same weight (Fig. 3B, lane 4). This result indicated that the over expression protein obtained after IPTG inducing was recombinant IL-1Ra. There was also an over expressed band of IL-1Ra in soluble fraction (Fig. 3A, lane 5) but not in precipitated one (Fig. 3A, lane 6) proved that IL-1Ra was expressed in soluble form in the host strain cytoplasm. This result was confirmed by western blot (Fig. 3B, lane 5 and 6).

Collectively, we successfully constructed *E. coli* BL21(DE3)/pETHis-IL1Ra strain with over expression of soluble human recombinant IL-1Ra protein.

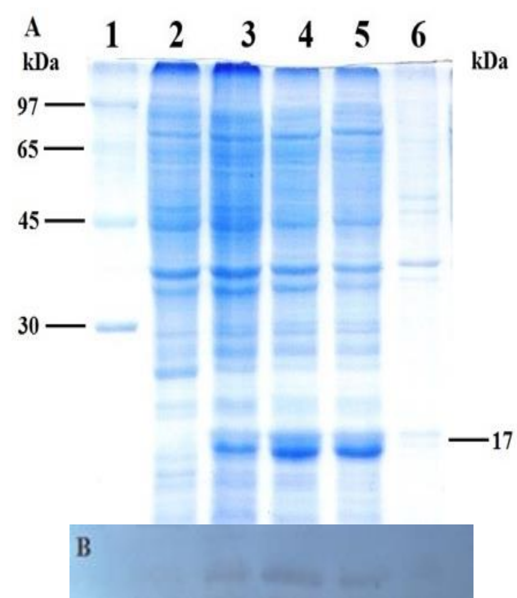


**Fig. 3:** Analyzing the expression of IL-1Ra in *E. coli* BL21(DE3) strain containing plasmid pETHis- IL1Ra by SDS-PAGE (A) and Western blot (B) 1: Protein marker (Low molecular weight); 2: *E. coli* BL21(DE3)/IPTG; 3: *E. coli* BL21(DE3)/ pETHis- IL1Ra non IPTG; 4-6: *E. coli* BL21(DE3)/ pETHis IL1Ra/IPTG (total; soluble and precipitated fraction)

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DOI: 10.21276/SSR-IIJLS.2020.6.3.1

**Fermentation of recombinant IL-1Ra using Jar Fermentor BioTron-LiFlusone-liter system-** For initial assessment of the expression of IL-1Ra of *E. coli E. coli* BL21(DE3)/pETHis-IL1Ra was pre-cultured, transferred into 1 liter of LB-Amp medium in the fermentation system (the v/v ratio was 10%). IPTG was added when $OD_{600}$ reached 0.76 and then fermentation took place for 22 hours. Samples were collected every 2 hours with a similar way for measuring $OD_{600}$ and the BL21(DE3)/pETHis-IL1Ra for generating the material for production of IL-1Ra, we fermented using the Jar Fermentor system.
expression of IL-1Ra (Fig. 4). The result showed that there was no significant difference in the growth rate of *E. coli* BL21(DE3)/pETHis-IL1Ra strain compared with non-pETHis-IL1Ra strain (Fig. 4A). Meanwhile, the expression of IL-1Ra in soluble form increased throughout the inducing period (Fig. 4B).

