ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF RECOMBINANT HUMAN IL-2

M.Sc. THESIS

Buse AKGÜN

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JANUARY 2022

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REKOMBİNANT İNSAN IL-2'NİN EKSPRESYONU, SAFLAŞTIRILMASI VE KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

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Buse Akgün, a M.Sc. student of ITU Graduate School student ID 521181103, successfully defended the thesis entitled "**EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF RECOMBINANT HUMAN IL-2**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my mom, grandma, and in loving memory of my dad,

FOREWORD

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TABLE OF CONTENTS

Page

ABBREVIATIONS

ACN : Acetonitrile
AMBIC : Ammonium Bicarbonate
APC : Antigen-presenting cell
APS : Ammonium Persulfate
BLAST : Basic Local Alignment Tool
BME : Beta-Mercaptoethanol
BSA : Bovine Serum Albumin
cDNA : Complement Deoxyribonucleic Acid
CD : Circular Dichroism
CE : Capillary Electrophoresis
DC : Dendritic Cell
DMSO : Dimethyl Sulfoxide
DNA : Deoxyribonucleic Acid
DTT : Dithiothreitol
E. coli : Escherichia coli
EDTA : Ethylene
FA : Formic Acid
FBS : Fetal Bovine Serum
FPLC : Fast protein liquid chromatography
IL : Interleukin
IL-2R : Interleukin 2 receptor
IPTG : Isopropyl β-D-1-thiogalactopyranoside
JAK : Janus Kinase
Kd : Equilibrium dissociation constant
LB : Luria Broth
MS : Mass Spectrometry
NCBI : National Center of Biotechnology Information
PCR : Polymerase Chain Reaction
PDB : Protein Data Bank
PH : Power of Hydrogen
PMSF : Phenylmethanesulfonyl fluoride
SDS : Sodium Dodecyl Sulfate
SDS-PAGE : Sodium Doceyl Sulfate-Polyacrylamide Gel Electrophoresis
STAT : Signal Transducers and Activators of Transcription
TBS : Tris-Buffered Saline
TBS-T : Tris-Buffered Saline-Tween 20
TEMED : Tetramethylethylenediamine
THP-1 : Human acute monocytic leukemia cell line
UV : Ultraviolet

SYMBOLS

°C	**:** Celcius
µg	**:** Microgram
µL	**:** Microliter
µM	**:** Micromolar
bp	**:** Base pair
g	**:** Gram
kDa	**:** Kilodalton
kb	**:** Kilobase
L	**:** Liter
M	**:** Molar
mg	**:** Miligram
min	**:** Minute
mL	**:** Mililiter
mM	**:** Milimolar
NaCl	**:** Sodium Chloride
OD	**:** Optical Density
RT	**:** Room Temperature
rpm	**:** Round per minute
v/v	**:** Volume to volume
3D	**:** Three-Dimensional

LIST OF TABLES

Page

LIST OF FIGURES

Page

EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF RECOMBINANT HUMAN IL-2

SUMMARY

Cytokines, which are small proteins secreted by the immune system, are in charge of directing the immune system. Through their formation, differentiation, and activation functions, cytokines govern the maintenance of innate and adaptive immune responses. They are primarily formed by mononuclear phagocytes, dendritic cells, and antigen-presenting cells.

Interleukin (IL) is a kind of cytokine that acts as an immunomodulatory protein. It induces a variety of cell and tissue responses. Interleukins mediate the interaction of leukocytes (white blood cells) and initiate a response by attaching to high-affinity receptors on the surface of the cells. They play a critical role in the regulation of cellular formation, differentiation, and activation that occurs over the course of inflammatory and immunological responses. Each family is assigned an IL based on sequence homology, receptor chain similarity, and functional qualities.

Interleukin-2 (IL-2) was the first cytokine discovered to stimulate the growth of T lymphocytes. T cells, B cells, natural killer (NK) cells, lymphokine-activated killer cells, and macrophages all require IL-2 to regulate their proliferation and differentiation. Mier et al. discovered the molecule and named it "IL-2" since it was produced by and acted on leukocytes. Its discovery is regarded as a milestone in immunology. However, there is one issue that is common to all lymphokines when it comes to the molecular and functional characterization of IL-2, and it is due to their production in small quantities. The cloning of cDNA for IL-2 was a significant turning point in 1983, precipitated by the discovery of IL-2. The Jurkat T cell leukemia cell line was employed for the IL-2 cDNA clone development.

IL-2 is a 15.5 kDa glycoprotein that belongs to the cytokine family four α-helical bundles. There are 153 amino acid residues in a single polypeptide chain of IL-2. IL-2 binds to and communicates with a receptor complex composed of three different subunits known as IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132). Different combinations of these three components bind to IL-2 with varying degrees of affinity. The αβγ heterotrimer, βγ dimer, and α chain monomer all bind to IL-2 with "high," "intermediate," and "low" affinity, respectively. Binding of IL-2 to the IL-2R heterodimer complex activates several pathways. In response to an interaction between interleukin-2 and its receptor, kinases connect to cytoplasmic areas of the receptor subunits, resulting in the tyrosine phosphorylation of many proteins and the activation of a number of signaling pathways, including JAK/STAT, PI-3K/AKT, and Ras/MAPK. IL-2 activity promotes cell survival, proliferation, cell cycle progression, and targeted gene transcription.

Due to its ability to activate both T and NK cells, IL-2 was the first cytokine to be successfully used in cancer treatment. The US Food and Drug Administration authorized high-dose IL2 for the treatment of melanoma and renal cell carcinoma in

1992 and 1998, respectively. Moreover, the use of recombinant IL-2 therapy may help researchers understand better the coronavirus disease 2019 (COVID-19), which is caused by a virus that leads to severe acute respiratory illnesses and has rapidly spread throughout the world. As a prospective treatment for this condition, the use of rIL2 may be beneficial for patients since it has the potential to accelerate disease recovery by increasing the number of lymphocytes in the body. A major difficulty is figuring out how to direct IL-2 activity toward Teffs and away from Tregs, which inhibit the immune system.

IL-2 is available in two recombinant forms derived from *E. coli*, but only aldesleukin is FDA-approved. Recombinant IL-2 differs structurally from its natural version. IL-2 recombinant is not glycosylated and lacks N-terminal alanine. To avoid the formation of an incorrect disulfide bond, serine has been substituted with cysteine at amino acid position 125. The pharmacological actions of endogenous and recombinant human IL-2 are similar.

In this study, *E. coli* Rosetta (DE3) was used as the host cell. Induction of protein expression was accomplished by the use of IPTG. Following that, inclusion bodies, which develop in the cell as a result of excessive protein expression, were separated and solubilized from cell lysates and refolded by step-wise dialysis. Anion exchange chromatography was used to separate the target protein from the rest of the protein mixture. After purification, the yield was determined to be 0.114 mg per liter of cell culture.

SDS-PAGE and immunoblotting methods were used to validate the effectiveness of the purification. The molecular weight is estimated using intact mass analysis through LC/MS. The CE-SDS analysis revealed that rIL-2 has a purity of around 80%. In addition, the pI value of the protein was determined as 7.31 using the capillary isoelectric focusing method. The peptide mapping on LC-MS/MS is used to figure out the main structure of the protein that has been purified. The secondary structure of pure human interleukin-2 (hIL-2) was investigated using circular dichroism (CD), and the results revealed that it included a high concentration of alpha helices. The biological action of our IL-2 is determined by phosphorylation of one of the MAPK pathway proteins, extracellular signal-regulated kinase 1/2 (ERK), on human monocytic cells, THP-1. An active protein has been produced as a result of this work.

The experimental results indicate that the procedures established for generating and purifying the rIL-2 protein may be employed to create a pure product that maintains its bioactivity**.**

REKOMBİNANT İNSAN IL-2'NİN EKSPRESYONU, SAFLAŞTIRILMASI VE KARAKTERİZASYONU

ÖZET

Bağışıklık sistemi, sitokin adı verilen bir dizi küçük protein tarafından kontrol edilir. Sitokinler, oluşum, farklılaşma ve aktivasyon işlevleri aracılığıyla, doğuştan gelen ve adaptif bağışıklık tepkilerinin korunmasını yönetir. Esas olarak mononükleer fagositler, dendritik hücreler ve antijen sunan hücreler tarafından oluşturulurlar ve hücreler arasında mediatör olarak görev yaparlar. Sitokin aracılığı ile hücreler arasında gerçekleştirelen etkileşimler otokrin (salgılandığı hücreye), parakrin (yakındaki hedef hücreye) ya da endoktrin (dolaşım aracılığıyla kana salınma ve uzak bir hücreye) etki şeklinde olabilir.

İnterlökin (IL), immünomodülatör protein görevi gören bir tür sitokindir ve immün sistemden salgılanan sitokinlerin önemli bir kısmını oluşturmaktadır. Çeşitli hücre ve doku tepkilerini indüklemekte görevlidir. İnterlökinler, lökositlerin (beyaz kan hücreleri) etkileşiminine aracılık ederler ve hücrelerin yüzeyindeki yüksek afiniteli reseptörlere bağlanarak immün yanıt oluştururlar. Enflamatuar ve immünolojik tepkiler sırasında meydana gelen hücresel oluşum, farklılaşma ve aktivasyonun düzenlenmesinde kritik bir rol oynarlar. İnterlökinler, dizi homolojisi, reseptör zinciri benzerliği ve fonksiyonel nitelikleri göz önünde bulundurularak isimlendirilmektedirler.

İnterlökin-2 (IL-2), T lenfositlerinin büyümesi ve farklılaşmasından sorumlu keşfedilen ilk sitokindir. T hücreleri, B hücreleri, doğal öldürücü (NK) hücreler, lenfokinle aktive olan öldürücü hücreler ve makrofajların tümü, çoğalmalarını ve farklılaşmalarını düzenlemek için IL-2'ye ihtiyaç duymaktadırlar. İnterkökin-2 molekülü Mier et al. tarafından keşfedilerek, lökositler tarafından üretildiği ve lökositler üzerine etki ettiği için moleküle "IL-2" adı vermiştir. İnterlökin-2'nin keşfi, immünolojide bir dönüm noktası olarak kabul edilmektedir. Ancak IL-2'nin moleküler ve fonksiyonel karakterizasyonu söz konusu olduğunda tüm lenfokinler için ortak olan, az miktarda üretilmesi sorun yaratmaktadır. Bu sebeple, 1983 senesinde IL-2 cDNA klonu geliştirmek için Jurkat T hücresi lösemi hücre hattı kullanılmıştır.

IL-2, 15.5 kDa moleküler ağırlığa sahip α-helix yapıda bir glikoproteindir. IL-2' nin tek bir polipeptit zincirinde 153 amino asit bulunur. İnsanda 4q26-28 kromozomunda bulunan IL-2 geni, dört ekzon ve üç introndan oluşmaktadır. IL-2, IL-2Rα (CD25), IL-2Rβ (CD122) ve IL-2Rγ (CD132) olarak bilinen üç farklı alt birimden oluşan bir reseptör kompleksine bağlanır. Bu reseptörün alt birimlerinin farklı kombinasyonları, değişen derecelerde afinite ile IL-2'ye bağlanır. Örneğin, αβγ heterotrimer, βγ dimer ve α zincir monomeri, sırasıyla "yüksek", "orta" ve "düşük" afinite ile IL-2'ye bağlanır.

IL-2'nin IL-2R heterodimer kompleksine bağlanması üç farklı yolağı aktive eder. IL-2 reseptörü ile etkileşime girdiğinde, kinazlar reseptör alt birimlerinin sitoplazmik bölgelerine bağlanır ve birçok proteinin tirozin fosforilasyonuna ve JAK/STAT, PI3K/AKT, ve Ras/MAPK gibi önemli yolakların aktive olmasına neden olur. Bu aktivasyon sonucunda IL-2, hücrenin hayatta kalmasını, çoğalmasını, hücre döngüsü ilerlemesini ve hedef gen transkripsiyonunu sağlar.

IL-2 kanser tedavisinde başarıyla kullanılan ilk sitokindir. ABD Gıda ve İlaç İdaresi (FDA), sırasıyla 1992 ve 1998'de melanom ve renal karsinoma tedavisi için yüksek doz IL2 kullanılmasına izin vermiştir. Düşük ve yüksek doz IL-2 tedavisi günümüzde hala denenmektedir. Örneğin, kanser hastalarında kemoterapi veya farklı sitokinlerle bir arada kullanılan IL-2 tedavisi, tek başına kullanılan IL-2'den daha yüksek etkinlik göstermiştir.

Rekombinant IL-2'nin aynı zamanda tüm dünyayı etkisi altına alan, şiddetli akut solunum yolu hastalığına neden olan ve 2020 mart ayında Dünya Sağlık Örgütü tarafından pandemi olarak ilan edilen 2019 koronavirüs (COVID-19) için tedavi yöntemi olarak kullanılması gündeme gelmiştir. İmmün sistemde ektin rol oynayan T ve NK hücrelerini aktive etme özelliği nedeniyle rhIL-2, hastalığın tedavisinde tercih edilebilmektedir. Bu sayede, vücuttaki lenfosit sayısında artış gözlemlenlenir ve hastaların iyileşmesini hızlandırma potansiyelinden dolayı rIL2 kullanımı hastalar için faydalı olabilir. IL-2 tedavisinde karşılaşılan en büyük zorluk, immün sistemi aktive eden Teff ile immün sistemi baskılayan Treg arasındaki dengenin sağlanmasıdır.

IL-2'nin *E. coli'*den türetilen, tecelelökin ve aldeslökin olmak üzere iki rekombinant formu mevcuttur. Ancak, yalnızca aldesleukin FDA onayına sahiptir. Rekombinant IL-2, doğal versiyonundan yapısal olarak farklılık göstermektedir ve glikolize değildir. Ayrıca, rekombinant IL-2 iki adet sisteine sahiptir. Yanlış sisteinler arasında kurulan disülfid bağının sebep olduğu yanlış katlanmanın önüne geçilmek için 125. pozisyondaki sistein amino asidi serin amino asidi ile değiştirilmiştir. Yine bu değiştirilmiş formdaki rekombinant IL-2, N-terminal alanine sahip değildir. Bu değişikliklere rağmen endojen ve rekombinant insan IL-2'sinin farmakolojik etkilerinin arasında herhangi bir fark görülmemektedir.

Bu çalışmada, farklı *E. coli* suşlarından Rosetta (DE3) ve BL21 (DE3)'te protein üretimi denenerek, nadir *E. coli* kodonlarına sahip olan, ökaryotik proteinlerin ekspresyonunu artırmak için geliştirilmiş bir varyant olduğundan, Rosetta (DE3) konak hücresinin kullanılması tercih edilmiştir. Ekpresyon vektörü olarak protein üretiminde yaygın olarak kullanılan pET vektörlerinden pET30 (a) kullanılmıştır. Protein ekspresyonu IPTG ile indüklenen lac operonunun kontrolü altındadır. Bunu takiben, üretim koşullarını optimize etmek için uygun IPTG konsantrasyonu, inkübasyon sıcaklığı ve süresi gibi parametreler denenerek, en yüksek verime sahip ortam şartları belirlenmiştir. Hücrede aşırı protein ekspresyonu sonucu inklüzyon cisimcikleri oluşur. Yapılan indüksiyon kontrolü ile rekombinant IL-2'nin hücre peletinde kaldığı ve çözünmüş halde bulunmadığı gözlemlenmiştir. İnklüzyon cisimcikleri sonikasyon ve ara yıkama işlemlerinden sonra 2 M üre içeren alkali tampon çözeltisi ile çözünür hale getirilmiştir. Sonrasında, denatüre halde bulunan protein diyaliz yöntemi kullanılarak üreden uzaklaştırılarak ve katlanmıştır.

