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Synthesis and Production of Recombinant Human Interleukin-2 via a Cell-Free Expression System

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ABSTRACT

Background and Objective: Cell-free protein expression system is a novel method for synthesizing recombinant proteins such as interleukin-2 (IL-2), which is of great importance in immune system responses in cancer and autoimmune patients. The aim of this study is to synthesize IL-2 using cell-free expression technique with high efficiency and lower cost.

Methods: In this experimental study, the bacterial host DH5α was used to amplify the IL-2 cDNA in the pcDNA3.1 expression vector. After culturing the eukaryotic CHO cell line and preparing the cell lysate, the cell cytoplasm containing protein expression factors along with the addition of essential substances required for amino acid expression was placed in the vicinity of the expression vector containing the IL-2 gene fragment. The negative control sample included all the materials of the tested sample without the presence of the expression vector containing the gene fragment. Both samples were incubated for 20 hours at 25 °C. The accuracy of the expression of recombinant human IL-2 was examined using SDS-PAGE and Dot Blot techniques with monoclonal antibodies against IL-2.

Findings: In the assessment of the expressed protein based on SDS-PAGE technique, a band corresponding to the IL-2 protein produced in the test sample measuring 15 kDa was detected, while no band was found in the vector-free cell lysate sample. In the dot blot technique using gold nanoparticles, the desired spot was obtained from the binding of IL-2 as an antigen to the monoclonal antibody specific to IL-2. Moreover, quantitative analysis of the expression level using Nanodrop was reported to be 17.8 µg/ml.

Conclusion: The results of the techniques showed that there is the possibility to synthesize and produce IL-2 using a cell-free expression method.

Keywords: *Recombinant Human Interleukin-2, Cell-Free Expression System, CHO Cell Line, Synthesis of Recombinant Human Interleukin-2.*

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Introduction

The glycoprotein interleukin-2 (IL-2), which is a cytokine with the role of T lymphocyte growth factor, is very important in the immune system (1, 2). The effect of IL-2 on T lymphocyte cells and the proliferation of cytotoxic T cells to induce cancer cell death, as well as the proliferation of regulatory T cells, leads to control of the immune system in autoimmune patients (3).

Recombinant protein synthesis is generally performed through two types of expression systems: intracellular and extracellular. Cell-free expression systems can be defined as infrastructures in which biochemical reactions occur independently of living cells, with transcription and translation occurring in cell extracts and in vitro (4). The Cell-Free Protein Synthesis (CFPS) system uses a minimal enzymatic machinery for transcription, translation, and energy regeneration, derived either from cell extracts or purified enzymes (5, 6). Designed as a simple and efficient reconstituted system, CFPS naturally overcomes the inherent limitation of a living cell and provides direct access to essential protein expression activities (7). CFPS is the first program to enable modular cell-like processes with support for active transcription and translation. In addition, due to the absence of a cytoplasmic membrane, it is possible to access and manipulate the protein and use non-standard amino acids to synthesize a wide range of simple or complex proteins, and the time for manipulation, synthesis, and purification is greatly reduced (8). Currently, the CFPS system has replaced traditional cell-based protein expression systems, making protein synthesis very cost-effective (5).

Eukaryotic cells are important for the expression of glycoproteins and complex proteins due to their ability to properly shape proteins, assemble them, and undergo post-translational modifications. Mammalian cell-based expression systems are the preferred choice for the synthesis of biologically active proteins (9). Among mammalian cell lines, Chinese hamster ovary (CHO) cells are currently the most common host cell line for biopharmaceutical production and are the most widely used for large-scale recombinant protein delivery, expression, and production (10, 11). Data on biopharmaceutical market approvals from 2014 to 2018 indicate that more than 80% of newly approved biopharmaceuticals were produced in CHO cells (12).

Since the therapeutic effect of human IL-2 has been studied and proven in patients with cancers, including kidney cancer and autoimmune diseases, this research was designed to express recombinant human IL-2 protein using a cell-free expression method that is more efficient and less expensive than traditional protein synthesis methods, in an attempt to accelerate the treatment and recovery of these patients.

Methods

Bacterial strain, cell line and plasmid: This experimental study was approved by the Ethics Committee of Babol University of Medical Sciences with the code IR.MUBABOL.HRI.REC.1400.124. In this study, the pcDNA3.1 plasmid was used to clone the IL-2 gene fragment, and the Escherichia coli DH5α strain was used to replicate and clone the plasmid containing the gene fragment, and the eukaryotic cell line CHO-K1 was used to prepare the cell lysate required for protein expression, which was obtained from the Pasteur Institute of Iran.

