Original Article

Design and Synthesis of Recombinant Murine Interleukin 4 (IL-4)

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Abstract

Background: Today's use of the recombinant cytokines in the fields of diagnosis, treatment and research is greatly considered. With regards to the important role of the Interleukin-4 cytokine in modulating and assessment of immune response, the availability of recombinant mouse IL-4 (mIL-4) cytokines will provide an important tool for researchers to further studies. **Method:** Total RNA was extracted from mouse spleen tissue. cDNA was then synthesized using RT-PCR. The target sequence was amplified by PCR, using designed primers which contains restriction sites. Analysis of PCR product was done by agarose gel electrophoresis. Amplified sequence was ligated in to the pET21-b (+) vector. E. coli Top10 was transformed with recombinant plasmid pET21-b (+) vector.The cloning was verified by PCR and gene sequencing. BL21(DE3)-CodonPlus E. coli bacteria was transformed with recombinant plasmid pET21-b (+) vector for expression of protein. The expression of protein was induced by IPTG. The expressed protein was analysed with SDS-PAGE. After use of the guanidine hydrochloride and dithiothreitol, the protein was refolded and purified by chromatography. To demonstrate the nature of the protein, Interleukin-4, western blot analysis was performed. **Results:** The length of visualized PCR product band on electerophoresis was correspond to the target fragment (360 bp). Analysis of PCR product which have done for screening colonies showed 591 bp band that indicated the desired ligation and transformation of colonies. SDS-PAGE of supernatant and sediment from the lysis of bacteria showed the 17.5 kDa insoluble recombinant protein. SDS-PAGE analysis of the eluted fraction of chromatography showed that recombinant protein was completely purified. The binding of anti-Interleukin-4 specific antibody with recombinant protein was confirmed by immunoblotting. **Conclusion**: Recombinant mouse IL-4 precursor is expressed as an insoluble molecule with 17.5 kDa molecular weight by pET-21b (+) and BL21(DE3)-CodonPlus E. coli bacteria, which can be change to soluble form by denaturation with guanidine hydrochloride. Using Inoue method to competent bacteria have a higher efficiency than other methods. In addition, in this study the production of IL-4 at optimum temperature of 25 °C showed higher expression. Expressed recombinant protein after confirmation of it's nature by western blotting and due to its high purity, could be used for diagnostic purposes.

Keywords: cloning, recombinant, Interleukin-4, BL21, pET-21b (+), Top10

Introduction

Cytokines are produced by many cells and their production and secretion are critical for the coordination between innate and acquired immune systems as the two main elements of the immune system [1]. IL4 / IL-13 cytokine family is considered as one of the standard type 2 cytokines in the immune system. Coping with parasitic infections, increasing IgE levels, and immune system responses shift to Th2 are some of the actions of these cytokines. Concerning gene structure, the gene locus of these cytokines is located on murine chromosome 11 and its human variant on chromosome 5. These two cytokines have a common receptor with two IL-4Rα and IL-13Rα1 subunits [1].

Murine interleukin-4 (IL-4) is a secreted monomeric glycoprotein with 140 amino acids after expression. Mostly produced by T lymphocytes and mast cells, basophils, and eosinophils to a lesser extent, IL-4 has many pleiotropic effects [2]. Among the functions of this cytokines, one can cite stimulation and differentiation of B lymphocytes, increasing MHC-II expression on B lymphocytes, proliferation and differentiation of T lymphocytes, shift of Th-1 response to Th-2 and synthesis of chemokines, isotype switching to IgE and IgG1 production, and regulation of CD23 expression on lymphocytes and monocytes. Hematopoiesis, parasitic infections, apoptosis, cancer, and many other diseases are important because of the above molecular roles of IL-4 in the

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pathology of allergic reactions [3-6]. Many biological applications have been described after the discovery of the cytokine IL-4 in 1982. Cytokine IL-4 was first described for its ability in increasing DNA synthesis in resting B lymphocytes stimulated with Anti-IgM. Thus, it was introduced as a B-cell growth factor (BCGF). After murine and human IL-4 cloning, access to the recombinant shape of this protein has enabled the possibility of further studies on the biological functions of IL-4 [3, 7, 8]. IL-4 function has been evaluated in many diseases, such as in the prevention of parasitic infections, the inhibition of inflammatory responses related to type I cytokines in autoimmune diseases like rheumatoid arthritis, the increased susceptibility to ischemic attacks, thrombosis, and cancer [9-11]. Three general roles are considered for this cytokine, including regulation of B cell growth and expression of specific isotypes of antibodies, stimulation of T cell growth, and reduction of NK cell killing capacity, and modulation of hematopoietic stem cell differentiation [3, 12, 13].