Hedef proteini konak hücre proteinlerinden ayırmak için anyon değişim kromatografisi kullanılmıştır. Saflatırma sonrasında total verim 1 litre bakteri kültüründe 0.114 mg olarak hesaplanmıştır. Saflaştırmanın etkinliğini doğrulamak için SDS-PAGE ve immünoblotlama yöntemlerinden yararlanılmıştır. Moleküler ağırlık, LC/MS aracılığıyla intak kütle analizi metodu kullanılarak belirlenmiştir. Bu analize göre, rhIL-2'nin monomer yapıda olup 15328 Da moleküler ağırlığa sahip olduğu doğrulanmıştır. Saflık tayini analizi kapiler elektroforez ile yapılmış ve proteinin yaklaşık olarak % 80 saflığa sahip olduğunu belirlenmiştir. Ayrıca proteinin pI değeri kapiler izoelektrik odaklama yöntemi kullanılarak 7.31 olarak belirlenmiştir. Proteinin amino asit sekansını doğrulamak için LC/MS ile yapılan peptit haritalama yönteminden yararlanılmıştır. Rekombinant interlökin-2'nin ikincil yapısı, dairesel dikroizm spektroskopisi kullanılarak araştırılmış olup, sonucunda yüksek konsantrasyonda α-heliks yapıda olduğu ortaya koyulmuştur. Proteinin aktif olduğunu göstermek için, THP-1 monosit hücreleri üzerindeki biyolojik etkisine bakılmıştır. MAPK sinyal yolağında bulununan proteinlerinden biri olan 1/2 (ERK) fosforile eden aktif bir protein olduğu belirlenmiştir.

Sonuç olarak, rekombinant interlökin 2'nin üst akım ve alt akım prosesleri geliştirildikten sonra karakterizasyonu yapılmıştır ve biyolojik etkinliğe sahip olduğu gözlemlenmiştir.

1. INTRODUCTION

1.1 Cytokine

The immune system is controlled by a series of small, secreted proteins called cytokines. Cytokines determine the sustenance of innate and adaptive immune responses through their development, differentiation, and activation functions. They are generated predominantly from mononuclear phagocytes, dendritic cells (DCs), and antigen-presenting cells (APCs) (Commins et al., 2010). Cytokines are classified according to their functions. For instance, some cytokines serve primarily as lymphocyte growth factors, whereas others behave as pro-inflammatory or anti-inflammatory molecules (Dinarello, 2007). Paracrine, autocrine, and endocrine signaling actions are all attainable for cytokines (Zhang et al., 2007). The summary of cytokine families is given in Table 1.1 (Commins et al., 2010).

Table 1.1 : Cytokine Families.

Family	Members
Hematopoietic	
Common γ chain	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21
Shared β chain (CD131)	IL-3, IL-5, GM-CSF
Shared IL-2β chain (CD122)	IL-2, IL-15
Other hematopoietic	IFN-γ, IL-7, IL-13, IL-21, IL-31, TSLP
IL-1 family	IL-1α, IL-1β, IL-1ra, IL-18, IL-33
gp130-utilizing	IL-6, IL-11, IL-27, IL-31, ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1), leukemia inhibitory factor (LIF), oncostatin M (OSM), osteopontin
IL-12	IL-12, IL-23, IL-35
IL-10 superfamily	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29
IL-17	IL-17A-F, IL-25 (IL-17E)
Interferons	
Type I interferons	IFN-α, IFN-β, IFN-ω
Type II interferon	IFN-γ (also a hematopoietic cytokine)
Type III interferons	IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B)
TNF superfamily	TNF-α, TNF-β, BAFF, APRIL

1.2 Interleukin

Interleukin (IL) is an immunomodulatory protein class of cytokine that causes a wide range of cell and tissue responses. There have been over 360,000 papers discussing interleukin since IL-1 was first discovered in 1977. Interleukins participate in contact with leukocytes (white blood cells) and activate a reaction by binding to high affinity receptors on the surface of cells (Akdis et al., 2016). They control cell proliferation, maturation, and migration. Therefore, interleukins play a major role in controlling cellular development, differentiation, and activation during the process of inflammatory and immune responses (Brocker et al., 2010). Helper CD4+ T lymphocytes, monocytes, macrophages, and endothelial cells are the primary producers of interleukins. Interleukins are denoted by the letters IL followed by a number. In the past, the same IL has been used under various names. Lymphocyte-activating factor, mitogenic protein, or T cell replacing factor III were all names for it. In 1979, the word "interleukin" was used for the purpose of standardization of the nomenclature in the Second International Lymphokine Workshop. Since that time, the interleukins have been named sequentially based on the date of their discovery (L. Ferreira et al., 2019). Up to now, 40 interleukins have been identified, with some of them being subdivided into subtypes such as IL-1α, IL-1β (Catalan-Dibene et al., 2017). Furthermore, IL-41 has been mentioned in recent studies as a novel immunomodulatory cytokine (Bridgewood et al., 2019). Each family is given an IL based on sequence homology, similarity of the receptor chain and functional properties (Akdis et al., 2011).

1.3 Interleukin 2

IL-2 is essential in the regulation of immune cell proliferation and differentiation, including T cells, B cells, natural killer (NK) cells, lymphokine-activated killer cells, and macrophages (Figure 1.1) (Geigert et al., 1993). The biological actions of interleukin-2 are described in Table 1.2 (Gaffen & Liu, 2004).

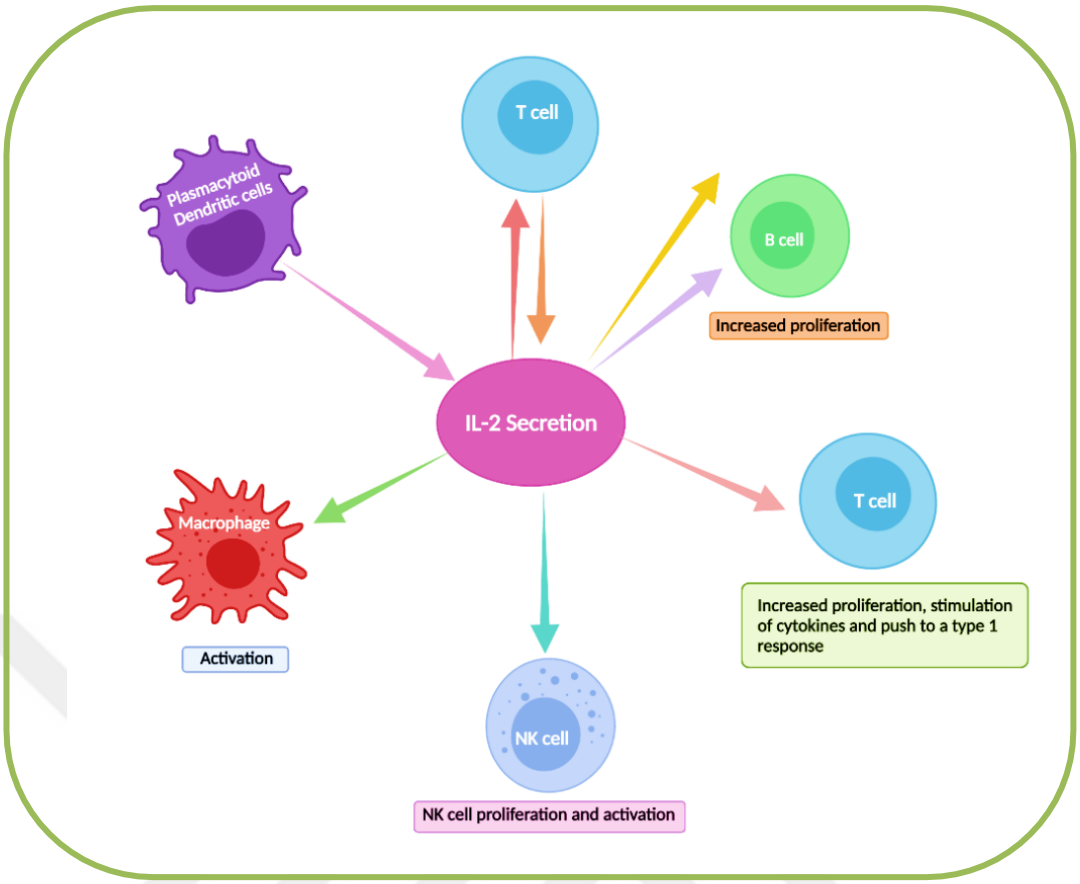


Figure 1.1 : Control of the immune system by Interleukin-2 (IL-2). Aside from favoring T cell proliferation, IL-2 produced by T cells and DCs also promotes the growth of B cells, T lymphocytes, T regulatory lymphocytes, and NK cells. IL-2 can also boost cytotoxicity by activating monocytes and NK cells. NK: natural killer cell; DC: dendritic cell; IL-2: interleukin-2. (Adapted from Talmadge, 2017).

Table 1.2 : Main biological activities of IL-2.

Cell type	Primary activities of IL-2
$CD4^+$ T cells	Induces expansion of antigen-specific clones via both proliferative and anti-apoptotic mechanisms Augments production of other cytokines Required for differentiation to Th1 and Th2 subsets Induces apoptosis of activated T cells via Fas/FasL signaling (activation-induced cell death) Involved in development of $CD4^+$ $CD25^+$ T regulatory cells (?)
$CD8^+$ T cells	Induces expansion of antigen-specific clones Augments cytokine secretion Augments cytolytic activity Induces proliferation of memory $CD8^+$ cells
B cells	Enhances antibody secretion Initiates immunoglobulin J chain transcription and synthesis Promotes proliferation
NK cells	Promotes proliferation Augments cytokine production Enhances cytolytic activity

The first cytokine characterized to promote the development of T-cells is interleukin-2. In 1976, Morgan et al. discovered interleukin-2 (IL-2) as a "T-cell growth factor"(Morgan et al., 1976). Later, Mier et al. identified the molecule and named it "IL-2" (Dhupkar & Gordon, 2017). Generally, the term "interleukin" refers to the molecule's role in signal transduction between leukocytes. Interleukin-2 was assigned this name because it was developed by and acts on leukocytes (Buchbinder & McDermott, 2014). IL-2 was the first human interleukin to be identified, completely characterized, and purified (Smith & Smith, 2016). Its discovery is considered an immunological breakthrough. However, there is a concern related to the molecular and functional characterization of IL-2 that is shared by all lymphokines: it is formed in very limited amounts (Geigert et al., 1993). A big turning point in 1983, led by the discovery of IL-2, was the cloning of cDNA for IL-2 (Rotte & Bhandaru, 2016). To develop an IL-2 cDNA clone, hybrid selection was used to isolate the Jurkat T cell leukemia cell line (Taniguchi & Minami, 1993).

1.4 The Biology of IL-2

IL-2 is a glycoprotein belonging to the four α-helical-bundle class of cytokines. The 153-amino-acid primary translation product of human IL-2 contains a 20-residue signal peptide and a 133-amino-acid mature protein which has a 15.5 kDa molecular weight. The human interleukin-2 (IL-2) gene is located on chromosome 4q26–28. This gene is composed of four exons and three introns (Fossiez et al., 2009).

1.5 Structural and Molecular Characteristics of the IL-2 Receptor (IL-2R)

IL-2 functions by attaching to the IL-2 receptor (IL-2R), which is made up of three subunits: alpha chain (IL-2Rα, CD25, Tac, p55), beta chain (IL-2Rβ, CD122, p70), and gamma chain (IL-2Rγ, CD132)(Olejniczak & Kasprzak, 2008). IL-2R complexes can alter in binding affinity to IL-2, with various subunit interactions. IL-2Rα, IL-2Rβ, and IL-2Rγ are three different components of the high-affinity (Kd = 10^{-11}M) form of IL-2R. Two different subunits are found in the intermediate-affinity IL-2R: IL-2Rβ and IL-2Rγ (Kd = 10^{-9}M). IL-2Rα binds to IL-2 with minimal affinity, hence it cannot trigger the significant signal transduction response (Kd = 10^{-8}M) represented in Figure 1.2 (Taniguchi & Minami, 1993).

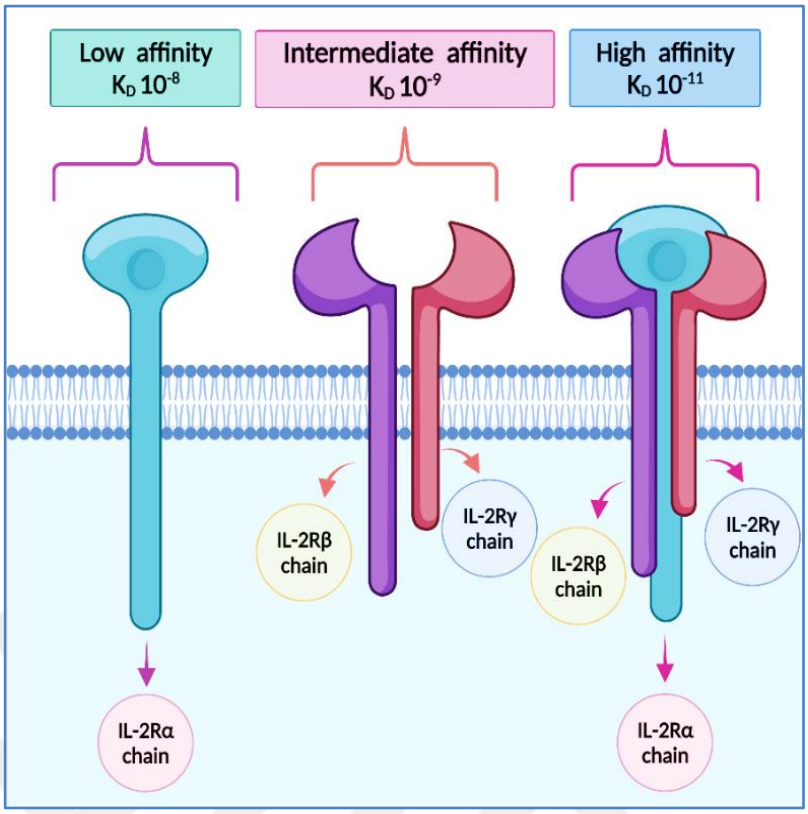


Figure 1.2 : Interleukin-2 receptor complex. (Adapted from Taniguchi & Minami, 1993).

1.6 Action Mechanism of IL-2

IL-2 modulates immunostimulation and immunosuppression through several pathways being activated by IL-2 binding to the IL-2R heterodimer complex (Overwijk et al., 2021). Whenever IL-2 interacts with its receptor, kinases attach to the cytoplasmic regions of the receptor subunits, causing tyrosine phosphorylation of many proteins. Janus kinase 1 (JAK1) interacts with the IL-2Rβ chain, whereas JAK3 interacts with the IL-2Rγ chain, resulting in three different pathways. Firstly, the recruitment of signal transducers and activators of transcription protein 1 (STAT1), STAT3, and STAT5 occurs. After phosphorylation, STAT proteins dimerize and translocate to the nucleus, where they control the transcription of genes required for T cell development and functioning. In the second pathway, to increase cell survival and proliferation, IL-2 triggers the PI3K—Akt—p70S6 kinase pathway. Additionally, the proto-oncogene tyrosine protein kinase SRC may bind to the phosphorylated receptor, activating the Ras/Erk/MAPK pathway and promoting cell cycle progression (Dhupkar & Gordon, 2017). Figure 1.3 demonstrates the downstream cascades of the mechanism.

