

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF SOLUBLE
RECOMBINANT TNFR1**



M.Sc. THESIS

Derya HATİPOĞLU

Department of Molecular Biology – Genetics and Biotechnology

Molecular Biology – Genetics and Biotechnology Programme

JUNE 2022

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF SOLUBLE
RECOMBINANT TNFR1**

M.Sc. THESIS

**Derya HATİPOĞLU
(521191107)**

Department of Molecular Biology – Genetics and Biotechnology

Molecular Biology – Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Gizem DİNLER DOĞANAY

JUNE 2022

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**ÇÖZÜNÜR REKOMBİNANT TNFR1'İN ÜRETİMİ, SAFLAŞTIRILMASI VE
KARAKTERİZASYONU**

YÜKSEK LİSANS TEZİ

**Derya HATİPOĞLU
(521191107)**

Moleküler Biyoloji – Genetik ve Biyoteknoloji Anabilim Dalı

Moleküler Biyoloji – Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Gizem DİNLER DOĞANAY

HAZİRAN 2022

Derya Hatipoğlu, a M.Sc. student of ITU Graduate School student ID 521191107, successfully defended the thesis entitled “**EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF SOLUBLE RECOMBINANT TNFR1**”, which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Prof. Dr. Gizem DİNLER DOĞANAY**
Istanbul Technical University

Jury Members : **Assoc. Prof. Dr. Halil BAYRAKTAR**
Istanbul Technical University

Prof. Dr. Özge Can
Acıbadem University

Date of Submission : 01 June 2022
Date of Defense : 28 June 2022





To my lovely family and friends,



FOREWORD

First of all, I would like to thank Mustafa Kemal ATATÜRK, the founder of our republic, who said "The supreme guide in life is science" and his comrades-in-arms. I am grateful to them.

Secondly, I would like to express my sincere gratitude to Prof. Dr. Gizem DİNLER DOĞANAY.

After that, a huge appreciation goes to all the previous and current GDLAB members, especially Baran DINGİLOĞLU, Latif AKBULUT, Ezgi BAŞTÜRK, Duygu YALÇINKAYA YAVUZ, Jale YILDIZ, Nisan Denizce CAN and Miray TÜRK, who helped me in my experiments, shared their expertise and provided me with knowledge.

Additionally, I would like to especially thank Buse AKGÜN, Kübra TÜRK, Nazlı Dilara ERDOĞDU, Meriona MEZANİ and Pemra BAKIRHAN for their enjoyable friendship and support.

A big thank you to Mira TAFA and Hayrun Nisa GENÇ for their help and friendship.

I would like to thank Tunahan KILIÇ for helping me with my transfer for my experiments.

I would also like to express my gratitude to TÜBİTAK (Turkish Scientific and Technical Research Council) for supporting me with the 1007 project and the 1001 project.

I sincerely thank Dr. Cezmi BAYRAM, who has always supported and motivated me with his wealth, and all my friends, especially Ahmet Oğuzhan BALCI and Samet KARAKIZ. I am so glad to have met them.

Lastly, I would like to express my deepest gratitude and love to my family, my mother Leyla HATİPOĞLU, my father SADIK HATİPOĞLU, my sister Büşra KESKİN and my brother METE HATİPOĞLU, who have been by my side during all my life, trusting, loving and helping me with great patience in this process.

June 2022

Derya HATİPOĞLU

TABLE OF CONTENTS

	<u>Page</u>
FOREWORD.....	ix
TABLE OF CONTENTS	xi
ABBREVIATIONS.....	xv
SYMBOLS	xvii
LIST OF TABLES	xix
LIST OF FIGURES	xxi
SUMMARY	xxv
ÖZET.....	xxvii
1. INTRODUCTION.....	1
1.1 Tumor Necrosis Factor-Alpha.....	1
1.2 Tumor Necrosis Factor Receptor Superfamily: TNFR1-TNFR2	2
1.3 Disulfide Bond Formation in <i>E. coli</i>	5
1.4 Dsb Proteins	10
1.4.1 DsbA	10
1.4.2 DsbB.....	10
1.4.3 DsbC.....	11
1.4.4 DsbD	12
1.5 Aim of The Study.....	13
2. MATERIALS AND METHODS	15
2.1 Materials.....	15
2.1.1 Equipments.....	15
2.1.2 Chemicals.....	16
2.1.3 Molecular biology kits	17
2.1.4 Antibodies	17
2.1.5 Buffers and solutions.....	18
2.1.5.1 Luria bertani (LB) medium	18
2.1.5.2 Luria bertani (LB) agar medium	18
2.1.5.3 ZY medium solution	18
2.1.5.4 50X 5052 solution.....	18
2.1.5.5 20X NPS solution.....	18