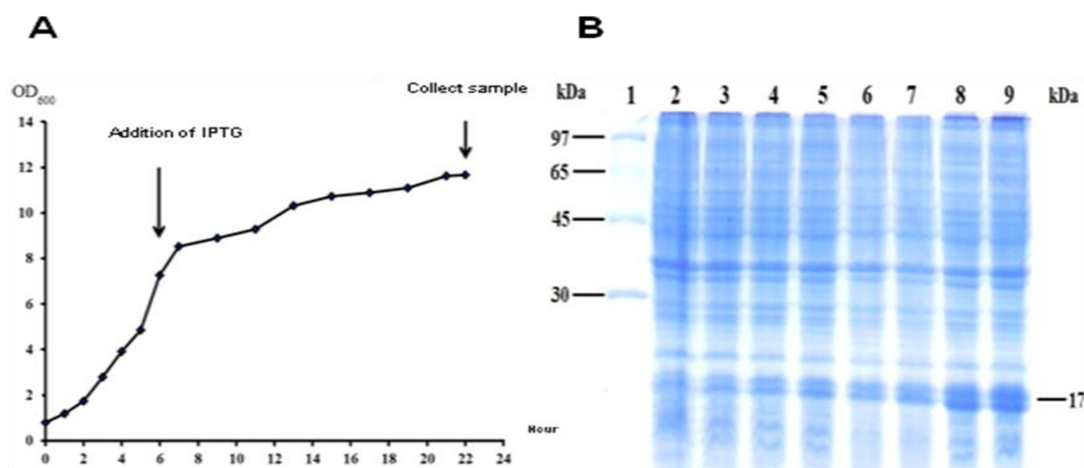


**Fig. 4:** Assessing the expression of IL-1Ra in *E. coli* BL21(DE3)/pETHis-IL1Ra. Growing curve of the strain (A) Analyzing with SDS-PAGE (B) 1: Protein marker; 2-9: protein in soluble form after 0, 2, 4, ect, and 14 hours of inducing, respectively

**Purification of IL-1Ra using cation exchange chromatography-** Protein IL-1Ra had the pI of 5.4 so at the pH=5 of buffer solution, IL-1Ra was positively charged. Therefore, we purified protein IL-1Ra with cation exchange chromatography by SP FF column. After the column was equilibrated and sample was loaded, we eluted the bound protein using two different ways: elution with pH (Tris-HCl solution pH=8 used) and elution with salt (Sodium acetate 20 mM and Sodium chloride 2 M solution pH=5 was used). The comparison of the two methods showed that elution with Tris-HCl pH=8 (Fig. 5A, lane 5) resulted in a higher protein concentration compared with elution with Sodium salts pH=5 (Fig. 5A, lane 9). The western blot result confirmed that we successfully purified IL-1Ra (Fig. 5B). Then, purification yield was analyzed with Quantity One software. The amount of protein IL-1Ra before purification accounted for 18.3% of the total soluble protein. In the flow-through solution, target protein only accounted 8.7% proven that IL-1Ra was able to bind to the column. In comparison, elution using Tris-HCl, pH=8 given a higher ratio of purification than that of using salt, although the difference was not significant (95.8 and 95.6%, respectively). However, the target protein was obtained with a higher amount when using Tris-HCl solution.

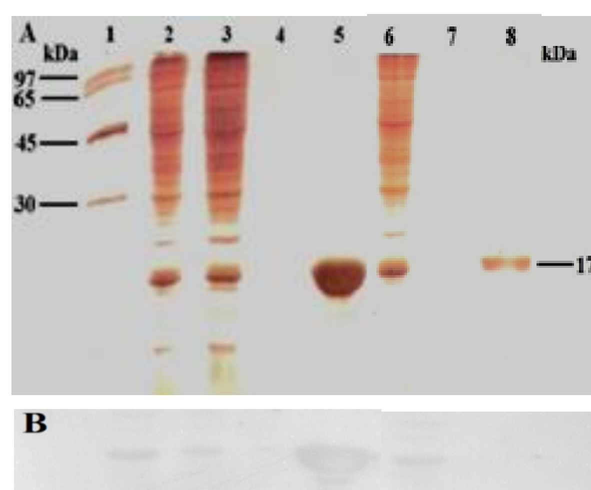


**Fig. 5:** Analyzing the purification of IL-1Ra using two different methods (A) and Western blot (B) 1: Protein marker; 2: Before purification; 3: Flow through; 4: Wash; 5: Elution with Tris-HCl, pH=8; 6: Before purification; 7: Wash; 8: Elution with Sodium acetate 20 mM and Sodium chloride (2 M); pH=5

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DOI: 10.21276/SSR-IIJLS.2020.6.3.1

Therefore, based on the recovery yield, we selected Tris-HCl, pH=8 as our elution buffer for further studies on IL-1Ra. Besides, protein concentration from fractions were also measured by Bradford method, then the amount of protein and recovery yield were calculated (Table 1).