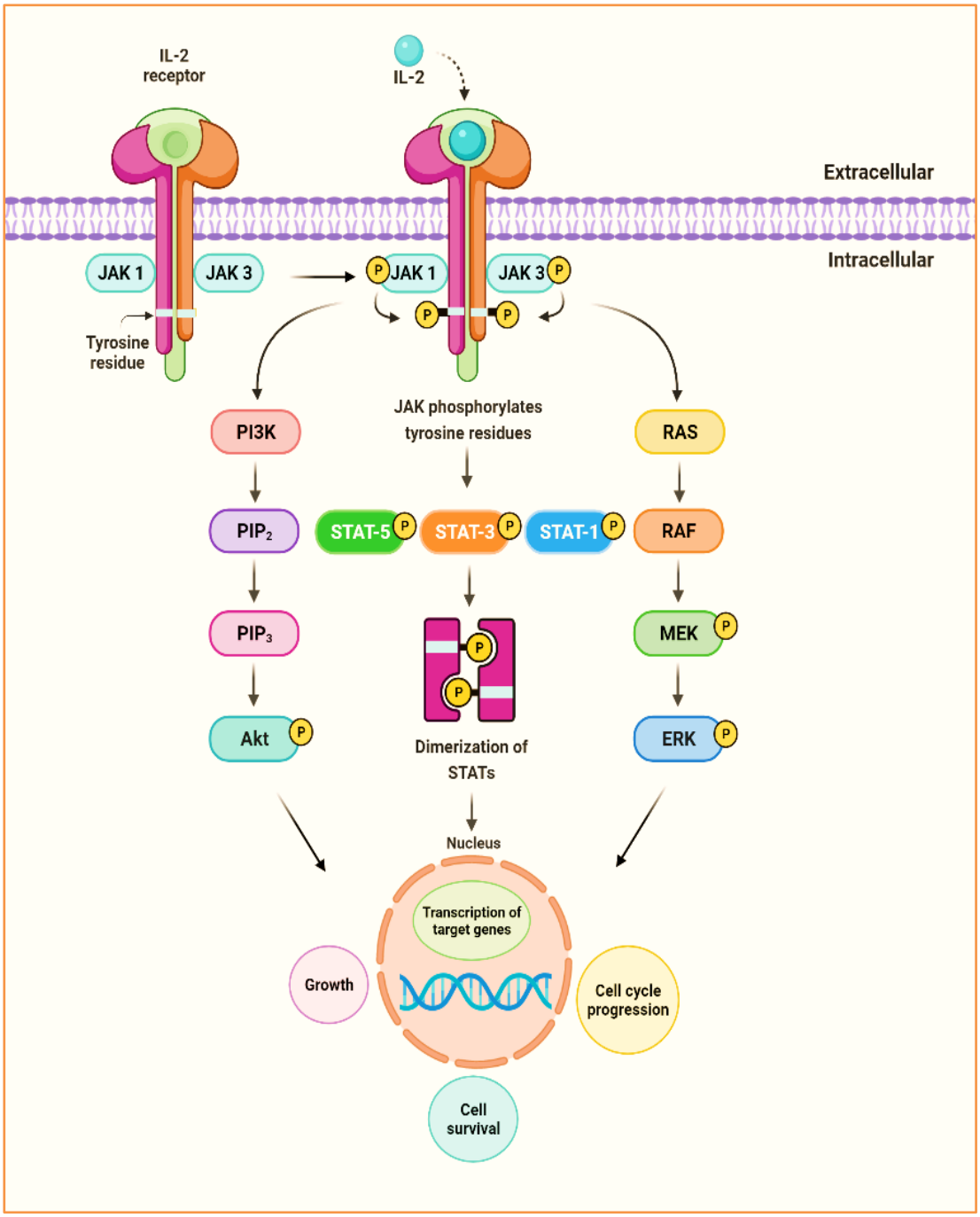


Figure 1.3 : The signaling pathway involving IL-2 and IL-2R. JAK1 and JAK3 are phosphorylated when they interact with IL-2, which in turn activates and dimerizes STAT proteins, which then move to the nucleus and start transcription of different genes. IL-2 stimulation might even activate the PI3K-Akt or MEK-Erk pathways, promoting cell survival and proliferation. (Adapted from Dhupkar & Gordon, 2017).

1.7 Clinical Use of IL-2

Several cell types within the immune system have the potential to promote or inhibit the beginning or progression of cancer. For example, Tregs (regulatory T cells) block

the tumor-killing effects of Teffs (T effector cells) and NK cells (Mitra & Leonard, 2018).

The first cytokine to be successfully employed in cancer therapy was IL-2, because it could stimulate both T and NK cells (Choudhry et al., 2018). Rosenberg et al.'s studies have shown that high-dose IL2 treatment for metastatic melanoma or renal cancer was a notable advance in IL-2 immunotherapy. Due to the persistence of responses shown in multicenter studies, use of high dose interleukin-2 (IL-2) in the treatment of melanoma and renal cell carcinoma has been approved by the US Food and Drug Administration in 1992 and 1998, respectively (Rosenberg, 2014).

Additionally, recombinant IL-2 treatment can provide a new perspective on the understanding of the coronavirus disease 2019 (COVID-19), which is caused by a virus that leads to severe acute respiratory syndrome and has been quickly spread across the world. Since Helper-T, Treg, and NK cells are important antiviral actors, they might be used as a therapeutic strategy in the battle against COVID-19 (Yazan, 2020). Current studies, upon evaluating the changes in peripheral blood WBC and lymphocyte count, together with the levels of IL6 and CRP in response to the immunostimulatory treatment, indicate that recombinant human IL2 (rIL2) can be used for the treatment of COVID-19. Patients with severe COVID-19 may benefit from the use of rIL2 as a prospective treatment since it has the potential to speed disease recovery by increasing the number of lymphocytes (Zhu et al., 2021). On the other hand, one of the most difficult challenges is determining how to guide IL-2 activity toward immune-supportive Teffs and away from immuno-suppressive Tregs, and another was toxicity linked to high-dose IL-2 (Dhupkar & Gordon, 2018; Overwijk et al., 2021).

IL-2 is available in two recombinant versions produced by *E. coli*: aldesleukin and teceleukin, but only aldesleukin is approved by the FDA (Huen & Vadhan-raj, 2021). Structurally, recombinant IL-2 (generic name: aldesleukin) varies from its native form. Glycosylation is not necessary for the full biological efficacy of several human lymphokines, including IL-2, which is why recombinant IL-2 generated in *E. coli* is not glycosylated (Malek, 2008). Another difference was the lack of N-terminal alanine in response to the plasmid's removal of the essential codon. Lastly, at amino acid position 125, serine has been replaced with cysteine in recombinant IL-2 in order to prevent the wrong disulfide bond formation (Lipiäinen et al., 2015). For complete

biological activity, an intramolecular disulfide connection between cysteine-58 and cysteine-105 is required (Wang et al., 1984; Yamada et al., 1987).

Furthermore, in native IL-2, the unbound cysteine at 125 is not required for IL-2 receptor recognition and contributes to the development of inclusion bodies (Ju et al., 1987).

Endogenous and recombinant human IL-2 have identical pharmacological effects. Hydrophobicity and the tendency to aggregate characterize recombinant interleukin-2. As a result, lyophilized Proleukin includes mannitol as a stabilizer and bulking agent, 0.02 percent sodium dodecyl sulfate, and a sodium phosphate buffer with a pH of 7.5. SDS is an ionic surfactant that binds to proteins and helps them become more soluble. Indeed, SDS is used in the formulation of Proleukin to facilitate IL-2 aggregation (Lipiäinen et al., 2015).

1.8 Aim of the Study

Interleukin-2 is a protein that facilitates cell-to-cell communication and plays a major role in controlling the immune response. Pharmacologically, recombinant human IL-2 is indistinguishable from endogenous human IL-2. It has been proven to have antitumor action against a variety of cancer types, and it has significant promise for use in cancer immunotherapy treatments. As a result, the study's primary goal is to generate a functioning recombinant version of human IL-2 that is suitable for biopharmaceutical use. In order to achieve this, different bacterial production conditions have been tried, and the optimal condition has been chosen. After that, the protein purification procedure was completed in a single step with the chromatographic method. The overall yield of bacterial culture after purification was estimated. Several analyses were performed to identify and characterize the pure protein. To control the purification technique, SDS-PAGE and immunoblotting analyses were done. Following that, via circular dichroism, the protein's secondary structure was determined, and CE was used to assess protein purity and isoelectric point. The amino acid sequence of the protein was confirmed using the peptide mapping technique. Finally, intact mass analysis was used to determine the molecular mass of recombinant human IL-2.

This study demonstrates that the qualitative and quantitative analysis of the drug substance of recombinant human IL-2 was obtained in our lab and will be further developed for industrial applications.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

The types of equipment used in the experiments are listed in Appendix A.

2.1.2 Chemicals and enzymes

All chemicals and enzymes used in the experiments are listed in Appendix B.

2.1.3 Buffers and solutions

Buffers and solutions used in the experiments are listed in Appendix C.

2.1.4 Bacterial strains

Several bacterial strains were used in this study. Escherichia coli Rosetta (DE3) F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE (Cam^R), BL21 (DE3) strain [B F– ompT gal dcm lon hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS)], and HST08 Stellar Chemically competent™ strain [F-, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ(lacZYA-argF) U169, Δ(mrr-hsdRMS-mcrBC), ΔmcrA, λ-].

2.1.5 Mammalian strains

In this study, recombinant hIL-2 activity was detected using the human monocytic cell line THP-1 (ATCC® TIB-202TM) from an acute monocytic leukemia patient.

2.2 Methods

2.2.1 Molecular cloning

Subcloning

The desired gene of interest was obtained from the pQE80L-IL2 C125S (plasmid # 104348), which was ordered from Addgene and sent in as an agar stab. The streak

plate technique was used to isolate a single colony of bacteria from a mixed population. The inoculum was streaked over the agar surface of a plate and spread with a loop.

Plasmid DNA isolation

A single colony of *E. Coli* pQE80L-IL2 C125S cells from an overnight grown plate was taken into a 20 mL LB medium containing 20 µL of kanamycin. The next day, after incubation, bacteria were transferred to sterile ultracentrifuge tubes and centrifuged at 4000 xg for 30 minutes at 4°C. Plasmid DNA isolation was performed using the NucleoSpin Mini Kit according to the manufacturer's instructions. Plasmid DNA concentration was measured at 280 nm using a Thermo Scientific NanoDrop 2000 spectrophotometer.

Oligonucleotide primers design

The NCBI/Primer-BLAST program was utilized to identify appropriate primers, and their Tm values for rIL-2 amplification were determined with the NEB Tm Calculator. Additional features of primers, including hairpins, dimers, and mismatches, were determined using the IDT DNA website. At the 5' ends of both forward and reverse primers, HindIII-HF® (NEB) and NdeI (NEB) restriction enzymes were introduced. Table 2.1 shows the features of the designed primers, which were synthesized by PRZ Biotech, Ankara.

Table 2.1 : Sequences, length, and Tm of primers designed for rhIL-2.

Primer Name	Primer Sequence (5'→ 3')	Lenght (bp)	Tm (°C)
IL2-Forward	GGTTCATATGCCTACTTCAAGTTCTACAAAG	31	67
IL2-Reverse	CACCAAGCTTTCAAGTCAGTGTTGAGATGAT	31	70

rhIL-2 amplification with Polymerase Chain Reaction (PCR)

The target DNA fragment was amplified using the Polymerase Chain Reaction (PCR) with restriction sites included in the primers. The reaction mixture and the procedure are given in Table 2.2 and Table 2.3 below.

Agarose gel electrophoresis

The charge migration of agarose gels allows for the identification, separation, extraction, and visualization of nucleic acids. In this study, agarose gels including 0.8% agarose in 1X TAE Buffer were prepared and the gel was run under 100 V for 45 minutes. The gel is visualized using UV light or the GelDocTM XR+ System (Bio-Rad, 1708195).

Table 2.2 : PCR reaction mixture composition.

Components	Amount
Q5 High-Fidelity 2X Master Mix	10 µL
10 µM Forward Primer	1 µL
10 µM Reverse Primer	1 µL
DNA Template (233 ng/µL)	4 µL
Nuclease-free Water	up to 20 µL

Table 2.3 : PCR cycling parameters.

Step	Temperature	Time	Number of Cycle
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	
Annealing	58°C	20 sec	33
Extension	72°C	20 sec	
Final Extension	72°C	2 min	1
Incubation	10°C	infinite	

DNA extraction from agarose gel & DNA purification

A PCR NucleoSpin® clean-up and gel extraction kit (MACHEREY-NAGEL) was used to accomplish DNA extraction from an agarose gel, as well as DNA purification and clean-up. This approach was based on the instructions provided by the manufacturer.

Restriction enzyme digestion

Table 2.4 : Reaction mixture of double digested plasmid.

Components	Amount
10X CutSmart Buffer	2.5 µL
NdeI	1.25 µL
HindIII	1.25 µL
Uncut pET30 (a) plasmid (80 ng/µL)	13 µL
Nuclease-free Water	up to 25 µL

Digestion of the PCR product and pET30a (+) plasmid was accomplished by double digestion with NdeI and HindIII-HF® restriction enzymes at 37°C for 4 hours. For 20 minutes at 65°C, restriction enzymes were inactivated. The reaction mixtures of double digested plasmid and double digested PCR product were given in Table 2.4 and Table 2.5, respectively.

Table 2.5 : Reaction mixture of double digested PCR product.

Components	Amount
10X CutSmart Buffer	2.5 µL
NdeI	1.25 µL
HindIII	1.25 µL
PCR product (48.8 ng/µL)	11.5 µL
Nuclease-free Water	up to 25 µL

DNA ligation

The amplified, digested, and purified PCR products, as well as the isolated, digested, and purified linearized vector, were ligated from their sticky ends for 1 hour at room temperature (24°C) using the T4 DNA ligase enzyme. Components are listed in Table 2.6 below. Ligated amplicons and plasmid backbone were transformed into the *E. coli* Stellar strain.

Table 2.6 : Reaction mixture of DNA Ligation.

Components	Amount
Digested DNA fragment (42 ng/µL)	2 µL
Linearized vector (39.3 ng/µL)	2.6 µL
T4 DNA Ligase	1.1 µL
T4 DNA Ligase reaction buffer	2 µL
Nuclease-free Water	up to 20 µL

Colony PCR

Table 2.7 : Colony PCR reaction mixture composition.

Components	Amount
Taq 2X Master Mix	10 µL
10 µM Forward Primer (T7)	1 µL
10 µM Reverse Primer (Bam)	1 µL
DNA Template	1 µL
Nuclease-free Water	up to 20 µL

The colonies from the transformed cell-containing plates were submitted to colony PCR using the following method: The chosen colony from the Kanamycin agar plate is dissolved in 20 µL of nuclease-free water and utilized as a template for the PCR process. The reaction mixture and the procedure are given in Table 2.7 and Table 2.8 below. A selected clony was sequenced to verify the presence of the desired gene of interest. DNA sequencing was performed by BMLabosis (Ankara, Turkey).

Table 2.8 : Colony PCR cycling parameters.

Step	Temperature	Time	Number of Cycle
Initial Denaturation	95°C	4 min	1
Denaturation	95°C	30 sec	
Annealing	52°C	30 sec	33
Extension	68°C	1.5 min	
Final Extension	68°C	5 min	1
Incubation	10°C	infinite	

2.2.2 Preparation of competent cells

The Inoue method was used to prepare competent cells for chemical transformation (Inoue et al., 1990). To summarize, frozen stock cells in glycerol solution were dispersed onto LB agar plates and incubated at 37°C for 16–20 hours. The next day, a single colony was picked and inoculated in an erlenmeyer flask in 5 mL of LB medium at 37°C with shaking at 180 rpm. Then, the overnight culture was placed in 100 mL of LB broth and allowed to grow at 37°C in an orbital shaker at 180 rpm. When the optical density (OD_{600}) reached 0.5-0.6 at 37°C, bacterial cells were transferred to sterile, disposable, precooled 50 ml falcon tubes and the cultures were embedded on ice for 10 minutes. Centrifugation at 2500 g for 10 minutes at 4°C was used to collect the LB medium. After removing the supernatant, the pellet was resuspended in 8 mL of ice-cold Inoue buffer (55 mM MnCl2, 10 mM PIPES (0.5M, pH 6.7), 15 mM CaCl2, 250 mM KCl). The sample was treated with 1 M DMSO (dimethyl sulfoxide). It was added at a concentration of 7% of the solution and swirled together before being incubated in an ice bath for 10 minutes. In the last step, either use the cells directly for transformation or divide them into 50 µL microfuge tubes and freeze them at -80°C until needed.

2.2.3 Plasmid transformation of competent cells

The competent cells Stellar, BL21 (DE3), and Rosetta (DE3) were transformed using the procedure described. The competent cells were taken from a freezer at -80°C and were thawed on ice. Approximately 1 µL of 100 ng/µL plasmid or 3 µL of ligation product was added to the 50 µL competent cell and the tube was flicked 4–5 times to ensure that the cells and DNA were well mixed. Then the tube was incubated on ice for 30 minutes. The heat shock was applied to the competent cells by putting the tube into the heat block at 42°C for 90 seconds, which immediately shocked the cells on ice

for 5 minutes. The 900 µL LB medium was then transferred to a sterile culture tube containing cells and shaken for 70 minutes at 37°C in an orbital shaker at 200 rpm. A further centrifugation at 4000 rpm for 5 minutes was performed on the cells. The remaining 800 µL of supernatant was discarded, and the pellet was dissolved in the remaining 100 µL LB. At the end of the process, the cells were spread on a LB agar plate, which contained antibiotics that were identified as being resistant to antibiotics, and the plate was incubated at 37°C overnight for 16–20 hours to allow colonies to develop.