The availability of recombinant cytokines and their inhibitors are important as they create conditions for scholars to evaluate their immune and inflammatory responses using them [9, 14]. Because of the significant and diverse roles of IL-4, its functions can be induced by the production of recombinant cytokines. Nowadays, given the widespread use of IL-4 in diagnosis, research, and treatment, recombinant cytokine production is done using different expression systems. Given the significance of the subject, the study was conducted to use the most cost-effective and efficient methods to produce a recombinant form of IL-4 protein.

Materials and Methods

Preparing IL-4 coding nucleotide sequence for binding plasmid

RNA extraction from mouse spleen cell tissue

RNA was extracted from mouse spleen cell tissue by a commercial kit (Pars Toos, Mashhad) with extraction steps were done according to kit approach with all steps done on ice to obtain murine IL-4 coding nucleotide-sequence.

Synthesis of cDNA from RNA

cDNA was synthesized by the RNA extracted from a commercial kit (Pars Toos, Mashhad) and cDNA was removed from the device and transferred to ice after the process was completed.

Designing primer

Firstly, by visiting uniprot.org website, information on murine IL-4 protein was obtained and information on murine coding sequence (CCDS) IL-4 from NCBI database. Then pET-21b (+) gene map was obtained by visiting NOVAGENE website. Then, the primers were designed using the Gene Runner program. The codons encoding the signal peptide and the end amino acid were deleted. The sequences of the fragment in question was examined for enzymatic cleavage and the two MCS cleavage enzymes and plasmid pET-21b (+) with the same buffering conditions not cutting the target coding sequence were selected. The two enzymes with these conditions are EcoRI and Xho I. The target fragment in the MCS plasmid was inserted between the cleavages sites of the two enzymes selected. A G nucleotide was added between EcoRI cleavage site and the target fragment sequence to maintain the ternary template of the codons. Based on GC percentage, primer length and Tm temperature of the primers were selected and analyzed in terms of forming hair pin and dimer structures by Gene Runner software. The primers designed were sent to SBS Genetech Co. China for constructing.

PCR to amplify the target fragment and insert the cleavage sites of EcoR I and Xho I enzymes

PCR with designed primers was used to amplify the desired fragment and to insert the cleavage sites to the two ends of the fragment. Prior to performing PCR, the required distilled water to obtain 100 pm/microliter concentration was added to the primers according to the manufacturer's instructions. For using the primers in the reaction, the concentration of the primers had to be 0.1. Hence, 5 µl of each primer was mixed with 45 µl of distilled water. PCR process was done using a thermocycler device. It has to be noted that the materials used without cDNA and with 10 µl of distilled water were prepared for negative control in another microtube. PCR product along with 100 bp marker with 1.5% agarose gel electrophoresis was checked for the accuracy of the reaction and gel was observed in Gel Documentation device.

Extraction and preparation of pET-21b (+) plasmid for binding to the target fragment

Extraction of plasmid in alkaline medium with SDS at low volume

The tube containing the culture medium already prepared was centrifuged at 4°C and 4100 rpm. The supernatant was gently and completely removed with the sampler as far as possible. Then 100 µl of solution 1 (containing 250 µl of Tris-HCl (2 M)), 400 µl of EDTA solution (0.5 M), 0.198 g of glucose powder and 16 ml of distilled water were added to the precipitate and vortexed. The solution was transferred into the microtube and 200 µL of solution 2 (containing 200 µL NaOH (1 M), 100 µL SDS solution (10%) and 700 µL distilled water were added to it. The microtube was shaken very slowly 2–3 times. Further shaking would release the genomic DNA of the bacteria. Then, it was placed on ice for 3-5 minutes. Later, 150 microliters of solution 3 (containing 6 ml potassium acetate (5 M), 1.15 ml acetic acid and 2.85 ml distilled water) were added to the microtube, mixed and placed on ice for 3–5 minutes. The microtube was centrifuged for 5 min at 13400 rpm. The supernatant was transferred to another sterile microtube and centrifuged again for 2 minutes at 13400 rpm. The resulting supernatant was again transferred to another microtube and the same as its volume isopropanol

was added and vortexed. The microtube was placed on ice for 5 minutes and then centrifuged at 13000 rpm for 5 min. The supernatant was removed and the resulting precipitate was rinsed twice with 500 µL of 70% ethanol. The microtube was left open for 5 minutes for the ethanol to evaporate completely. The precipitate was dissolved in 50 µl of TE buffer at pH = 8 for 20 minutes at 37°C. To examine the quality of the plasmid extracted, 2 µL of the sample along 2 µL of 1 kbp marker were observed on 1% agarose gel electrophoresis and gel with Gel Documentation device.