Media, enzymes, chemicals and kits: RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum from Gibco, and Pen-Strep 1X from Sigma, Germany, were used to grow the CHO-K1 cell line, while Brain Heart Infusion (BHI) Broth purchased from Merck, Germany, was used to grow Escherichia coli DH5α. Ampicillin antibiotic powder for injection at a concentration of 50 µg/ml was used to select the transformed bacteria on Luria-Bertani agar (LB) medium with ampicillin. Anti-Interleukin-2 monoclonal

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antibody at a concentration of 0.5 µg/ml was used to observe the spot in the Dot Blot technique. Denazist Asia kit (Iran) was used for plasmid extraction.

Transformation of pcDNA3-IL-2 in prokaryotic host DH5α and plasmid extraction: After receiving the pcDNA3.1 plasmid containing the IL-2 gene fragment, in order to propagate the vector containing the desired gene fragment, the Escherichia coli DH5α strain was prepared for plasmid acceptance after culturing it in BHI broth using 100 mM calcium chloride and heat shock. The plasmid containing the IL-2 gene fragment was transferred by heat shock at 42 °C. In order to prove the entry of the vector into the bacteria, transformed and antibiotic-resistant colonies were examined in LB agar medium containing the antibiotic ampicillin. Using a plasmid extraction kit, the plasmid containing the fragment was extracted from a number of colonies grown on LB agar medium containing the antibiotic ampicillin, which indicates the presence of the vector in the colony. In order to confirm the presence of the cloned fragment in the recombinant construct donated by Dr. Kardar, the obtained plasmid solution was used as a template with plasmid-specific primers designed on both sides of the inserted gene fragment, and amplified by PCR at an annealing temperature of 58 °C for 35 cycles.

Cell culture: For this purpose, CHO-K1 cells were cultured in RPMI-1640 medium with 10% FBS and PenStrep 1X in a 37 °C incubator with 5% CO_2 and 95% humidity. This cell line is epithelial-like and adheres and grows at the bottom of the cell culture flask.

Cell extract: The cells obtained from the cell flask were collected and centrifuged at 100 g for 10 minutes. In the next step, they were washed with a solution prepared from Shafa Zist (Shafamel Co., Amol, Iran). Then, the cells were immersed in an extraction solution prepared from Shafamel Company, which had previously reached room temperature. After completing this step, the cell supernatant was collected. The collected supernatant was stored as a cell extract for the next steps in a -80 °C freezer.

Expression stage: Eukaryotic cell lysate of CHO-K1 cells was incubated with pcDNA3.1 vector containing IL-2 gene that had been amplified in previous stages. The obtained cell extract was incubated in equal ratio with expression solution consisting of 18 essential substances required for expression, such as amino acid mixture. The expression was performed in a cell-free system. The negative control sample was also considered as expression mixture and cell extract solution without vector. The microtubes of the tested sample and negative control sample were incubated for 20 hours at room temperature of 25 °C. The level of protein expression in the microtube was measured with Nanodrop, so that the desired blank for reading was the expression mixture and cell extract solution without vector.

Dot blot technique: In order to ensure the presence of IL-2 protein, the dot blot technique was used with monoclonal antibody against IL-2 and antibody conjugated with gold nanoparticles. To perform the dot blot technique, first two areas were determined on the nitrocellulose substrate (PVDF, Roche) for control and test. Each area covered a length and width of one centimeter. For the control area, 2 µl of Goat anti-mouse antibody containing 0.4 µg of antibody was first loaded onto the control area using a sampler. Then, in the second area, which was related to the test area, 2 µl of anti-IL-2 antibody Mouse containing 2 µg of antibody was loaded onto the test area using a sampler. After 30 minutes, both areas of the substrate were washed with phosphate buffer containing Tween 20. Then, the cell-free expression mixture was poured onto the test area and phosphate buffer was poured onto the control area, and after one hour of incubation and washing the plate to remove unbound proteins, 2 µl of the conjugate solution containing anti-IL-2 antibody with gold nanoparticles (at a concentration of 2 µg/ml) was poured onto both areas. After washing the plate to remove unbound antibodies, the colors resulting from the reaction were examined.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique: SDS-PAGE was used to visualize the protein expressed by cell-free expression and to assess the molecular size of IL-2.

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According to the table below, the percentage of separating gel in SDS-PAGE for the separation of IL-2 is 12.5%, which was used in this study (Table 1).