2.2.4 Bacterial growth

The single colony was picked from the overnight grown plate and was transferred into an Erlenmeyer flask with LB medium containing the appropriate antibiotic (30 mg/ml kanamycin). Then, it was incubated overnight at 37°C in an orbital shaker at 200 rpm. The next day, after incubation, the culture was inoculated in a 1:100 ratio into fresh Luria Bertani (LB) medium at 37°C and 200 rpm. Cell growth was controlled with OD_{600} and the cells were induced with an appropriate amount of IPTG when OD_{600} reached a value of between 0.6 and 0.7. The optimal inoculation culture conditions were identified by determining the duration of induction (1 to 6 hours), growth temperature (37°C, 30°C, 27°C, and 18°C), and IPTG concentration (0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM, and 1 mM) following induction.

2.2.5 Isolation, solubilization, and refolding of rhIL-2

Total protein was extracted from Rosetta (DE3) cells containing the pET30a (+):: recombinant hIL-2 plasmid using a 1:20 (v/v) lysis buffer. Then, sonication was applied at 50 power for 7 minutes, roughly 10 bursts and 20 seconds of rest on ice. Sonication should result in cleaner sonicated cells with buffer. After cell disruption, the sample was centrifuged at 23000 xg for 30 minutes at 4°C. The supernatant was discarded, and the inclusion body-containing pellet was resuspended in wash buffer. Cells were centrifuged at maximum speed (23,000 xg) for 30 minutes at 4°C. This washing step was repeated two times. The supernatant was removed while the pellet was dissolved in solubilization buffer. Cells were incubated at room temperature for 2 hours on a shaker. The pellet was discarded after 30 minutes of centrifugation at 23000 xg at 4°C. The supernatant containing solubilized inclusion bodies was dialyzed for 16 hours against 50 mM Glycine-NaOH pH 10.4 using MWCO 3.5K (Thermo

Scientific, 68035). After dialysis, refolded proteins were collected and filtered with a 0.45 μm syringe filter separated for the purification step.

2.2.6 Purification of rhIL-2

The separation of rIL-2 from total protein was accomplished by the use of anion exchange chromatography (AEX) and the ÄKTA avant 25 system (Cytiva, 28930842).

It is a purification technique that uses an ion-exchange resin with positively charged groups to separate compounds based on their charge. The content of the binding and elution buffers used for purification is given below. The 1 ml HiTrap® Q-FF anion exchange column was equilibrated by binding buffer with a 1 ml/min flow rate for 10 column volumes (CV). After that, a filtered protein sample was loaded onto the column. Nonspecific proteins were removed from the column with 50 mM Glycine-NaOH and 2 CV wash buffer. Lastly, rIL-2 was eluted with salted elution buffer. The elution step was performed as a linear gradient for 30 CV. The fractions were collected and analyzed by 15% SDS-PAGE and immunoblotting assays.

2.2.7 Ultrafiltration

Ultrafiltration is a membrane filtration method that uses hydrostatic pressure to drive water through a semi-permeable membrane, comparable to reverse osmosis. After the anion exchange chromatography step, selected fractions were pooled and transferred into Amicon® Ultra-15 Centrifugal Filters with a membrane containing a 30 kDa molecular weight cut-of (30 kDa MWCO) and centrifuged at 10,000 xg for 15 minutes at 4°C. The part under the filter was collected and the sample was loaded into the Amicon® Ultra-15 Centrifugal Filters with a membrane containing a 3 kDa molecular weight cut-of (3 kDa MWCO) and centrifuged under the same conditions. Then, rIL-2 was concentrated until it reached the required volume for the next step, and particles with a high molecular weight were removed after two stages of ultrafiltration.

2.2.8 Desalting and buffer exchange of rhIL-2

The desalting and buffer exchange steps were performed using the Äkta Avant 25 FPLC system (Cytiva, 28930842). A 5 mL HiTrap® Desalting column was used for desalting instead of the dialysis method. After ultrafiltration, a concentrated protein sample up to a volume of 1.5 mL was loaded onto the desalting column and buffer exchange occurred. Salts were removed from the sample and replaced with a

formulation buffer that contained an appropriate amount of sodium phosphate, SDS, and mannitol to a pH range of 7.2 to 7.8.

2.2.9 Characterization of rhIL-2

2.2.9.1 SDS-PAGE and immunoblotting

SDS-PAGE is a technique to separate proteins in an electrical field based on their size as they migrate towards the positive electrode by denaturing them and giving them a uniform negative charge. Protein samples were prepared by combining them with 4X Laemmli buffer and boiling them for 10 minutes at 95°C. A 15% SDS gel was used for both SDS-PAGE and immunoblotting. Gels were loaded with denatured protein samples and running was performed at 90 V until the marker passed the stacking gel, then it was increased to 120 V. Following the completion of the run, the gel was incubated with Coomassie Brilliant Blue staining solution for overnight. Destaining solutions were then used to remove the Coomassie Brilliant Blue staining from the gel and reveal the proteins.

Immunoblotting is a very sensitive technique for protein identification. The technique uses a combination of gel electrophoresis and antigen–antibody interaction. Under an electric field of 100 V for 90 minutes, molecules on the gel were transferred into the nitrocellulose membrane. After wet transfer was performed, the membranes were blocked using 5% milk prepared in TBS-T for 1 hour. The membrane was then incubated with a primary antibody (Anti-IL-2 mouse monoclonal antibody) in a 5% BSA solution for 16 hours at +4°C while being rotated. TBS-T was used to clean the membranes three times for five minutes each time. Then, they were treated for two hours at room temperature with a secondary antibody (Mouse IgG antibody) conjugated with HRP. The membrane was visualized using the Clarity Western ECL Substrate (Bio-Rad, 1705060).

2.2.9.2 Visualization and data analysis

The ChemiDoc Imaging System (Biorad, USA) was used to image each gel and membrane. The ImageLab software (Bio-Rad) was used to carry out the analysis of the data. A protein marker was used to determine the molecular weight of each protein.

2.2.9.3 Circular dichroism spectrometry

Circular dichroism (CD) is a method for determining the secondary structure of proteins and polypeptides that is highly sensitive and fast. The secondary structure of purified recombinant IL-2 proteins was identified as α-helix in far-UV CD spectra. The J-1500 Circular Dichroism Spectrophotometer (Jasco, Easton, MD) was used to perform all CD spectrophotometer measurements using a 1 mm cuvette.

In this study, secondary structure studies were carried out at wavelengths ranging from 200 to 250 nm. One nm step size, 1 nm bandwidth, a 50 nm/min speed, and 2 seconds of reaction time at 25°C were the CD scanning conditions.

2.2.9.4 Capillary electrophoresis analysis

Capillary electrophoresis is a high-resolution analytical method that uses an applied voltage to separate ions depending on their electrophoretic mobility. Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) and capillary isoelectric focusing (CIEF) are examples of CE procedures that were used in this study. The PA800 Plus CE equipment was used for all capillary electrophoresis analysis (Beckman Coulter). Samples were prepared and analyzed according to the Beckman Coulter manual. Protein analysis by CE-SDS is based on the separation of SDS-labeled protein variants by a filtering matrix in a continuous electric field. An SDS-MW buffer sample was added to the concentrated sample in order to bring the total volume to 95 µL. The sample was treated with 5 µL of 250 mM Iodoacetamide (IAM) and 2 µL of an internal standard (10 kDa). For 10 minutes, the mixture was heated at 70°C. Once the samples and necessary buffers were placed on the tray, the process was started.

Capillary isoelectric focusing (cIEF) is a way to partition protein/peptide combinations, protein glycoforms, and other charge changes based on their isoelectric point (pI). To separate molecules according to their pI, ampholyte mixtures create a pH gradient inside the gel. When the molecule of interest reaches the pH corresponding to its pI, it has a net charge of zero and stops to moving along the electrical field. After that, separation proceeds under the impact of UV absorbance across the neutral capillary. The samples and required buffers were carried out in compliance with the manual. In addition, pI markers with values of 10, 9.5, and 5.5 were used.

2.2.9.5 Peptide mapping analysis

Peptide mapping is an analytical approach for verifying the amino acid sequence and chemical changes of a protein. Mass spectrometry was used to identify specific protein fragments generated by enzyme digestion using Acquity UPLC and Waters Synapt G2-Si HDMS (Waters Corp). Analyzed rIL-2 samples were obtained from SDS-PAGE gels by employing an in-gel digestion procedure mentioned below.

- Each slice of gel was cut into small pieces and placed in tubes.
- 100 µL of double distilled water was added to cover the gel pieces and vortexed for 5 minutes. After that, double distilled water was discarded from the gels.
- 100 µL of 100 mM AMBIC/100% ACN solution was added to cover the gel pieces and vortexed for 10 minutes. After that, the AMBIC/100% ACN solution was discarded from the gels. This step was repeated until the gel pieces were colorless.
- 100 µL of 100% ACN solution was added to cover the gel pieces and vortexed for 5 minutes. After that, the ACN solution was discarded from the gels and gels were dried under vacuum for approximately 8 minutes.
- 100 µL of 10 mM DTT in 100 mM AMBIC solution was added to cover the gel pieces and they were incubated at room temperature for 45 min. Then the supernatant was discarded.
- 100 µL of 50 mM IAM in 100 mM AMBIC solution was added to cover the gel pieces and the reaction proceeded in the dark at room temperature for 45 min. Then the supernatant was discarded.
- The processes described in 2-4 were repeated and gels were dried under a vacuum for approximately 8 minutes.
- 30-40 µL of 20 ng/µL in 50 mM AMBIC solution were added to cover the gel pieces and incubated on ice.
- On ice, 50 µL of 50 mM AMBIC was added to cover the gel pieces and incubated for 15 minutes. After that, it was incubated for 16 hours at 37°C in an orbital shaker at 180 rpm.
- The next day, the supernatant was collected and it was placed into a different tube.
- 1 µL of formic acid was added into the supernatant and the vortex for a short time. Then, it was kept at 4°C.

- To cover the gel pieces, a 100 L mixture of 45% ddH2O/50% ACN/5% formic acid was added and vortexed for 5 minutes**.** Then the supernatant was collected and transferred to the tube stored at 4°C.
- The processes described in 11–12 were repeated until the gel pieces were colorless and the peptides were dried under a vacuum for approximately 2 hours.
- Then, dried peptides were dissolved in 40 µL of 89% ddH2O/1% ACN/10% formic acid mixture.

Peptide samples were placed onto an ACQUITY UPLC® Peptide BEH C18, 300Å, 1.7 µm, 2.1 mm x 100 mm (Waters, 186003686) analytical column after being digested. The column temperature was maintained at 65°C throughout the experiment. Positive electrospray ionization and resolution mode were used for all analyses.

2.2.9.6 Intact mass analysis

Intact mass analysis is the determination of a protein's complete molecular weight using mass spectrometry (MS) without the molecule having been digested previously. The molecular mass of recombinant human IL-2 was determined using SYNAPT G2-Si HDMS. For this study, a BEH C4 300, 1.7 m, 2.1 mm x 50 mm column (Waters, 186004495) was utilized and the flow rate was kept constant at 0.3 ml/min.

2.2.9.7 Biological activity analysis

In order to determine whether purified recombinant human IL-2 is biologically active or not, the protein of interest was administered to human monocyte THP-1 cells. $1x10^6$ cells/ml were planted into a 12-well plate. After varying periods of incubation (10 min, 20 min, 30 min, 1 h, 2 h) at 37°C with 5% CO_2, cells are extracted. After that, centrifugation at 300 x g for 5 minutes at +4°C, THP1 cells were separated from their suspensions. Once the pellet was rinsed with 1X PBS, the cells were dissolved in the mammalian lysis solution and incubated on ice for 25 minutes. The sample is then centrifuged for 20 minutes at a speed of 17000 xg at a temperature of +4°C. The supernatant was maintained at -80°C until further usage, while the pellet was thrown away. The Bradford assay was used in this experiment to determine protein concentration using BSA standards.

3. RESULTS

3.1 Amplification, Enzyme Digestion, Ligation and Verification of rhIL-2

The target gene of interest was taken from pQE80L-IL2 C125S (plasmid # 104348), and it was amplified using polymerase chain reaction (PCR) with suitable restriction sites inserted in the primers specific to rhIL-2. Figure 3.1 displays the PCR results after they were performed on an agarose gel electrophoresis apparatus.

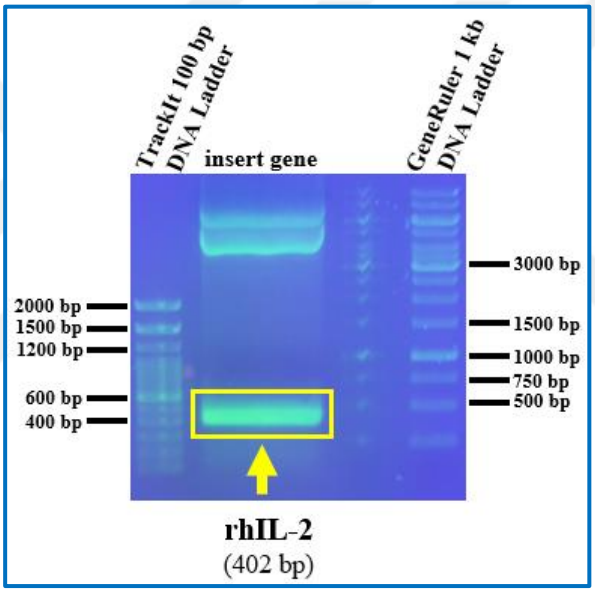


Figure 3.1 : The rhIL-2 PCR results. 20 µl PCR product with 4 µl 6X DNA Loading Dye. 0.8% Agarose / TAE.

Figure 3.1 indicates the amplified DNA fragment was expected to be the size of 402 base pairs. After the PCR product was purified, both the PCR product and the pET30a (+) plasmid were double digested with NdeI and HindIII-HF® restriction enzymes. Ligation was performed on both the digested and linearized vectors as well as the digested insert. Then, the ligated plasmid construct was transformed into the *E. coli* Stellar strain. Several colonies were thereafter chosen from the LB agar growth to check for colonies with successfully ligated constructs.

In order to verify the presence of the desired gene of interest, colony PCR and sequencing were performed on selected colonies. DNA sequencing was performed via

sanger sequencing by BM Laboratory Systems (Ankara, Turkey). SnapGene Viewer 5.0.7 from GSL Biotech was used to create the plasmid map (Figure 3.2).

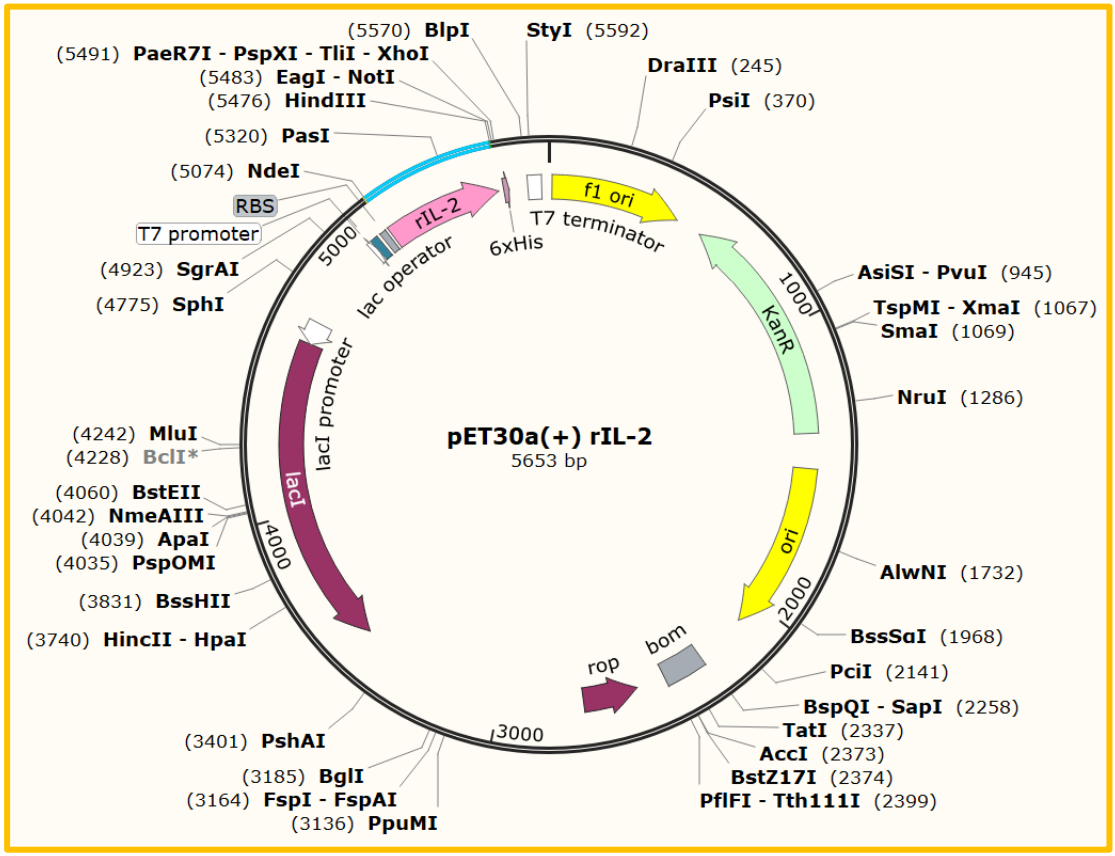


Figure 3.2 : Plasmid map of pET30a(+) rIL-2 was generated with SnapGene.