Binding of the target fragment to the plasmid

Binding of the fragment in question to the plasmid

Materials with 5µL plasmid, 1µL fragment, 1µL T4 DNA ligase (30 units per µL), 1.2µL 10x ligase buffer and 3.8µL distilled water were mixed in a microtube for 4 hours at 22 ° C.

Making bacteria competent by Inoue method

Two strains of E. coli Top10 and BL21 (DE3)-CodonPlus - had to be made competent to receive plasmid, which was done by Inoue method. The media containing the bacteria (OD = 0.5) was transferred to a 50 ml Falcon under sterile conditions and was placed on ice for 10 minutes. Then, it was centrifuged for 10 minutes at 3900 rpm at 4°C. The supernatant was completely discharged and 8 mL of the transformer buffer (listed in Appendix 5) was added to the precipitate. After the dissolution of the precipitate, it was centrifuged at 4°C for 10 min at 3900 rpm. The supernatant was removed, and 2 ml of the transformer buffer was added to the precipitate and 150 µl of DMSO was added drop by drop to the falcon on ice while shaking. It was placed on ice for 10 minutes. The above solution was transferred to 16 microtubes and placed in a nitrogen tank and then stored at -70°C. All the steps were done on ice under sterile conditions.

Transferring recombinant plasmid to bacteria and proliferating it

Transferring recombinant plasmid to Top10 E. coli by heat shock method

Heat shock method was used to insert the recombinant plasmid into the Top10 E.coli. According to this method, keeping the bacteria on the ice neutralizes the charge on the membrane surface and DNA approaching it. Then, with a slight heat shock, DNA enters the bacterium through the shattered membrane, and by placing it on the ice, DNA movement reduces and stays in the bacterium. The procedure was done by adding 45 µL of competent Top10 E. coli to 5 µL of the recombinant plasmid in a microtube and then slowly stirring it. This was done on ice and then placed on ice for 30 minutes again. In the next step, it was put on ice for 42 minutes at 42°C for 2 minutes. Ultimately, for the bacteria to recover, 200 microliters of liquid LB medium was added to it that had been placed at 37°C and incubated for 1 hour in a shaker incubator at 37°C. The whole volume was transferred to sterile LB containing ampicillin at a concentration of 100 µg / ml in sterile conditions and spread with a glass loop at ambient temperature. After complete adsorption, the plate was incubated at 37 ° C for 24 hours. Bacteria receiving the ampicillin resistance gene pET-21b (+) plasmid were expected to grow on the ampicillin-containing antibiotic medium.

PCR for screening grown colonies in terms of the existence of recombinant plasmid

Given the presence of T7 operon in pET-21b plasmid (+) and the fragment being inside it, the recombinant bacteria receiving plasmid were screened by PCR with T7 primer. Then, 10 colonies were randomly selected, numbered, and a microtubule containing 10 µL Taq 2X Premix, 1 µL T7 forward primer (10 pmol / µl), 1 µL T7 reverse primer (10 pmol / µl), 8 µl H2O with a total volume of 20 µl was prepared for each colony. They were transferred to the respective microtubule by once and flame from each colony and cultured on a new plate. The cultures were incubated at 37 ° C and transferred to the refrigerator after 18 hours. PCR was done using thermocycler. Screening results were analyzed by 5 µl of PCR product with 5 µl of 100 bp marker on 1.5% gel.

Extracting amplified plasmid from E. coli Top10

The colonies receiving plasmid were cultured in 3 µL of ampicillin LB medium at a concentration of 100 µg / ml and incubated in the incubator at 37 ° C to prepare the bacterium for plasmid extraction. The recombinant plasmid was extracted using Pars Tus extraction kit (Mashhad, Iran), according to the manufacturer's instructions. The final extraction solution was subjected to 1% agarose gel analysis. In addition, the extraction product was digested with XhoI and EcorI restriction enzymes, and was then subjected to agarose gel analysis, again. In addition, the pET-21b (+)-IL-4 recombinant plasmid was further verified by gene sequencing (Macrogen, Seoul, South Korea) using a pair of primers specific for the insertion region of IL-4 fragment into the pET-21b (+) plasmid and nearby regions. The oligonucleotide sequence of these primers follows as: forward,5′-TGAGA GAATTCG CATAT CCAACG GATGCG ACAA-3′ and reverse, 5′-ACTGA CTCGAG CGAGTAATCCATTTGCATGA-3′.