Table 1. Different values of polyacrylamide gel electrophoresis percentage

	Percentage of gel				
	5%	**7.5%**	**10%**	**12%**	**15%**
Weight of the desired band according to the marker protein size (kilodaltons)	>250	120-250	40-120	15-40	<15

Results

Transformation, extraction and PCR of the gene fragment inserted into the plasmid: After the electrophoresis of the plasmid extracted from the host bacterium Escherichia coli DH5α, a band around 6 kb, which was the same size as the vector and the desired gene fragment, was observed on a 1% agarose gel (Figure 1). The vector extracted from the bacteria was amplified by PCR using specific primers for the IL-2 gene fragment as a template, and by electrophoresis of the product, a band of 462 bp was observed, which corresponded to the size of the desired gene (Figure 2).

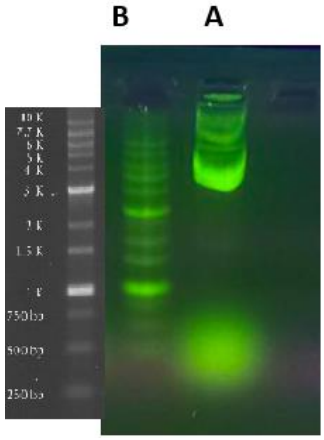

Figure 1. Plasmid band containing the IL-2 gene, which was observed on the electrophoresis gel after extraction of the plasmid from the transformed bacteria, was approximately 6 kilobases (column A: sample, column B: ladder).

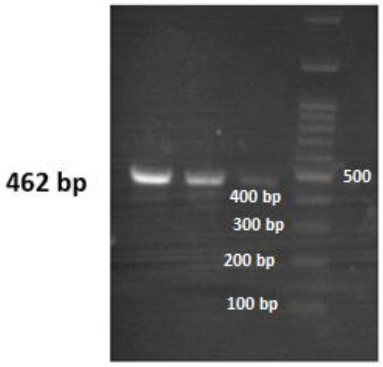

Figure 2. Gel electrophoresis of the IL-2 gene PCR product with specific primers, resulting 462 bp band and 100 bp DNA ladder

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Culture of the eukaryotic cell line CHO-K1: After reaching a cell density of 80%, the eukaryotic cells of the CHO-K1 cell line were examined in terms of morphology and number at different stages (Figure 3). Finally, an extract of 50 million cells was used to prepare the cell extract.

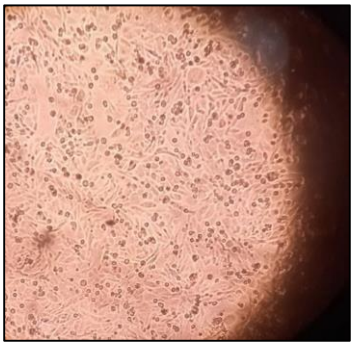

Figure 3. Microscopic image of CHO-k1 cell line cultured in complete medium at 10x magnification

Quantitative and qualitative evaluation of the concentration of expressed protein in the cell-free expression system: After combining the lysed CHO-K1 cells with the expression solution and adding the vector containing the IL-2 gene to this mixture, incubation was performed at 25 degrees centigrade for 20 hours, and the protein concentration was read by the Nanodrop device at a concentration of 17.8 µg/ml, which was obtained after subtracting the number read in the expression mixture without the vector (Figure 4). It is worth noting that the ELISA method is used to measure the quantitative value of expressed protein; ELISA was used in this study, but it did not yield any results and the kit did not work properly, so the above numbers were obtained indirectly from the difference in protein in the reaction mixture with blank tubes. The protein expressed in the expression solution and cell lysate were used for SDS-PAGE analysis, and the presence of a 15 kDa band confirmed protein synthesis of this size (Figure 5). To prove the expression of IL-2 in the expression solution and in the 15 kDa band observed in the SDS-PAGE technique, the desired expression solution was tested by the dot blot technique using the gold nanoparticle method. The use of monoclonal IL-2 antibody conjugated with gold nanoparticles in this technique and its binding to IL-2 as an antigen and the creation of a colored spots proves the expression of IL-2 in this expression solution (Figure 5). The color of the dots in the dot blot technique was analyzed with ImageJ software (Figure 6).

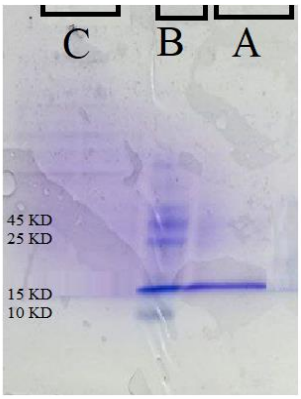

Figure 4. Polyacrylamide gel. In this image, A is synthesized IL-2, B is the size marker protein, and C is the cell extract solution as a negative control.