3.2 rhIL-2 Expression Under Optimal Conditions

First, studies were conducted to optimize bacterial protein production. The focus was on determining the best bacterial strain, IPTG concentration for induction, time length of IPTG induction, and temperature at which IPTG induction should take place to ensure optimum production.

To accomplish control of leaky expression of the target protein, the *E. coli* strains BL21 (DE3) and Rosetta (DE3) were tried with or without IPTG at 37°C for 4 hours. Figure 3.3 and Figure 3.4 show the SDS-PAGE and the immunoblotting results.

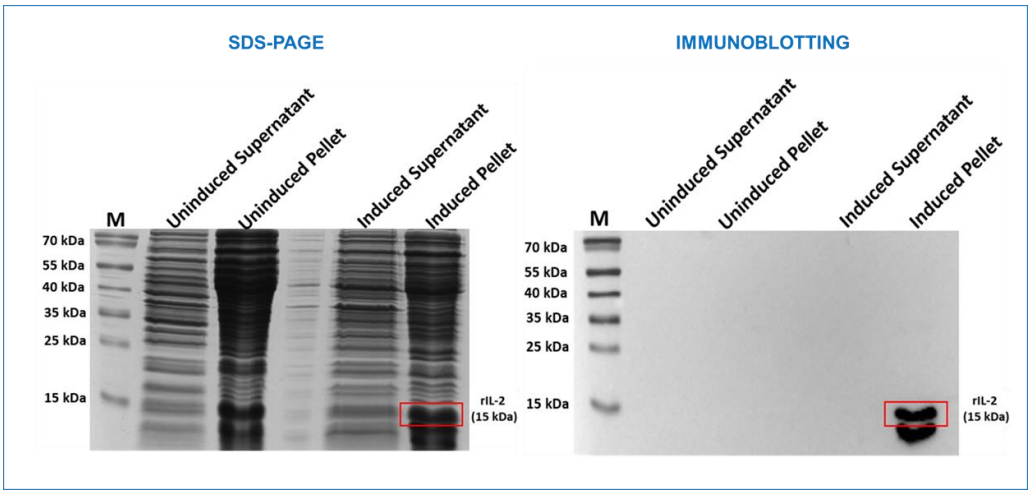


Figure 3.3 : The IPTG induction results of the optimization of hIL-2 protein production in *E. coli.* The SDS-PAGE (15%) findings are shown on the left, while the Immunoblotting results are shown on the right. M is PageRuler protein marker, Thermo Scientific.

The occurrence of the target hIL-2 band in the total protein extract but not in the soluble lysate, demonstrated that insoluble IBs had been formed. The following figure shows that rhIL-2 was successfully expressed in both BL21 (DE3) and Rosetta (DE3) competent cells. Since Rosetta host strains are BL21 variants developed to boost the expression of eukaryotic proteins with rare *E. coli* codons, Rosetta (DE3) competent cells were selected in this study.

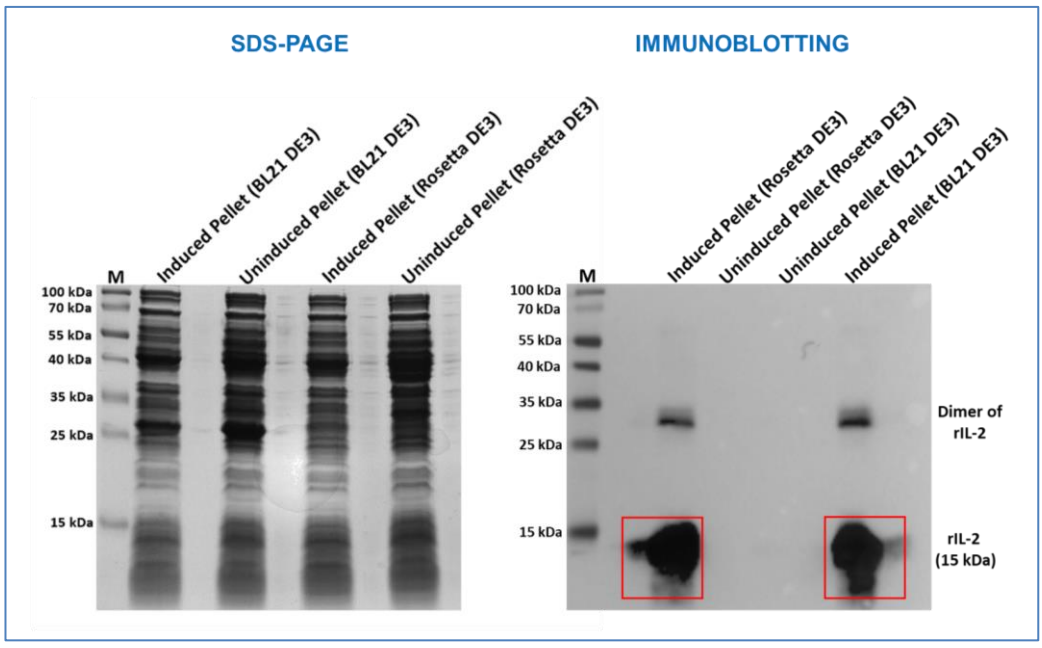


Figure 3.4 : The result of competent cells trial. The SDS-PAGE (15%) findings are shown on the left, while the Immunoblotting results are shown on the right M is PageRuler protein marker, Thermo Scientific.

The optimal IPTG induction concentration for *E. coli* Rosetta (DE3) cells expressing pET-30a(+)::hIL-2 was determined by comparing the amount of target protein produced at five different IPTG concentrations: 0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM, and 1.0 mM. Figure 3.5 shows the SDS-PAGE and immunoblotting findings. Consequently, a concentration of 0.25 mM IPTG was chosen as it was sufficient for optimal induction.

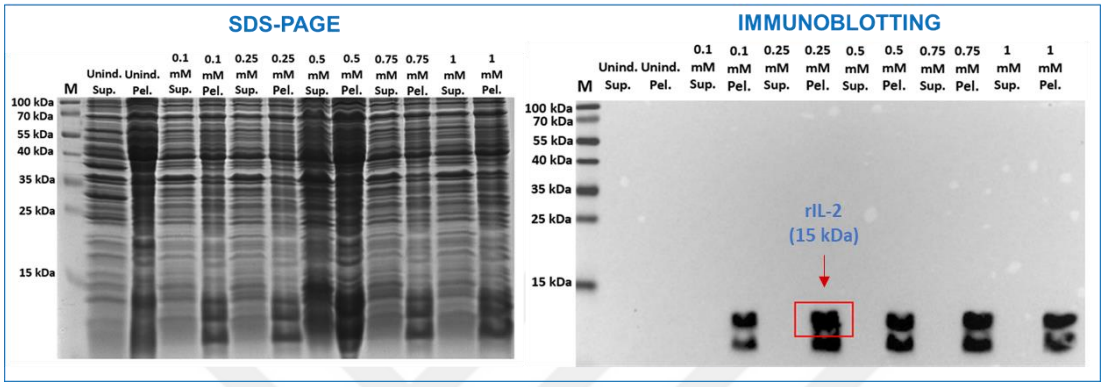


Figure 3.5 : The IPTG concentration results. The SDS-PAGE (15%) findings are shown on the left, while the Immunoblotting results are shown on the right. M is PageRuler protein marker, Thermo Scientific. Unind. is uninduced-control. Sup. is supernatant. Pel. is pellet.

Four different temperatures were tried after induction to increase the solubility level of protein. Temperatures ranged from 18°C overnight to 27°C for 8 hours, 30°C for 7 hours, and 37°C for 4 hours. Its SDS-PAGE and immunoblotting results are given in Figure 3.6. According to the figure below, 37°C was chosen as the incubation temperature because of the high expression level of rhIL-2.

The optimum incubation time following induction was determined after examining the quantity of rhIL-2 produced for six hours. Figure 3.7 demonstrates the SDS-PAGE and immunoblotting findings. In this case, the appropriate incubation time was estimated at 4 hours taking into account the amount of target protein taken every hour during the incubation.

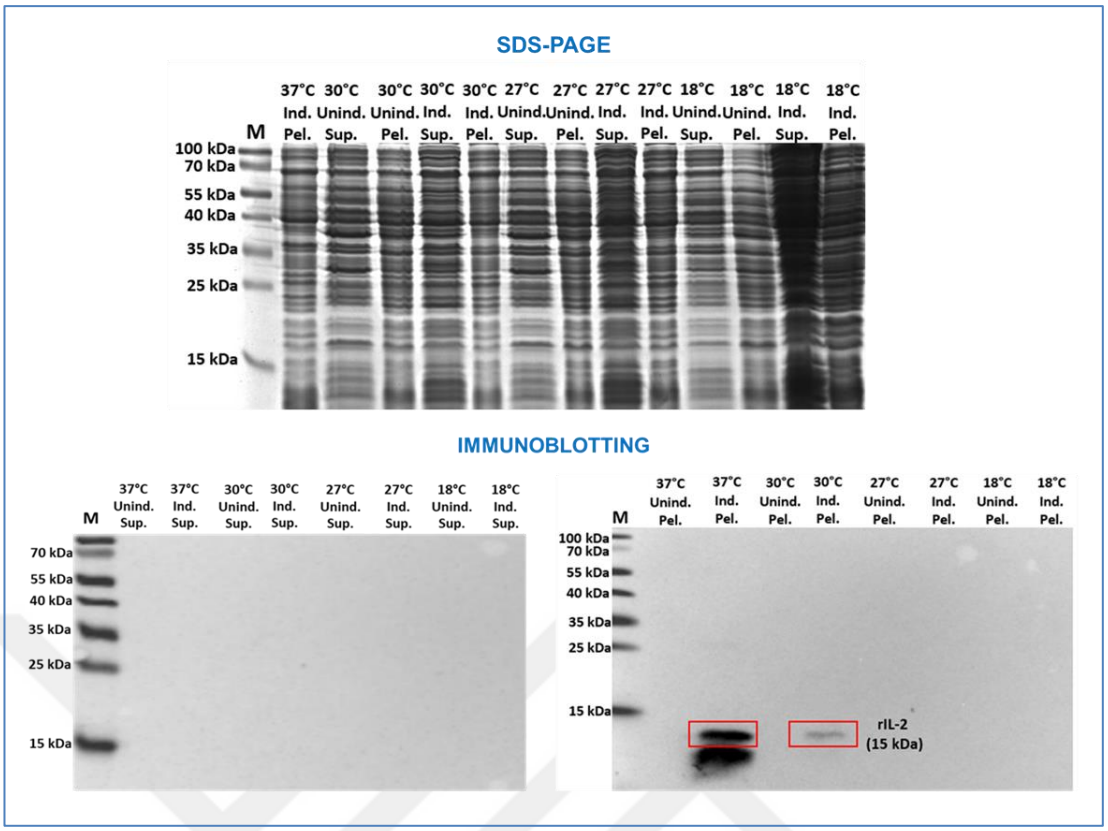


Figure 3.6 : The result of incubation temperature trial. The SDS-PAGE (15%) findings are shown on the above, while the Immunoblotting results are shown on the below. M is PageRuler protein marker, Thermo Scientific. Unind. is uninduced-control. Sup. is supernatant. Pel. is pellet.

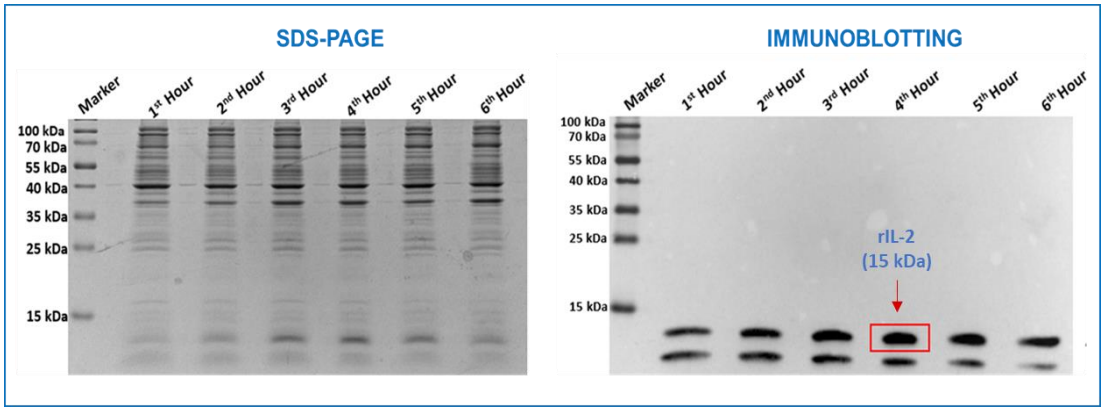


Figure 3.7 : The result of incubation duration after induction. The SDS-PAGE (15%) findings are shown on the left, while the Immunoblotting results are shown on the right. M is PageRuler protein marker, Thermo Scientific.

3.3 Recovery, Solubilization and Refolding of rhIL-2

To begin purifying human recombinant IL-2 from the pellet, the first cell pellet was dissolved in a lysis buffer and disrupted by sonication. The pellet containing lysed cells, insoluble cellular proteins and IL-2 inclusion bodies was then washed three times with wash buffer. The final step was the solubilization of the inclusion bodies with a

combination of alkaline buffer and 2 M urea after 2 hours of room temperature incubation. The solubilized protein was refolded using a one-step dialysis method. As a result, no aggregation of the solubilized rhIL-2 was observed during dialysis for 16 hours against binding buffer. The SDS-PAGE and immunoblotting results are given in Figure 3.8.

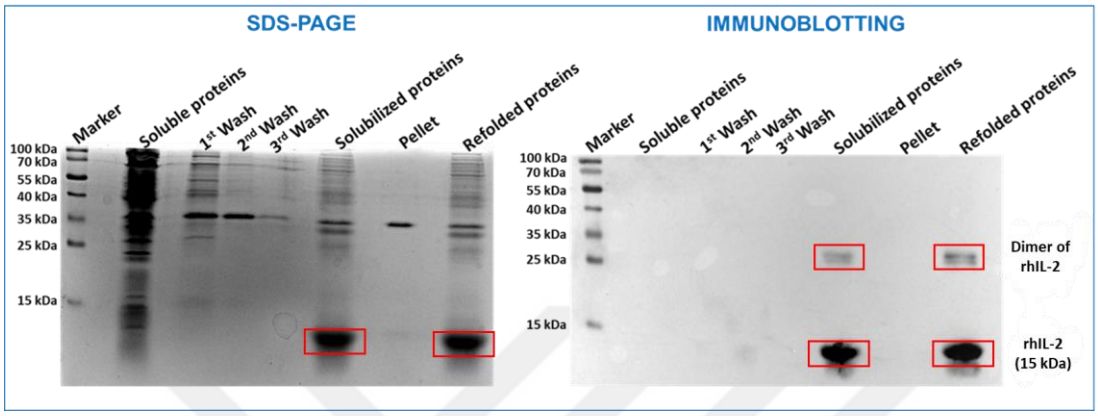


Figure 3.8 : The SDS-PAGE and immunoblotting results of the solubilized and refolded rhIL-2. Marker is PageRuler protein marker, Thermo Scientific.

3.4 Purification and Buffer Exchange of rhIL-2

Purification of refolded proteins was carried out following dialysis. It was achieved by the use of anion exchange chromatography (AEX) applied in a bind-and-elute mode to bind the target molecule and remove host cell proteins. rIL-2 was eluted using salted elution buffer. A HiTrap® Q-FF anion exchange column was used for purification, and Unicorn 7.0 software was used for chromatograms. Following that, fractions were collected and subjected to SDS-PAGE (15%) and immunoblotting analysis. Figure 3.9 and Figure 3.10 display the chromatogram and SDS-PAGE (15%) and immunoblotting analysis results of three biological replicates of rhIL-2, respectively.