Low volume protein expression (2 ml)

Transferring the plasmid extracted from Top10 strain to E. coli BL21 strain by heat shock method

The same method used to transfer the plasmid into E. coli Top10 was used. To 50 µL of the susceptible E. coli BL21 bacterium, 1 µl of plasmid extracted from Top10 E. coli was added and mixed slowly. This was done on ice and then placed on ice for 30 minutes. In the next stage, it was placed on a device with a block at 42°C for 90 seconds and 2 minutes on ice. The whole volume was transferred to a sterile LB containing ampicillin at a concentration of 100 µg / ml under

sterile conditions and spread over the culture medium using a glass slide. After complete adsorption, the plate was incubated at 37 ° C for 24 hours. Bacteria receiving the ampicillin resistance gene pET-21b (+) plasmid were expected to grow on the ampicillin-containing antibiotic medium.

Adding IPTG to stimulate protein expression in low volume (2 ml)

E. coli BL21 (DE3) -CodonPlus bacteria receiving plasmid were cultured in a 2 ml Falcon tube containing LB medium with ampicillin at a concentration of 100 µg / ml and placed at the incubator for 4 hours at 37 ° C to prepare for stimulating expression. Then, the culture was transferred to Shaker–Incubator at 37 ° C for 18 hours. The next day, 200 µL of the culture medium was added to the vial containing 2 ml of LB medium containing ampicillin at 100 µg / ml, and placed in Shaker–Incubator. After OD reaching 0.5, IPTG at a concentration of 0.2 mM was added and placed in a Shaker–Incubator at 150 rpm and 22 ° C for 18 hours.

Evaluation of protein expression by E. coli BL21 in low volume (2 ml)

The bacteria were first lysed and then S Sodium dodecyl sulfate polyacrylamide gel electrophoresis (DS-PAGE) method was used to examine the expression of the recombinant protein by E. coli BL21 (DE3)-CodonPlus. The medium with E. coli BL21 was transferred to the microtubule and centrifuged at 1,400 rpm for 1 minute and complete supernatant was removed to lyse the bacteria. All 2 ml of the medium was centrifuged thus the precipitate was removed. Then 300 ml of lysis buffer was added to the precipitate and was thoroughly mixed and vortexed. Then, it was sonicated for 5 minutes on ice using a Sonicator. It was then centrifuged at 13,000 rpm for 1 minute and the supernatant was transferred to another microtube and the precipitate was kept in the same microtube. For SDS-PAGE, confirmation of protein expression by E.coli BL21 was the first stage of sample preparation and marker. In doing so, 5 µL of 5 µL of 4X sample buffer was added to 15 µL of the supernatant solution of sonicite and placed at 97 ° C for 4 minutes. The sample was added to the precipitate of the bacterial sonicite with 50 µL of sample buffer and placed at 97 ° C for 5 min after vortexing and homogenization. Vertex was performed once every 1 minute. Six µL of 2x sample buffer was added to 6 µl of protein marker and placed at 97 ° C for 2 minutes. Ten µl of marker, 10 µl of supernatant and 5 µl of precipitate on stacking gel were electrophoresed on 5% separating gel and 15% acrylamide (listed in Appendix 8). First, the samples were subjected to 120 volts of separating gel first, and then 160 volts. To prepare 45 ml of distilled water, 45 ml of methanol and 10 ml of acetic acid were mixed with 0.1 g of Coomassie Brilliant Blue. Then, 45 ml of distilled water and 45 ml of methanol were mixed to prepare the dye. The gel was incubated in color for 45 minutes and then, in color after completion of SDS-PAGE to view the bands.

Expression of the protein in high volume (100 ml)

Adding IPTG to stimulate protein expression in high volume (100 ml)

E. coli BL21 was cultured in 3 ml LB medium containing ampicillin at a concentration of 100 µg / ml and incubated for 24 hours at 37 ° C to prepare for stimulation of expression. The next day, 1 ml of the culture medium was added to 100 ml of LB medium containing ampicillin at a concentration of 100 µg / ml and placed in 37 ° C. After OD reached 0.5, IPTG was added at a concentration of 0.2 mM. The culture medium was then placed in a shaker incubator at 150 rpm and 22 ° C for 18 hours.

Evaluation of protein expression by E. coli BL21 in high volume (100 ml)

The culture medium was centrifuged for 5 minutes at 5500 rpm at 4 ° C. After removing the supernatant, 5 ml of lysis buffer was added to the precipitate and vortexed. Then, 50 µL of it was used for SDS-PAGE and the remainder for further steps. The SDS-PAGE steps were performed in the same manner and in the low volume order. At this stage, 5% stacking gel and 12.5% acrylamide separator were used.