**Table 1:** Summarizing the recovery yield of IL-1Ra

| Sample | Volume (mL) | Protein concentration (mg/mL) | Total protein (mg) | Purification ratio (%) | Amount of IL-1Ra (mg) |
|---|---|---|---|---|---|
| Before purification | 250 | 1.32 | 335.28 | 18.3 | 61.18 |
| IL-1Ra containing fraction | 125 | 0.36 | 45.0 | 95.8 | 43.11 |
| Yield (%) | – | – | 70.5 | – | – |

## DISCUSSION

Rheumatoid arthritis (RA) is a musculoskeletal inflammation which affects peripheral joint [12]. The prevalence of RA is low when considering worldwide data, which is around 0.5% [13] but had severe effects on health-related quality of life [14]. IL-1Ra, a natural occurring inhibitor of IL-1 was reported to have the ability to decrease the inflammatory response in animal models with arthritis diseases [15-17]. In patients with RA, the level of both IL-1 and IL-1Ra showed elevation, which meant IL-1Ra was produced and secreted when having RA. However, the amount of IL-1Ra still lower compared to IL-1 as the ratio of IL-1Ra/IL-1 was low [18]. Even though, we could have inferred from that IL-1Ra was secreted in order to reduce the rising of IL-1. Meanwhile, the administration of IL-1Ra into RA patients was reported to have anti-inflammatory effects and was able to slowdown joint damage [19]. Thus, IL-1Ra proved to be a solution for RA patients. To that generating an IL-1Ra production source to supply for the treatment of RA was essential. To meet this demand, in this study, we constructed recombinant plasmid pETHis-il1ra and cloned into *E. coli* BL21(DE3) strain to assess the expression, fermentation and purification of human IL-1Ra. Based on the results of sequencing and Western blot using anti-IL-1Ra antibody, we could confirm our recombinant protein was human IL-1Ra.

There were studies on cloning and expressing of IL-1Ra and other IL-1Ra fused protein. In 2008, Liu et al cloned and expressed IL-1Ra fused with IgE in inclusion bodies using pBV220 vector and purified by two-steps purification. On the other hand, human IL-1Ra cloned and expressed by Chang *et al*. [20] and Liu *et al*. [21] using pBV220 vector resulted in obtaining soluble form of hIL-1Ra. Taken together, recombinant IL-1Ra could be expressed in soluble form in different kind of vectors. Although, the amount of IL-1Ra expressed in our study was quite low comparing with Chang *et al*. [20] (18.3% and 40%, respectively), the purification ratio was almost equal (over 95%). By using pET-His vector as a carrier, the expression of IL-1Ra in *E. coli* BL21(DE3) strain was controlled by T7 promoter, which could be induced by adding IPTG. Therefore, to obtain a higher amount of IL-1Ra, other concentration of IPTG should be assessed. Furthermore, we tested one-step purification process instead of a well established two-step protocol, which resulted in reducing the cost when applying into larger scale, but still retrieved an enough amount of protein for future studies.

## CONCLUSIONS

We successfully constructed *E. coli* BL21(DE3) strain carrying recombinant plasmid pETHis-IL1Ra encoding for human IL-1Ra protein and induced expression with IPTG. The expression and purification of recombinant IL-1Ra were assessed through SDS-PAGE, Western blot and calculated by Quantitive One software. The fermentation process was carried out by Jar Fermentor BioTron-LiFlusone-liter system resulted in 61.8 g of IL-1Ra in one-liter cultured medium. The purification ratio of recombinant IL-1Ra using cation exchange chromatography was 95.8% and recovery yield was 70.5%.

This project laid groundwork for further study on human IL-1Ra and also provided a source of material for pharmaceutical research on rheumatoid arthritis.

ISSN (O): 2581-8740 | ISSN (P): 2581-8732
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**ACKNOWLEDGMENTS**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**CONTRIBUTION OF AUTHORS**

**Research concept-** Hieu Tran-Van

**Research design-** Kim-Hang Thi Ngo, Thanh Nguyen-Phuoc, Hieu Tran-Van

**Supervision-** Hieu Tran-Van

**Materials-** Hieu Tran-Van, Thuoc Linh Tran

**Data collection-** Kim-Hang Thi Ngo, Thanh Nguyen-Phuoc, Hieu Tran-Van

**Data analysis and Interpretation-**Kim-Hang Thi Ngo, Thanh Nguyen-Phuoc, Hieu Tran-Van

**Literature search-** Kim-Hang Thi Ngo, Duy Nguyen-Le

**Writing article-** Kim-Hang Thi Ngo, Duy Nguyen-Le, Hieu Tran-Van

**Critical review-** Hieu Tran-Van

**Article editing-** Duy Nguyen-Le, Hieu Tran-Van

**Final approval-** Hieu Tran-Van

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ISSN (O): 2581-8740 | ISSN (P): 2581-8732
Thio-Ngo *et al*., 2020
DOI: 10.21276/SSR-IIJLS.2020.6.3.1

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