The second process was ultrafiltration. Figure 3.10 shows that recombinant hIL-2 was eluted between fractions 1 to 12. The chosen fractions were pooled and transferred to the cut-off filter.

A concentrated protein sample was put onto the desalting column (5 mL HiTrap® Desalting column) for the following ultrafiltration, and buffer exchange occurred. The salts in the sample were removed and replaced with a formulation buffer. Then, the ultimate rhIL-2 product's yield per liter of bacterial growth is estimated at around 0.1 mg for all three biological replicates.

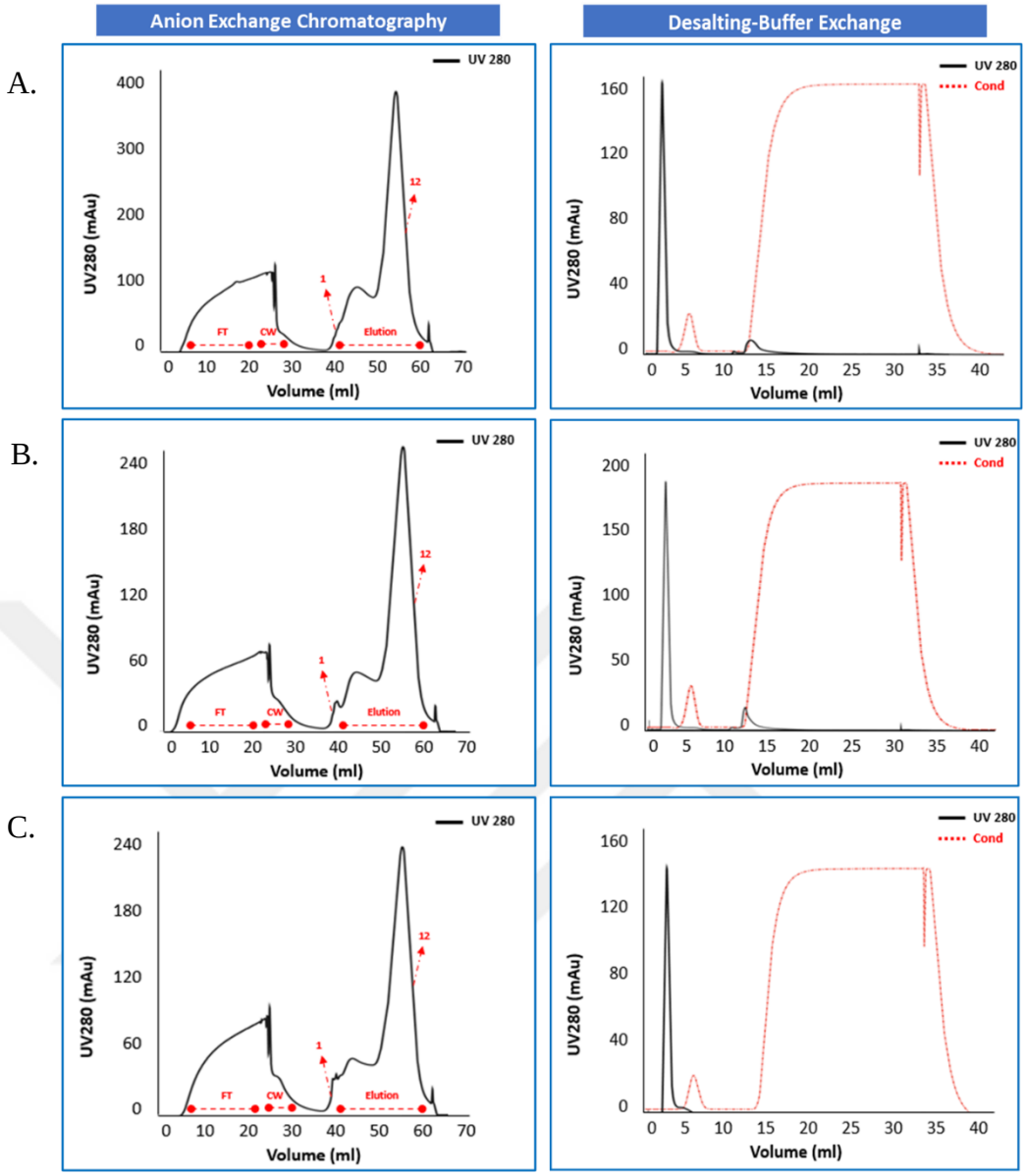
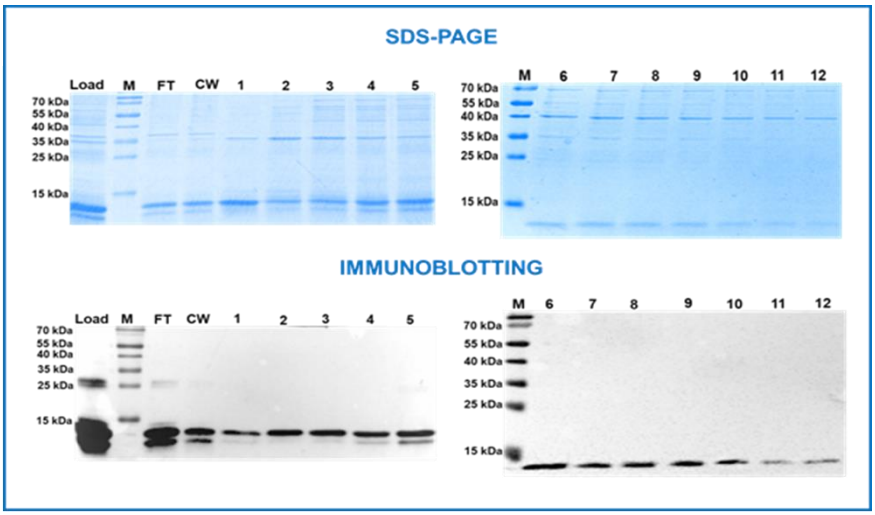
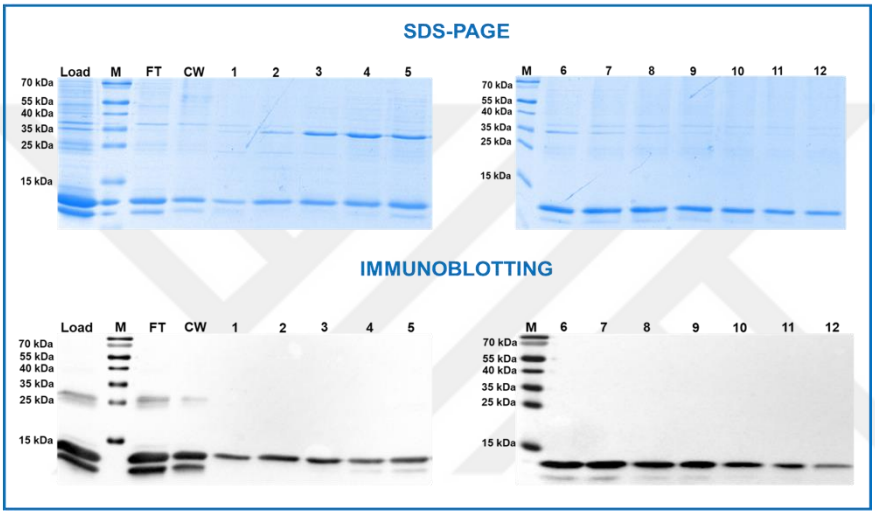


Figure 3.9 : A., B., and C. represent chromatograms of three biological replicates of rhIL-2. The elution fractions collected are indicated numerically. FT is Flow-through, CW is Column Wash.

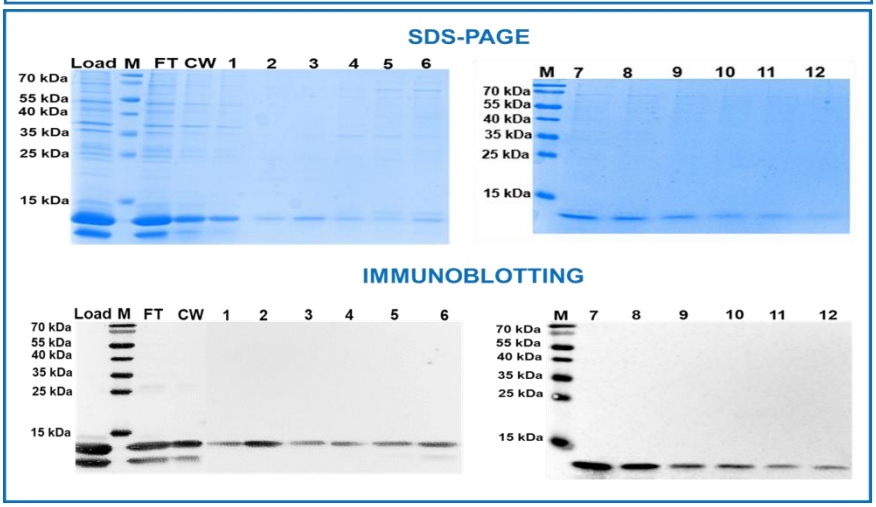
A.



B.



C.



. **Figure 3.10 :** A., B., and C. represent SDS-PAGE and immunoblotting analysis result of three biological replicates of rhIL-2. The elution fractions collected are indicated numerically. FT is Flow-through, CW is Column Wash, and M is PageRuler marker

3.5 CD Spectrum Analysis of rhIL-2

The target protein forms an α-helix structure when it folds. Circular dichroism is one way of controlling the correct folding process. In this study, far-UV CD spectra were applied at wavelengths ranging from 200 to 250 nm. According to the literature (Yamada et al., 1987), the circular dichroism spectrum of three biological replicates of refolded rhIL-2 was found to be similar to the various forms of rIL-2. A related figure was given in Figure 3.11.

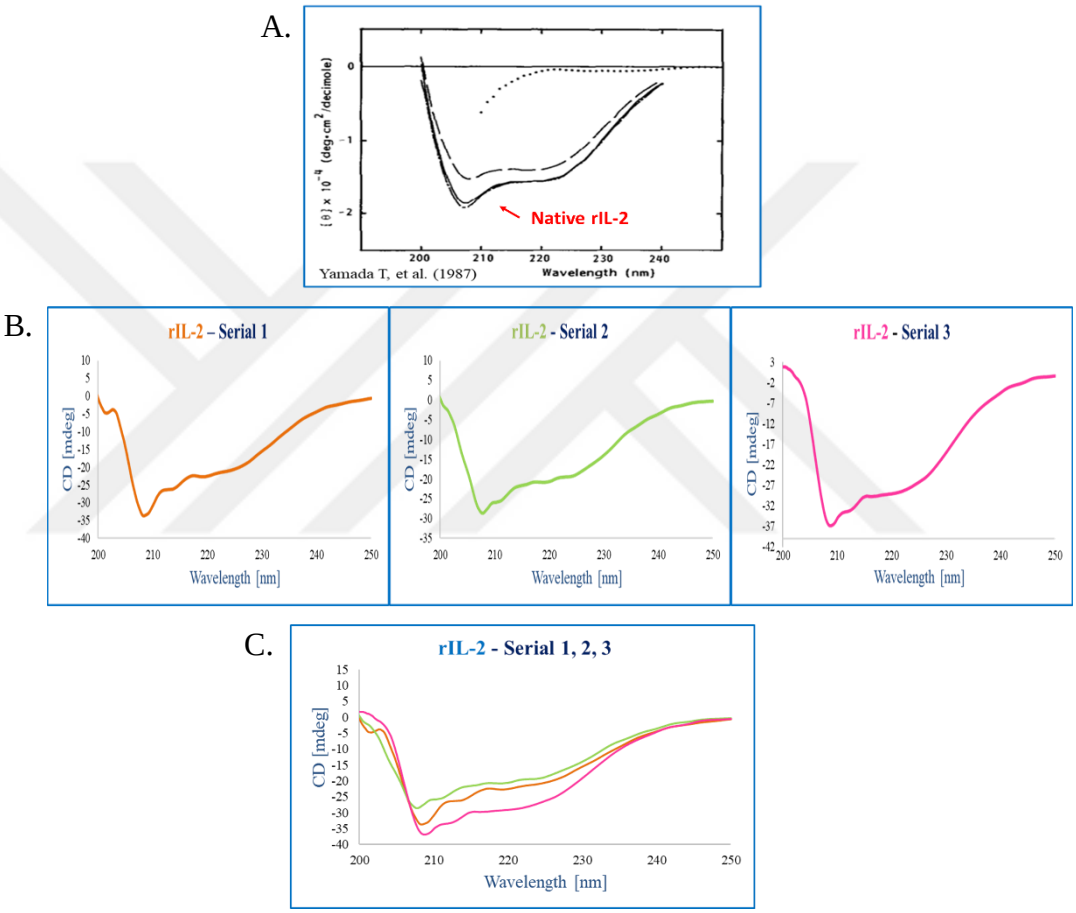


Figure 3.11 : The results of the CD spectra. A. belongs to the reference, B. gives three replicates of rIL-2, and C. shows the comparison of three replicates.

3.6 Capillary Electrophoresis Analysis of rhIL-2

Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) was used to determine and compare the impurity and purity of human IL-2. The purity percentages of the three different biological replicates are 78.71, 83.42, and 83.48, respectively.

In addition, the migration times of the peaks are consistent with each other. Figure 3.12 shows the CE electropherograms of the non-reduced rIL-2 replicates.

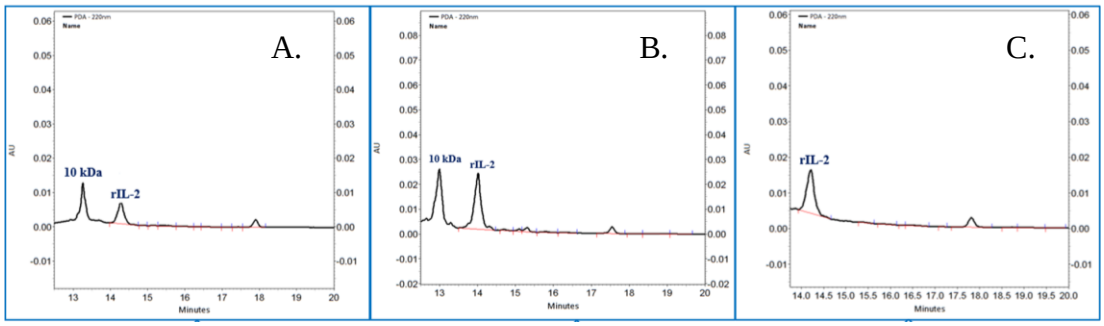


Figure 3.12 : Electropherograms of the non-reduced rIL-2 replicates.

Capillary isoelectric focusing is required for separating proteins or peptides depending on their isoelectric points (pI). Regarding the theoretical pI calculation for rIL-2, we employed values of 10, 9.5, and 5.5 as pI markers for this study. As a result, the pI of the rIL-2 protein was found to be 7.31. Moreover, neither acidic nor basic variations were discovered during cIEF analysis. The cIEF electropherograms of the pI marker and rIL-2 are shown in Figure 3.13.

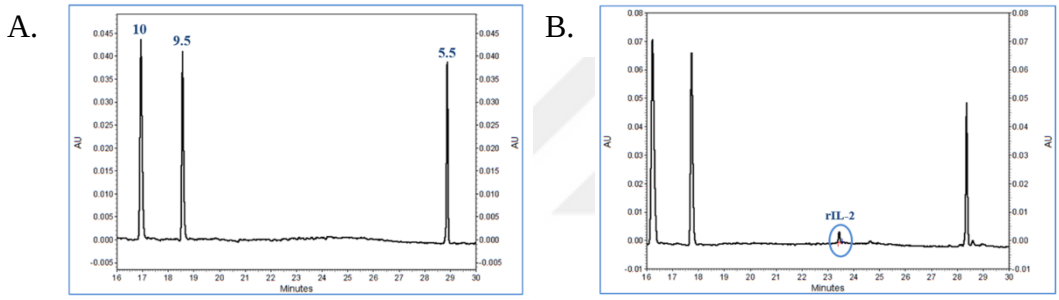


Figure 3.13 : The results of the cIEF electropherograms A. belongs to the pI marker (10, 9.5, 5.5) and B. shows the pI of rIL-2.