Protein solubilization

Guanidine hydrochloride and DTT were used to solubilize the expressed protein as storage and insoluble bodies. Then 2.39 g of guanidine hydrochloride was gradually added to the sample prepared from the previous stage and vortexed to a concentration of 5 mM. EDTA was added to 1 mM and vortexed. After adding 50 µl of DTT and Vertex, it was sonicated for 5 minutes. Then, it was placed in the refrigerator for 24 hours at 4°C.

Purification

Purification of protein by nickel affinity chromatography

Affinity chromatography with nickel column was used for purification because of using pET-21b (+) plasmid to amplify the fragment and consequently addition of histidine tag to the recombinant protein. NaCl was first added to the protein solution to reach 100 mM. The sample was cloudy after adding the salt; this, it was centrifuged for 10 minutes at 8000 rpm at 10°C and the precipitate was removed. For chromatography, 10 ml Ni-IDA gel column was used. It is important to keep the gel dry at all stages. The column was first washed with 30 ml of distilled water at medium speed. Then, the column was rinsed with 30 ml buffer. The output was set to minimum speed and the sample was passed through the column. Recombinant protein that binds to nickel ions via a histidine tag is fixed on the column. Then, the sample was passed through 40 ml of buffer at the start and then 100 ml of wash buffer, respectively, to remove the remaining unbound proteins. Output rate was minimally adjusted and 10 ml of 500 mM imidazole buffer and 1 mM extraction buffer were used to separate the recombinant

protein from the column. All column outputs were collected in microtubes. All fractions collected were evaluated for the presence of recombinant protein.

Western blotting

Western blotting was used to examine antigenicity and the specific reaction of the protein with the antibody. The procedure was done on SDS-PAGE sample as performed in the preceding steps. After electrophoresis, the protein must be transferred from the acrylamide gel to polyvinylidene difluoride (PVDF) membrane. For this purpose, first, 6 pieces of 7 × 9 cm filter paper were prepared. The sponge was soaked in transfer buffer and placed on the holder. Three pieces of paper were soaked in transfer buffer, respectively, and placed on the sponge. The transfer buffer between each paper was removed and the bubbles created between the papers were removed. After the papers, acrylamide gel was placed on the paper. The membrane was immersed in methanol for 30 seconds and then immersed in a transfer buffer and placed on the gel. Again, three equal pieces of filter paper were placed on the membrane in the same order and the sponge soaked in transfer buffer was placed on the paper. The clamp holder and assembly were positioned in the tank so that the membrane was at the anode side and the gel at the cathode side. Next to it was a piece of ice. Transmission was done for 15 minutes at a current of 300 mA. After the transfer, the membrane was dyed with Coomassie Brilliant Blue and after ensuring the band transfer to the membrane, bleaching was performed. By viewing the bands, the membrane was cut, numbered, and 1500 microliters of methanol were poured on each line so that the membrane was fully bleached. Then, rinsing was done twice with 150 mM PBS. Then, to block the vacancies on the membrane, 1000 ml of 2 BSA was added on each membrane slice and placed on sealed nylon, and placed in a refrigerator at 4°C for 18 hours. The antibody was washed 5 times with 150 mM PBS solution for 5 minutes each time. The antibody conjugated with biotin was diluted 1% in BSA with 1:1000 dilution, and 1000 µL of it was added to each line and incubated for 2 hours at room temperature on a rocker. Then it was washed 4 times with 150 mM PBS solution for 5 minutes. After washing, 1000 µl of streptavidin-bound HRP enzyme was diluted 1% in BSA with 1: 20,000 dilution and incubated for 1 h at room temperature on a rocker. Four 4 times rinsing was done with 150 mM PBS solution for 5 minutes each. Then 200 µl of solution A and 200 µl of solution B were mixed with a little luminescence substrate and washed with membrane for 2 minutes. The result of the reaction of the membrane slices was observed with the Gel Documentation device.

Results

Preparing IL-4 coding nucleotide sequence for plasmid binding

RNA extraction from mouse spleen cell tissue

RNA extracted from mouse spleen tissue was electrophoresed on 1% agarose gel to examine the quality. The presence of two bands s18 and s28 confirmed the extraction accuracy.

PCR to amplify the target fragment and insert the cleavage sites of the two EcoR I and Xho I enzymes

After extracting RNA from the spleen tissue and ensuring its quality, construction was done by RT-PCR. After designing the suitable primers, PCR was performed to amplify the target fragment and insert the cleavage sites of the two EcoR I and Xho I enzymes. The product was electrophoresed on 1.5% gel to examine the accuracy of PCR. As is seen in Figure 1, a band of 360 bp appeared on the gel equal to the desired fragment length.