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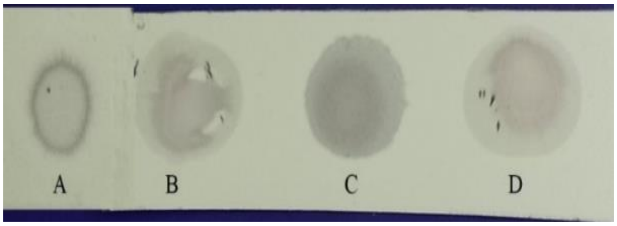

Figure 5. Image of dot blot technique with gold nanoparticles. A: Control and B: IL-2 specific antibody and expression solution and conjugated IgG-anti goat antibody, C: Specific antigen and IL-2 antibody and IgG-anti goat conjugate, D: IgG-goat antibody and expression solution and conjugated IL-2 antibody

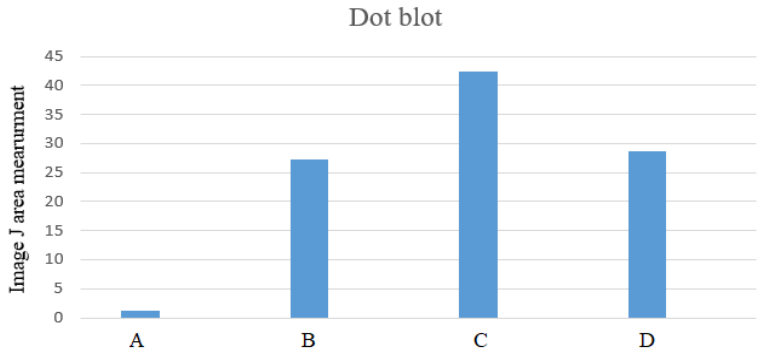

Figure 6. Diagram of protein concentration in dot blot technique using Image J software. A, B, C, and D are based on the spots in Figure 5, and spot C has the highest color intensity.

Discussion

The findings of the study showed that the cell-free expression system with lysate of 50 million CHO cells along with expression solution and addition of an appropriate concentration of expression vector containing the IL-2 gene fragment at 25 °C for 20 hours is capable of synthesizing and expressing IL-2 protein.

IL-2 was expressed with an intracellular expression system and in the bacterial cell E. coli BL21 in some Iranian studies (13). Expression and production of recombinant human IL-2 by the prokaryotic intracellular method results in the formation of inclusion-body aggregates, which subsequently require difficult steps of protein purification using chromatography methods (14). However, the cell-free protein synthesis method does not form inclusion-body aggregates due to the lack of the host cell cytoplasmic membrane, and protein purification is carried out more easily and at a higher speed. The pharmaceutical sample of IL-2 (Proleukin) and the biosimilar sample are produced by Shafamel Company, they are produced in a prokaryotic system and are functional. IL-2 is produced by eukaryotic cells such as Pastoris Pichia and CHO (Peprotech Company) and are used to propagate T lymphocytes in culture. E. coli cell-based expression often results in lower yields of soluble protein, especially for complex proteins, compared to E. coli cell-free expression (7). Synthesis of the murine scFv RIT domain in the E. coli BL21 StarTM cell-free expression system is less efficient than cell-free expression based on CHO cell lysates (7).

Today, 70% of recombinant protein drugs on the market are produced using eukaryotic CHO cells. CHO cells grow rapidly and perform the post-translational modifications required for biological activity well (10). In the chemical method of IL-2 synthesis, glycosylation of this protein does not occur. Although glycosylation does not have a significant effect on the activity of the protein, its lack of glycosylation

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reduces the half-life of IL-2, which is one of the problems of using IL-2 by chemical synthesis, while the use of cell-free expression methods of eukaryotic cell lysates such as CHO allows for protein glycosylation (15). Due to the high similarity of proteins expressed in eukaryotic CHO cells to human proteins, as well as the high activity of proteins produced in a cell-free expression system based on CHO cells compared to lysates of Sf21 cells, rabbit reticulocytes, and wheat germ, lysates of these cells were used in the present study as a suitable medium for IL-2 synthesis (10, 16, 17).

In this study, IL-2 produced in the extracellular system based on CHO cell lysate had a suitable structure in terms of molecular weight and SDS-PAGE analysis and reacted with a specific antibody against IL-2 in the dot blot technique with gold nanoparticles, which indicated the presence of IL-2 in the examined expression solution.

Acknowledgment

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