3.7 Intact Mass Analysis of rIL-2

The molecular weight of recombinant human IL-2 was determined using intact mass analysis by Liquid Chromatography–Mass Spectrometry (LC-MS/MS). The mass spectra and deconvolute mass data of three different products are given in Figure 3.14.

Every replication had the same estimated molecular weight of recombinant hIL-2, which was 15328 Da and consistent across the board. As a result of intact mass analysis, the moleculer weight of rhIL-2 replicates was confirmed via calculated deconvolute mass.

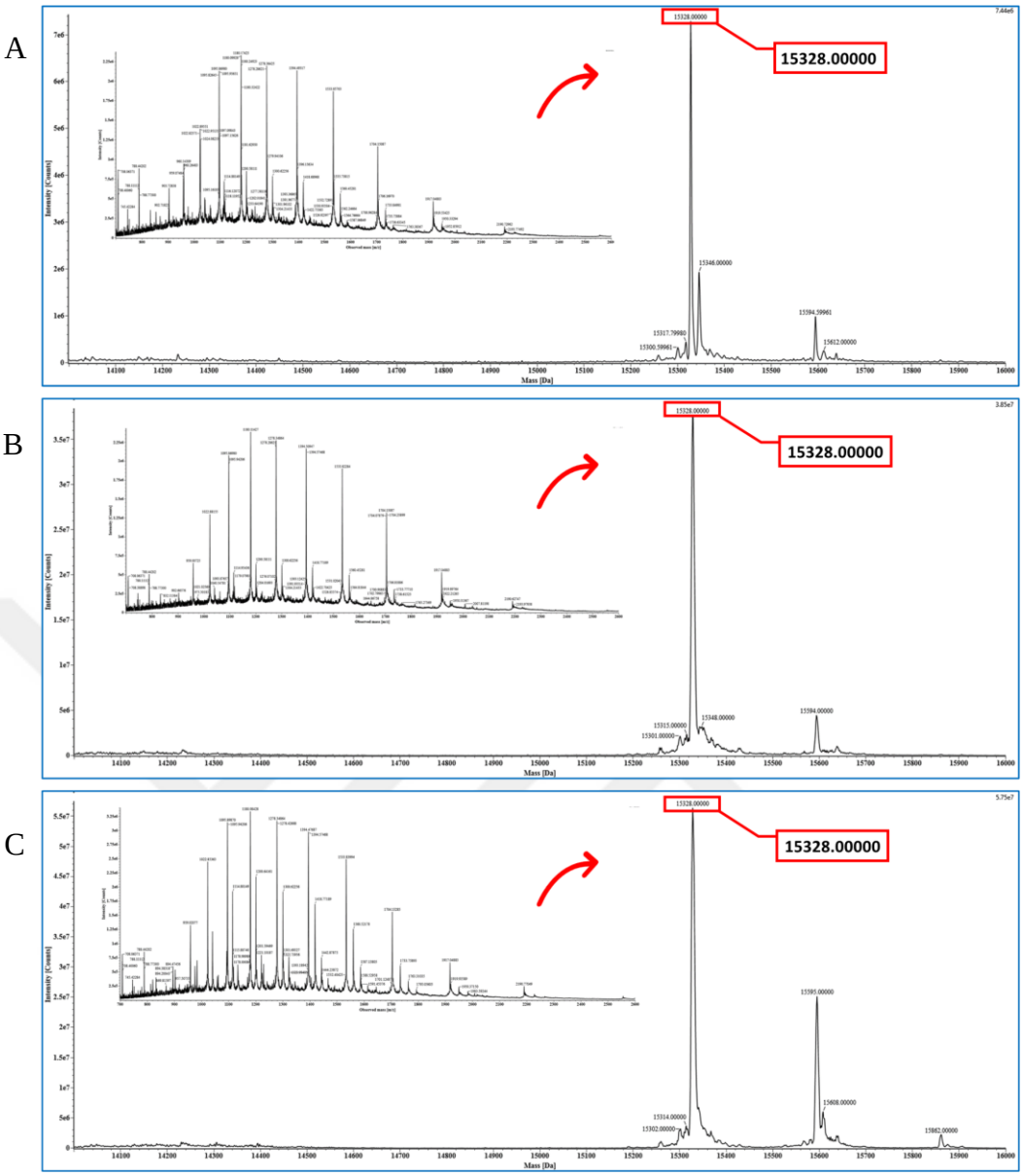


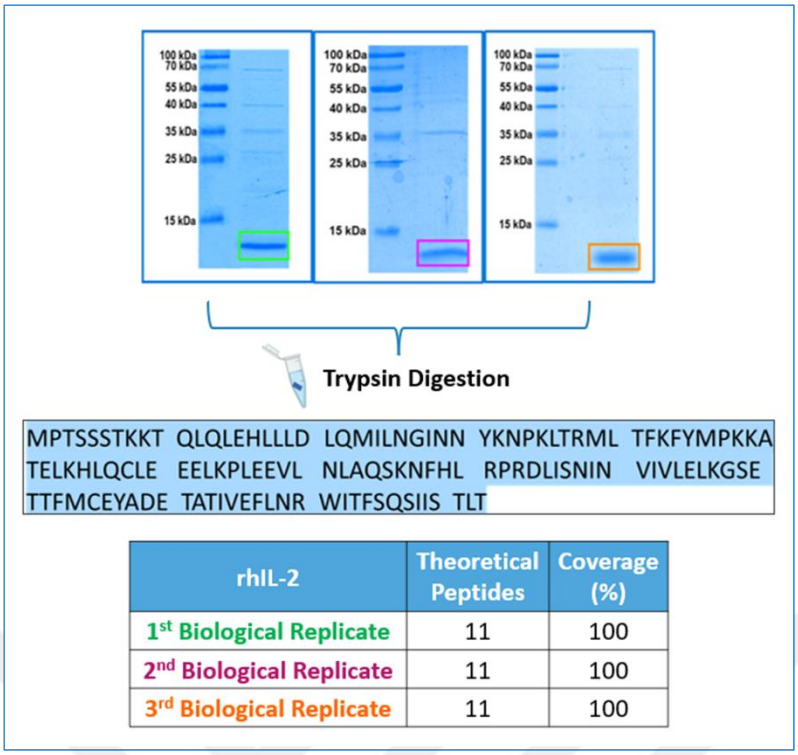
Figure 3.14 : LC/MS spectrum of recombinant human IL-2 (left) and the deconvoluted mass spectra of peaks corresponds to rhIL-2 peak (right).

3.8 Peptide Mapping Analysis of rhIL-2

The peptide mapping technique using LC-MS/MS is used to determine the main structure of the protein bands found on SDS-PAGE gels of purified products.

The sequence (CS373812.1) was downloaded in FASTA format from the GenBank database.

In order to facilitate spectrum processing and peak identification via database comparison, Waters Corporation's Progenesis QI software V4.1 was utilized. As a result, the rhIL-2 amino acid sequence was perfectly matched (100%) to the database (Figure 3.15).



rhIL-2	Theoretical Peptides	Coverage (%)
1st Biological Replicate	11	100
2nd Biological Replicate	11	100
3rd Biological Replicate	11	100

Figure 3.15 : Peptide mapping outcomes from three replicates of proteins. They are digested with the trypsin enzyme from SDS-PAGE.

3.9 Biological Activity of rhIL-2

Biological activity of the rhIL-2 product is investigated in human monocytic THP-1 cells in addition to purification and characterization. The total protein content of these monocytes is utilized to determine the biological activity of the rhIL-2 product. The amount of p-ERK is studied in this experiment after stimulation of the response generated when recombinant interleukin 2 was administered to THP-1 cells at different dosages for 2 hours. IL-2 cells were exposed to a wide range of doses, starting with the lowest 10 IU mL^{-1} concentrations and reaching 150 IU mL^{-1}. After several trials, 50 IU mL^{-1} and 100 IU mL^{-1} data were chosen due to meaningful results.

After 30 minutes of stimulation, the levels of phosphorylated ERK 1/2 began to fall. Figure 3.16 depicts the findings.

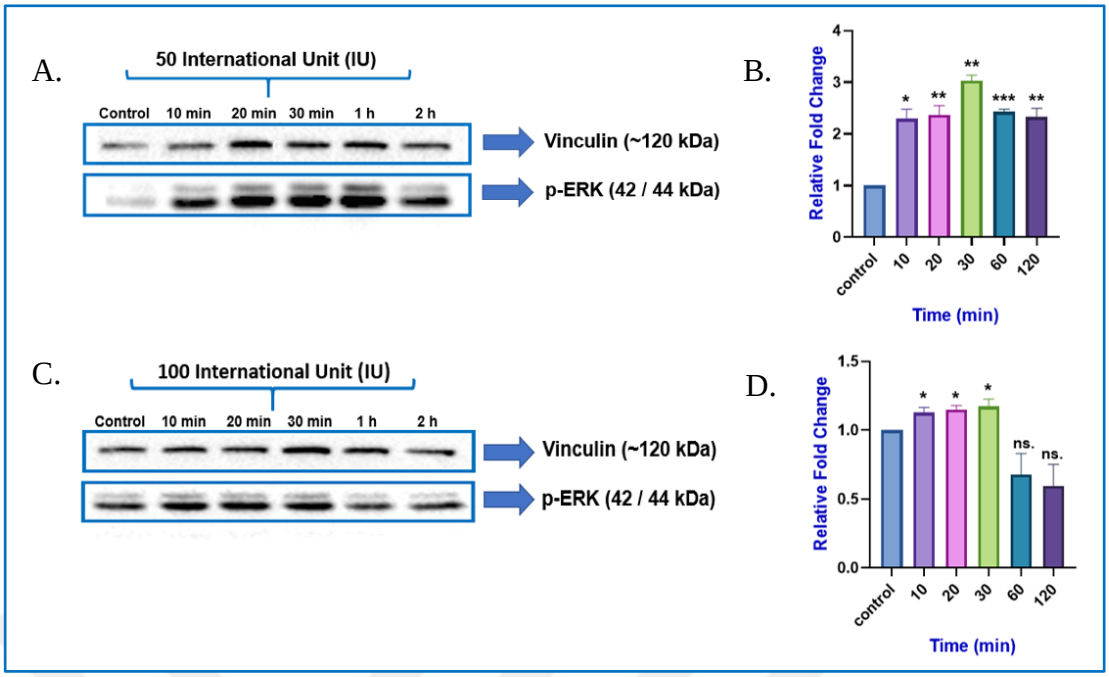


Figure 3.16 : The increase in p-ERK levels following activation of 50 IU (A and B.) and 100 IU rhIL-2 (C. and D.).

4. DISCUSSION

Cytokines are small, soluble proteins that give guidance and assist immune and non-immune cells in communicating with each other. IL-2 turns out to be a crucial cytokine that controls cell survival, growth, and differentiation. This contributed to the development of immunotherapy for cancer and viral infections (Sim & Radvanyi, 2014). As a therapeutic protein, the natural human version of interleukin 2 is glycosylated, which is a control mechanism for protein folding. Aldesleukin has been shown to be bioactive in vivo and in vitro despite not being glycosylated after production in *E. coli*. Thus, it is critical for the pharmaceutical industry to develop, purify, and characterize recombinantly generated interleukin 2 as a therapeutic protein.

Human interleukin 2 was inserted into the pET-30(a) vector in this study, and it was expressed in E. coli Rosetta (DE3) as a cloning host because it can produce large amounts of the recombinant protein. The cloning procedure was completed successfully, as seen in Figure 3.2. Appendix A also contains the sequencing findings.

Additional advantages of using *E. coli* as an expression system include a rapid transformation process and a lower overall cost of production when compared to using alternative host organisms (Pliego-Arreaga et al., 2013). After that, protein synthesis was controlled by IPTG induction. During the process, IPTG will enter the cells and remove LacI from the LacO location. T7 RNA polymerase then attaches to the T7 promoter and starts gene transcription. It ultimately causes the formation of the target protein. Next, optimized growth conditions were determined, and it resulted in a high amount of rhIL-2. Consequently, over-expression of proteins in the cytoplasm causes the development of insoluble aggregates of the target protein known as inclusion bodies (IBs) (Singh et al., 2015). According to previous reports, the majority of the produced IL-2 in *E. coli* was insoluble (formed IBs); nevertheless, the recombinant IL2 maintained its biological activity even after being extracted from SDS polyacrylamide gel (Schutz et al., 1977).

There are four phases involved in recovering bioactive therapeutic proteins from inclusion bodies: separation of inclusion bodies from *E. coli* cells; solubilization of

protein aggregates, refolding and purification of the solubilized proteins. The use of buffers with high pH (> 12) in conjunction with 2 M urea has been described as a mild solubilization approach and has been utilized effectively for inclusion body solubilization due to its ability to destabilize both the ionic and hydrophobic interactions responsible for protein aggregation in inclusion bodies (Singh et al., 2015). In this research, mild solubilization techniques were employed to recover and refold proteins from inclusion bodies as given in Figure 3.8. The use of 2 M urea did not entirely unfold the protein, preserving the native-like secondary structure. Moreover, the use of high pH aids in improved solubilization since it is far from the isoelectric point of rhIL-2, which is 7.31 as confirmed by cIEF and shown in figure 3.13. After removing chaotropic chemicals by dialysis, solubilized rhIL-2 is refolded back into its natural state.

Anion exchange chromatography (AEX) in bind-and-elute mode was used to purify the samples. Following that, the selected fractions were pooled, and the protein sample was loaded onto the desalting column for buffer exchange. To retain the natural form of rhIL-2, the salts in the sample were removed and replaced with a formulation buffer. Figure 3.9 illustrates the chromatogram result. Purification fractions are collected and evaluated using various characterization procedures. Figure 3.10 depicts the purity and estimated molecular weight of the target protein in fractions via SDS-PAGE and immunoblotting. As a result, the remaining cell lysate proteins are effectively isolated from rhIL-2.

Circular dichroism is one method of ensuring that the right folding process takes place. Understanding the protein's secondary structure under natural circumstances requires the use of this characterization approach. In this research, we used far-UV CD spectra with wavelengths spanning from 200 to 250 nm to examine the secondary structure. According to the literature, the organized structure contains two significant negative peaks in the native rIL-2 far-UV CD spectra. One should be a minimum of around 208 nm, while the other should be a shoulder of about 222 nm (Yamada et al., 1987). Similar to navite rhIL-2, the target protein produces α-helix shape, as seen in Figure 3.11.

The molecular weight of recombinant human IL-2 was measured using Liquid Chromatography–Mass Spectrometry (LC-MS/MS). Figure 3.14 depicts the mass spectra and deconvoluted mass data. The molecular weight of rhIL-2 was calculated

to be 15328 Da as a result of intact mass analysis, which is consistent with the literature (Rotte & Bhandaru, 2016). Noises in the deconvoluted mass spectra seen in the LC chromatography inset arise depending on concentration and formulation buffer components.

Peptide mapping is an analytical technique for confirming the amino acid sequence and chemical changes in a protein. In this study, particular protein fragments were produced by trypsin enzyme digestion. As a result, the rhIL-2 amino acid sequence was exactly (100%) matched to the database shown in Figure 3.15.

Capillary electrophoresis is a very effective separation method that is often utilized in pharmaceutical research and development (R&D), quality control (QC), and stability investigations. CE has a lot of advantages over high-performance liquid chromatography (HPLC). Simpleness, speed of analysis, automation, robustness, a variety of procedures for selection, and low cost are only a few examples of why it is used in a lot of biomedical and pharmaceutical tests (Ahuja, 2008). The purity of rhIL-2 in formulation buffer was tested using non-reducing capillary electrophoresis in this investigation. The electropherogram in Figure 3.12 reveals that the protein recovered after purification is approximately 80% pure, indicating that it was extremely pure considering the one-step purification procedure.

In addition to purification and characterization, the biological activity of the rhIL-2 product is being examined in human monocytic THP-1 cells. According to the literature, when IL-2 binds to its receptor, it initiates several signaling pathways in the immune system, including JAK/STAT, RAS-MAPK, and PI3K/AKT. Within immune cells, interleukin-2 (IL-2) activates the extracellular signal-regulated protein kinase (ERK) family, which is a prominent kinase in this pathway. ERK is composed of two closely related isoforms, ERK1 and ERK2, which have a molecular weight of 42 and 44 kDa, respectively. An investigation revealed that IL-2 exposure increased p-ERK in monocytes (Kondadasula et al., 2008).