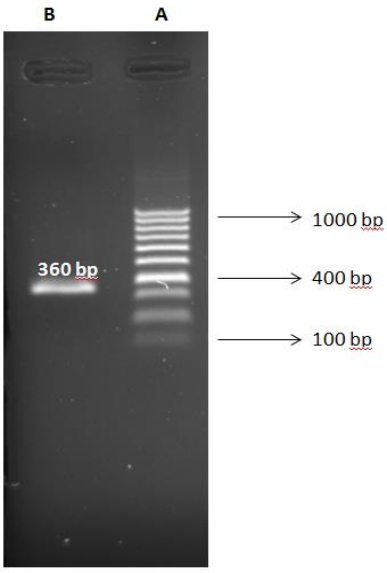

Figure 1: PCR product electrophoresis on 1.5% gel A: bp 100 marker. B: PCR product

Double-cut of the fragment with EcoR I and Xho I enzymes and extracting it from the liquid

The fragment was cut from PCR product by EcoRI and XhoI enzymes and then extracted from the liquid. The extracted product was electrophoresed on 1.5% gel for quality analysis. The accuracy of the process was confirmed by observing sample gel electrophoresis after cutting.

Extracting and preparing plasmid for binding to the desired fragment

Plasmid extraction

Plasmid pET-21b (+) was extracted from Top10 E. coli using the method stated. The extracted plasmid was electrophoresed on 1% agarose gel for quality evaluation. The 5442 bp bands corresponding to plasmid pET-21b (+) were observed on the gel (Figure 2).

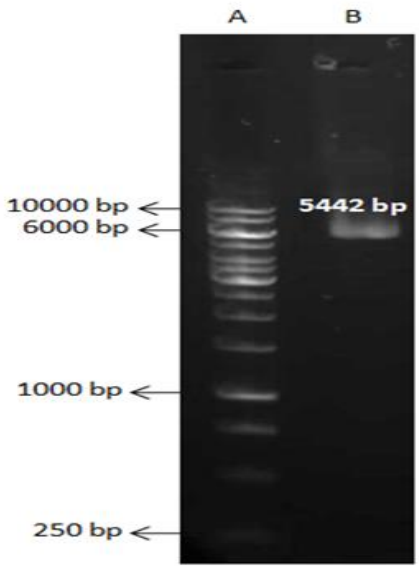

Figure 2: Plasmid electrophoresis extracted on 1% agarose gel
A: kbp marker 1, B: Extracted plasmid

Double-cut of plasmid with EcoR I and Xho I enzymes and extracting from liquid

The pET-21b (+) plasmid was cut with the help of EcoRI and XhoI enzymes and the cut plasmid was extracted. Then 5 µl of it and 5 µl of uncut plasmid on 1% agarose gel were electrophoresed to ensure the extraction of plasmid. The 5408 bp bands of the cleaved plasmid are placed below the cleaved plasmid in the gel and the cleaved fragment is not visible (Figure 3).

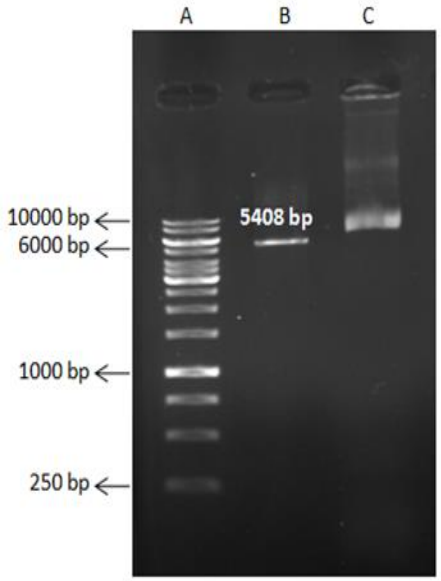

Figure 3: Cleaved plasmid electrophoresis on 1% agarose gel
A: kbp marker 1, B: cleaved plasmid, C: uncut plasmid

Transmission of recombinant plasmid to Top10 E. coli and its proliferation

Transmission of recombinant plasmid E. coli Top10

The recombinant plasmid was transferred to Top 10 susceptible bacterium and cultured on LB medium containing ampicillin. Plasmid pET-21b (+) contained the antibiotic resistance gene ampicillin, so colonies lacking this plasmid did not grow on the culture medium.

PCR for screening colonies grown on the media in terms of having recombinant plasmid

PCR was performed with T7 primers to screen the colonies grown on the media for recombinant plasmid. PCR product was electrophoresed on 1.5% gel for examining the colonies. The band length observed is the sum of the length of the segment in question and the remainder of the T7 operator. As Figure 4 of the gel shows, the colonies tested had received the recombinant plasmid among the colonies grown on the culture medium. In addition, the result of gene sequencing further confirmed the accurate replication of IL-4 fragment.