In this study, to detect biological activation, phosphorylated ERK 1/2 levels were controlled after stimulation of THP-1 cells with rhIL-2. As a result, phosphorylated ERK1/2 was upregulated and, after 30 minutes, its level began to fall due to its short half-life.

Finally, when all of the studies have been completed, it is possible to conclude that functional recombinant human IL-2 was successfully produced in this study.

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APPENDICES

APPENDIX A **:** Laboratory equipments

APPENDIX B **:** Chemicals, enzymes, and commertial kits

APPENDIX C **:** Buffers and solutions

APPENDIX D **:** 3D structure of rhIL-2

APPENDIX E **:** Sequencing data of rhIL-2

APPENDIX A

Table A.1 indicates all the laboratory equipments used in this study.

Table A.1 : Used equipments in this study.

Equipment	Supplier Company
Agarose Gel System	Bio-Rad Wide Mini-Sub Cell GT
Analytical Balance	OHAUS AX-224
Autoclave	Zealway GR110DF
Automatic Cell Counter	Nanoentek EVE
Biosafety Cabinet	HEDLAB X-BİO CLASS-II
Capillary Electrophoresis	Beckman Coulter PA 800 Plus
Centrifuge	VWR Mega Star 3.0R
Chromatographic System	GE Healthcare Äkta Avant25
Circular Dichroism	Jasco J-1500
CO_2 Incubator	ESCO (CCL-170B-8)
Deepfreeze (-20ºC)	Bosch
Electrophoresis Power Supply	Bio-Rad
Electrophoresis Power Supply	GE Healthcare
Freezer (-80°C)	Aucma
Heat Block	BioSan Bio TDB-100
Ice Flaker	Scotsman AF 80
Imaging System	Bio-Rad ChemiDoc™ MP
Imaging System	Bio-Rad GelDoc™ XR+
Inverted Microscope	LEICA DMi1
Laminar Flow	Faster BH-EN 2003
Magnetic Stirrer	DragonLab MS-H-S
Mass Spectrometry	Waters Synapt G2-Si
Microcentrifuge	Beckman Coulter Microfuge 20R
Micropipette	Eppendorf
Micropipette	Thermo Fisher
Microplate Spectrophotometer	Bio-Rad Benchmark Plus
Minispin	Weightlab WN-CMV6000
Orbital Shaker	Lab Companion™ ISF-7200
pH Meter	Mettler Toledo
Platform Shaker	DLAB SK-R1807-E
Refrigerator (+4ºC)	Arçelik
Rotator Mixer	DLAB MX-RL-Pro
SDS-PAGE Gel Electrophoresis System	Bio-Rad Mini Protean Tetra
Sonicator	Bandelin Sonopuls
Ultrasonic Bath	Bandelin Sonorex
Ultrapure Water System	Merck Direct-Q®-3UV
UV-Visible Spectrophotometer	Thermo Fisher Nanodrop 2000
Vortex	DragonLab MX-F
Wet/ Tank Blotting System	Bio-Rad Mini Trans-Blot® Cell

APPENDIX B

Table B.1 and Table B.2 indicate all the chemicals used in this study.

Table B.1 : Used chemicals in this study.

Chemical	Supplier Company
Acetic Acid	Sigma-Aldrich
Acetonitrile	Merck
Acrylamide/Bis-Acrylamide (40%)	Bio-Rad
Agar Bacteriological Grade	MultiCell
Agarose Molecular Biology Grade	MultiCell
Amicon Ultracel 3K Membrane	Merck Millipore
Amicon Ultracel 30 K Membrane	Merck Millipore
Ammonium Bicarbonate (AMBIC)	Merck
Ammonium Persulfate (APS)	Sigma-Aldrich
Bromophenol Blue	Sigma-Aldrich
Bradford Reagent (1X)	Bio-Rad
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Calcium Chloride Anhydrous	Merck
Di-sodium Hydrogen Phosphate Dihydrate	Merck
Dimethyl Sulfoxide (DMSO)	BioMatik
Dithiothreitol (DTT)	Fermentas
Ethanol Absolute	Merck
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich
Fetal Bovine Serum (FBS)	Thermo Fisher
Formic Acid	Merck
Glycine	Sigma-Aldrich
Glycerol	MultiCell
HindIII- HF® Restriction Enzyme	NEB
HiTrap® Q-FF	GE Healthcare
HiTrap® Desalting	GE Healthcare
Hydrochloric Acid (HCl)	Sigma-Aldrich
Iodoacetamide	Bio-Rad
Isopropanol	Merck
Kanamycin	Sigma-Aldrich
Mannitol	Merck
Methanol	Merck
NdeI Restriction Enzyme	NEB
Nitrocellulose Membrane (0.45µm)	Bio-Rad
Non-Fat Dry Milk	Santa Cruz
PhosSTOP	Roche
Phosphate-Buffered Saline (PBS) Tablets	Thermo Fisher
Peptone	Merck
Prestained Protein Ladder (PageRuler)	Thermo Fisher
Q5 High-Fidelity 2X Master Mix	NEB
RedSafe 20000X	Intron Biotech.
RPMI 1640	Lonza
Sodium Chloride (NaCl)	Sigma-Aldrich
Sodium Dihydrogen Phosphate Dihydrate	Merck
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich
Sodium Hydroxide (NaOH)	Multicell
Taq 2X Master Mix	NEB
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Tris Base	MultiCell
Triton X-100	Sigma-Aldrich
Trypsin-EDTA	Thermo Fisher
Trypsin	Sigma-Aldrich
Tween-20	BioShop

Table B.2 : Used chemicals in this study (continued).

Chemical	Supplier Company
Urea	Sigma-Aldrich
Yeast Extract	Sigma-Aldrich
2-Mercaptoethanol	Sigma-Aldrich
10X CutSmart Buffer	NEB
6X Loading Dye Solution	Intron Biotech.
100 bp DNA Ladder	Invitrogen
1 kb DNA Ladder	Thermo Scientific

Table B.3 indicates all the molecular biology kits used in this study.

Table B.3 : Used molecular biology kits in this study.

Name of the Kit	Supplier Company
Clarity Western ECL Substrate	Bio-Rad
NucleoSpin Gel and PCR Clean-up Kit	Macherey-Nagel
NucleoSpin Plasmid Kit	Macherey-Nagel

Table B.4 indicates all the antibodies used in this study.

Table B.4 : Used antibodies in this study.

Antibody	Type	Dilution
Anti-Rabbit IgG (CST, 7074S)	Polyclonal HRP-linked	1:3000 (5% Milk)
Anti-Mouse IgG (CST, 7076S)	Polyclonal HRP-linked	1:3000 (5% Milk)
Anti-IL-2 Antibody (SC-133118)	Monoclonal Mouse	1:1000 (5% BSA)
GAPDH	Monoclonal Rabbit	1:500 (5% BSA)
Phospho-p44/42 MAPK (Erk1/2)	Monoclonal Rabbit	1:500 (5% BSA)
Phospho-PI3 Kinase p85 /p55	Monoclonal Rabbit	1:500 (5% BSA)
p44/42 MAPK (Erk1/2) Antibody	Monoclonal Rabbit	1:500 (5% BSA)
Vinculin	Monoclonal Rabbit	1:500 (5% BSA)

APPENDIX C

Buffers and solutions are given in Table C.1 to Table C.23.

Table C.1 : Used lysis, wash, and solubilization buffers in this study.

Lysis Buffer	Wash Buffer	Solubilization Buffer
50 mM Tris-HCI (pH: 8.0)	50 mM Tris-HCI (pH: 8.0)	50 mM Tris-HCI (pH: 12.5)
1 mM PMSF	1 mM PMSF	1 mM PMSF
5 mM EDTA	5 mM EDTA	1 mM EDTA
	1% Triton X-100	2 M Urea, 5 mM BME

Table C.2 : Used anion exchange buffers in this study.

Binding Buffer	Elution Buffer
50 mM Glycine-NaOH (pH: 10.4)	50 mM Glycine-NaOH (pH: 10.4) 1 M NaCI

Table C.3 : Used formulation buffer in this study.

Formulation Buffer
Appropriate amount of Sodium phosphate (pH range 7.2 to 7.8), SDS, Mannitol

Table C.4 : The 50X TAE buffer is composed of the following components.

Compound	Amount
2 M Tris Base	242 g
1 M Acetic Acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL
dH2O	up to 1 L

Table C.5 : The 1X TBS buffer is composed of the following components.

Compound	Amount
10X TBS Buffer	100 mL
dH2O	up to 1 L

Table C.6 : The Luria Bertani (LB) medium is composed of the following components for 1 liter.

Compound	Amount
Bacterial Peptone	10 g
Yeast Extract	5g
NaCl	10 g

Table C.7 : The Luria Bertani (LB) agar medium is composed of the following components.

Compound	Amount
Bacterial Peptone	10 g
Yeast Extract	5g
NaCl	10 g
Agar	15 g
dH2O	up to 1 L

Table C.8 : The 10X running buffer is composed of the following components.

Compound	Amount
Tris Base	30.3 g
Glycine	144 g
SDS	10 g
dH2O	up to 1 L

Table C.9 : The 10X transfer buffer is composed of the following components.

Compound	Amount
Tris Base	30.3 g
Glycine	144 g
dH2O	up to 1 L

Table C.10 : The 1X transfer buffer is composed of the following components.

Compound	Amount
10X Transfer Buffer	100 mL
Methanol	200 mL
dH2O	up to 1 L

Table C.11 : The 10X TBS buffer is composed of the following components.

Compound	Amount
Tris Base	24.22 g
NaCl	87.66 g
dH2O	up to 1 L
pH	adjust to 7.6 with HCl

Table C.12 : The 1X TBS-T buffer is composed of the following components.

Compound	Amount
Tween-20	1 mL
1X TBS	up to 1 L

Table C.13 : The Coomassie Brilliant Blue stain solution is composed of the following components.

Compound	Amount
Coomassie Brilliant Blue	1 g
Methanol	400 mL
Acetic Acid	100 mL
dH2O	up to 1 L

Table C.14 : The Destaining solution is composed of the following components.

Compound	Amount
Methanol	400 mL
Acetic Acid	100 mL
dH2O	up to 1 L

Table C.15 : The 4X laemmli sample buffer is composed of the following components.

Compound	Amount
1 M Tris-HCl (pH 6.8)	2.4 mL
Glycerol	4 mL
Bromophenol Blue	4 mg
SDS	0.8 g
dH2O	up to 10 mL

Table C.16 : The 10% Ammonium Persulfate (APS) solution is composed of the following components.

Compound	Amount
APS	1 g
dH2O	up to 10 mL

Table C.17 : The 5% non-fat dry milk solution is composed of the following components.

Compound	Amount
Non-fat dry milk	2.5 g
1X TBS-T	up to 50 mL

Table C.18 : The 5% BSA solution is composed of the following components.

Compound	Amount
BSA	1.5 g
1X TBS-T	up to 30 mL

Table C.19 : The stock 500 mM isopropyl-β-D-thiogalactopyranoside (IPTG) is composed of the following components.

Compound	Amount
IPTG	1.19 g
dH2O	up to 10 mL

Table C.20 : The stock 200 mM PMSF is composed of the following components.

Compound	Amount
PMSF	0.348 g
Isopropanol	up to 10 mL

Table C.21 : The 30 mg/mL Kanamycin stock solution is composed of the following components.

Compound	Amount
Kanamycin	0.3 g
dH2O	up to 10 mL

Table C.22 : The components of 10% and 15% SDS polyacrylamide separating gels.

Content	Amount	
	10% Gel	15% Gel
dH_2O	4.79 mL	3.54 mL
40% Acrylamide/Bis-acrylamide	2.5 mL	3.75 mL
1.5 M Tris-HCl (pH 8.8)	2.5 mL	2.5 mL
10% SDS	100 µL	100 µL
10% APS	100 µL	100 µL
100% TEMED	10 µL	10 µL

Table C.23 : The components of 4% SDS polyacrylamide stacking gel.

Content	Amount
dH_2O	2.92 mL
40% Acrylamide/Bis-acrylamide	540 µL
1.5 M Tris-HCl (pH 6.8)	500 µL
10% SDS	40 µL
10% APS	40 µL
100% TEMED	4 µL

APPENDIX D

3INK – Sequence of Interleukin-2.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATE
LKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMC
EYADETATIVEFLNRWITFAQSIISTLT

PDB ID: 3INK – Crystal structure of Interleukin-2.

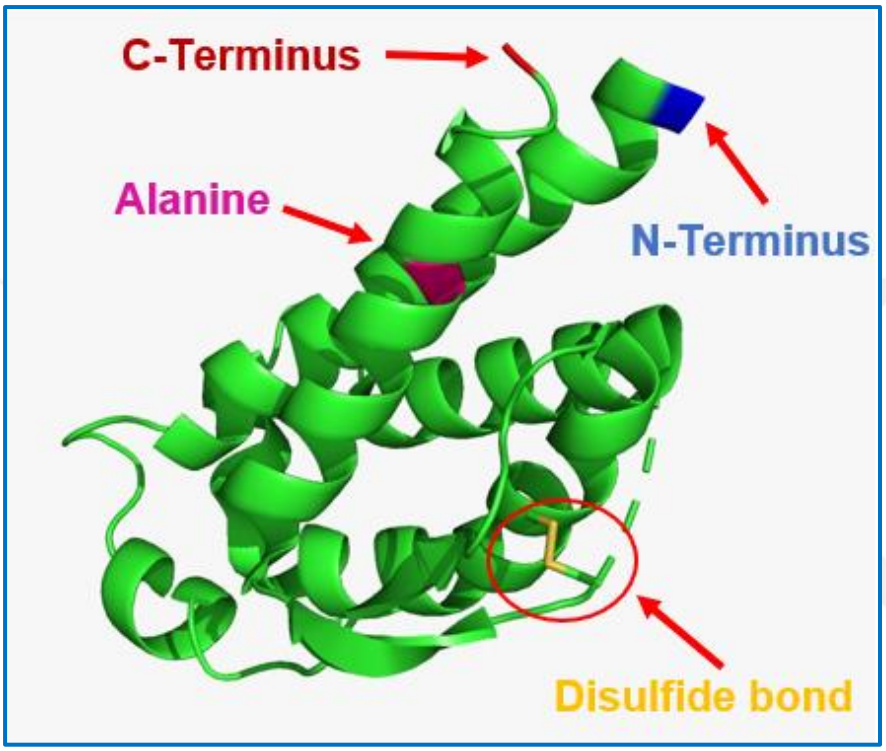

Figure D.1 : 3INK – The 3D structure of Interleukin-2.

APPENDIX E

Human IL-2 Sequence (UniProtKB - P60568) – Query_10001

MYRMQLLSCIALSLALVTNSAPTSSSTKKTQLQLEHLLLDLQMILNGINNYK
NPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRD
LISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT

Recombinant IL-2 Sequence – Query_10002

MAPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA
TELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFM
CEYADETATIVEFLNRWITFCQSIISTLT

Drugbank Sequence of Aldesleukin – Query_10003

MPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATE
LKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMC
EYADETATIVEFLNRWITFSQSIISTLT

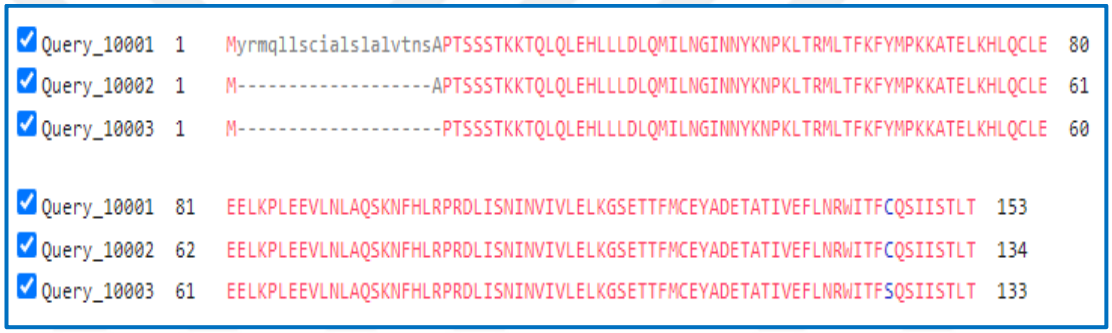

Figure E.1 : Multiple alignment of the amino acid sequences of IL-2 using NCBI COBALT.

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