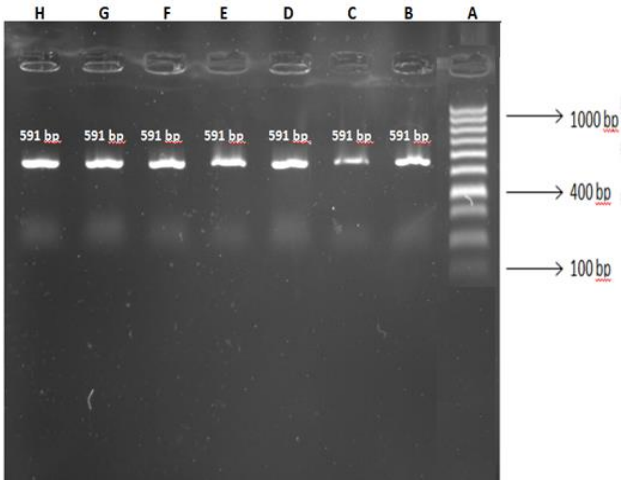

Figure 4: PCR product electrophoresis for screening on 1.5% agarose gel
A: bp 100 marker, B-H: PCR product

Protein expression

Low volume protein expression (2 ml)

One of the colonies grown on LB medium was selected and cultured in liquid LB medium. IL-4 protein expression was stimulated by IPTG as described in the work procedure. After removing the culture medium and adding lysis solution to the precipitate and sonicating, the precipitate and supernatant were separated. Protein expression was evaluated by SDS-PAGE in both liquid and sediment phases. The marker used was between 14 and 97 kDa. At this step, a 17.5 kDa band of recombinant IL-4 was observed in the sediment (Figure 5).

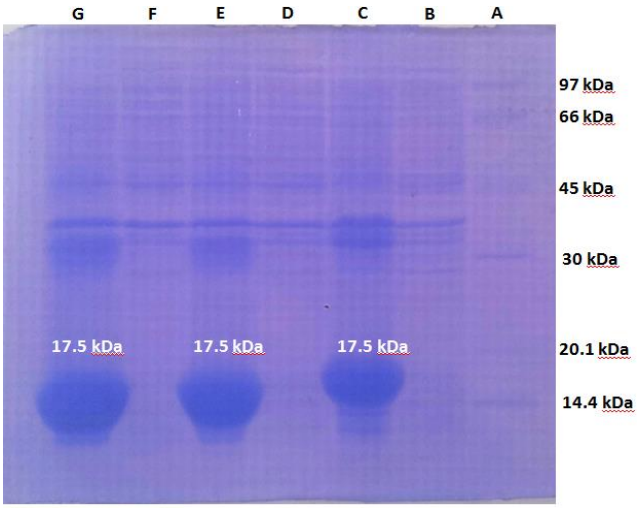

Figure 5: The result of low volume expression: 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue A: Marker. D, F, B: Bacterial lysis solution, C: E, G Bacterial lysis deposition

Immunoblotting

Western blotting was done to confirm the nature of the protein and to examine the antigenicity. The observation was done with the help of a slightly luminescence substrate and a Gel Documentation device. As is seen in the figure, a 17.5 kDa band appeared. The appearance of 17.5 kDa band shows the binding of IL-4-specific polyclonal antibody to the recombinant protein produced (Figure 6).

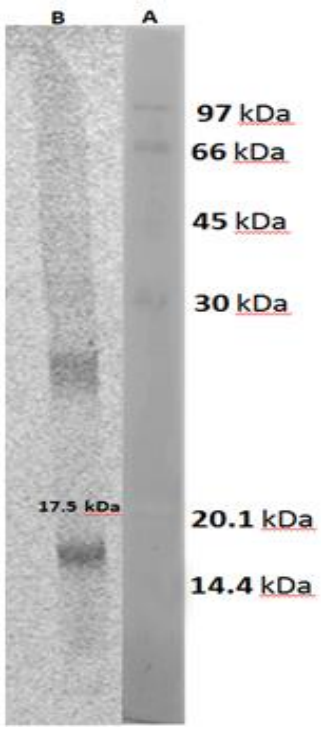

Figure 6: Western blotting result A: Marker, B: Recombinant protein bound to specific IL-4 antibody after dialysis

Discussion

E. coli prokaryotic system was used in the study: a system with simple, inexpensive growth and culture conditions and high proliferation rate. One of the potential problems due to using E. coli prokaryotic system in the study was the lack of glycosylation in the protein and its production as storage matters. The pET-21b (+) system was used for proliferation and expression because of its ability to produce large-volume protein, T7 promoter specificity for T7 RNA polymerase, and the ability to control the rate and timing of protein production. Using this plasmid facilitated the screening of colonies for protein expression and purification. The pET-21b (+) plasmid has a beta-lactamase gene (Ampicillin antibiotic resistance agent), a key feature for screening plasmid-receiving colonies because only colonies can grow on LB medium containing ampicillin that have received the plasmid. The site of entry of the target gene fragment in this plasmid is among the T7 operons. This feature facilitates screening of colonies for recombinant plasmids using PCR and T7 primers. PCR product of T7 promoter has longer recombinant plasmids compared to non-recombinant plasmids. Moreover, the existence of histidine amino acid codon 6 in the pET-21b (+) plasmid sequence produces a histidine tag at the carboxyl end of the protein being produced, facilitating protein purification.

Inoue method was used in the study to make bacteria competent. This method provides higher efficiency and stability for susceptibility to bacteria and transformation than the calcium chloride method (used in previous studies). E.coli BL21 (DE3) CodonPlus was used to express the recombinant protein in the present study. BL21 (DE3) strain is one of the ideal hosts for T7 promoter expression systems including pET. This is because CodonPlus contains T7 RNA polymerase gene as well as tRNA gene; it identifies codons of arginine, leucine, isoleucine and proline, which are rarely expressed in E. coli. This strain brings about a high level of expression of stable recombinant proteins. IPTG is a lactose analog, and a lactose activator of the Lac promoter. Given that the Lac promoter is located at the beginning of T7 operon in pET-21b (+) and at the beginning of the T7 RNA polymerase gene on the CodonPlus chromosome, IPTG induces the expression of both. As the target fragment is located among T7 operon, adding 0.2 mM IPTG resulted in a favorable level of recombinant protein expression.

In this study, 17.5 kDa was predicted by considering deleting and adding related to signal sequences, plasmid, and histidine sequences. Expression of recombinant protein with the predicted weight was confirmed by performing SDS-PAGE. Due to the existence of 6 cysteines involved in insoluble intracellular disulfide bond, the formation of insoluble and storing bodies was predicted. By performing SDS-PAGE, this prediction was confirmed and the expression of the recombinant protein occurred by bacteria insolubility. The proposed methods of the studies conducted for solubilization of accumulated proteins in storage are using 5-6 mM concentrations of chitropic agents such as urea and guanidine hydrochloride and detergents like SDS and N-acetyl

trimethyl ammonium chloride. Moreover, using mercaptoethanol and di-trithiol is useful in preventing disulfide bond formation. The use of chelating agents lie EDTA prevents cysteine oxidation. Hence, 5 mM guanidine hydrochloride, 1 mM DTT and EDTA were used to make the expression protein. Using guanidine hydrochloride and DTT leads to protein denaturation. It is used to restore the normal shape of the protein. A mixture of thiol oxide and reducing agents like glutathione with concentrations 5-15 mM is recommended. Thus, the buffer with 0.3 mM glutathione oxide and 3 mM reductive glutathione was used to recover the original form of the protein and suitable folding was reached [15].

In the studies conducted regarding the eukaryotic system, several steps including gel filtration, ion exchange chromatography, and HPLC have been used, but purification has done well in prokaryotic systems using a method like gel filtration or chromatography. In this study, given the existence of histidine tag in the protein produced, using nickel affinity chromatography was useful as a method for purification. Histidine tag tends to bind to nickel-charged ions on the column; hence, it leads to binding of the protein produced to the column. Imidazole solution was used to wash and separate the protein bound to the column that is separated from the column in competition with histidine bound to nickel and the protein. The use of this method proved highly efficient in this project so that the results of SDS-PAGE after chromatography indicated a complete purity of the protein [16]. Western blotting - which is a significant and powerful method for the immunological identification of proteins, especially low-concentration proteins - was used to confirm the protein and to evaluate its immunogenicity in the study. Western blotting and binding of murine specific IL-4 polyclonal antibody confirmed the accuracy of recombinant IL-4 and its desired antigenicity. Also, the gene cloning process was confirmed by PCR and gene sequencing, and the expressed rIL-4 protein was verified and characterized by SDS-PAGE and western blot analyses.

Conclusion

Murine recombinant IL-4 protein weighed 17.5 kDa was expressed insolubly by pET-21b (+) and BL21 (DE3) - CodonPlus E. Coli expression systems. The protein expressed using guanidine hydrochloride was transformed into a soluble form. Despite expression in E. coli and lack of glycolyzation, the expressed protein had a good folding and reaction with a specific polyclonal antibody. Hence, it can be used in producing specific antibody.

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