2.1.5.6 ZYP-5052 autoinduction medium	18
2.1.5.7 10X tris-buffered saline (TBS) buffer	18
2.1.5.8 50X TAE buffer.....	18
2.1.5.9 10X running buffer.....	19
2.1.5.10 10X transfer buffer.....	19
2.1.5.11 4X laemmli sample buffer	19
2.1.5.12 Coomassie brilliant blue stain solution	19
2.1.5.13 Destaining solution	19
2.1.5.14 1X mild strip buffer	19
2.1.5.15 10% Ammonium persulfate (APS) solution	19
2.1.5.16 5% non-fat dry milk solution	19
2.1.5.17 SDS polyacrylamide stacking and seperating gels	20
2.1.5.18 5% bovine serum albumin (BSA) solution	20
2.1.5.19 Lysis and purification buffers	20
2.1.5.20 Storage buffer	21
2.1.5.21 Bacterial strains.....	21
2.2 Methods	21
2.2.1 Vector and primer design	21
2.2.2 Molecular cloning.....	21
2.2.2.1 Subcloning and plasmid DNA isolation.....	21
2.2.2.2 DsbC amplification with polymerase chain reaction (PCR)	22
2.2.2.3 Agarose gel electrophoresis and DNA extraction from the gel	22
2.2.2.4 Restriction enzyme digestion and DNA ligation.....	23
2.2.3 Competent cell preparation	23
2.2.4 Plasmid transformation	24
2.2.5 Colony PCR.....	24
2.2.6 Bacterial growth.....	25
2.2.7 Expression of DsbC-TNFR1	25
2.2.8 Purification of DsbC-TNFR1	26
2.2.9 Buffer exchange of DsbC-TNFR1 via ultrafiltration.....	26
2.2.10 SDS-PAGE and immunoblotting.....	26
2.2.11 Circular dichroism analysis.....	27
2.2.12 Intact mass analysis.....	27
2.2.13 Peptide mapping via liquid chromatography – mass spectrometry.....	27
2.2.14 Purity analysis by capillary electrophoresis	29
2.2.15 Isoelectric point determination by capillary electrophoresis.....	29
2.2.16 <i>In-vitro</i> pull down assay.....	30

2.2.17 Blue-native PAGE	31
3. RESULTS	33
3.1 Analysis of DsbC-TNFR1: PCR Amplification, Restriction Enzyme Digestion, Ligation	33
3.2 Protein Expression Optimization	35
3.3 Purification of DsbC-TNFR1 Fusion Protein	39
3.4 Secondary Structure Determination with Circular Dichroism	42
3.5 Purity Analysis by Capillary Electrophoresis	43
3.6 Isoelectric Point Determination by Capillary Electrophoresis	43
3.7 Intact Mass Analysis	44
3.8 Blue Native PAGE Analysis	45
3.9 <i>In-vitro</i> Pull-Down Assay	45
4. DISCUSSION	47
REFERENCES	51
APPENDICES	57
CURRICULUM VITAE	65



ABBREVIATIONS

ACN	: Acetonitrile
AEX	: Anion Exchange Chromatography
AMBIC	: Ammonium Bicarbonate
APS	: Ammonium Persulfate
BSA	: Bovine Serum Albumin
CD	: Circular Dichroism
CE	: Capillary Electrophoresis
CIAP	: Cellular Inhibitor of Apoptosis Protein
CRD	: Cysteine Rich Domain
DD	: Death Domain
DMEM	: Dulbecco's Eagle Modified Medium
DMSO	: Dimethyl Sulfoxide
DRP	: Disulfide Rich Protein
DTT	: Dithiothreitol
<i>E. coli</i>	: Escherichia coli
FA	: Formic Acid
FADD:	: Fas associated death domain
ICD	: Intracellular Domain
IgG	: Immunoglobulin G
IMAC	: Immobilized Metal Affinity Chromatography
LB	: Luria Broth
LC-MS/MS	: Liquid Chromatography – Mass Spectrometry
MS	: Mass Spectrometry
PCR	: Polymerase Chain Reaction
PDB	: Protein Data Bank
PLAD	: Pre-Ligand Assembly Domain
SDS	: Sodium Dodecyl Sulfate
SDS-PAGE	: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SODD	: Silencer of Death Domains
TACE	: TNF- α converting enzyme

TBS	: Tris-Buffered Saline
TBS-T	: Tris-Buffered Saline-Tween 20
TEMED	: Tetramethylethylenediamine
TEV	: Tobacco Etch Virus
TNF-α	: Tumor Necrosis Factor Alpha
TNFR	: Tumor Necrosis Factor Receptor



SYMBOLS

μg	: Microgram
μl	: Microliter
μM	: Micromolar
bp	: Base pair
pmol	: Picomole
g	: Gram
HCl	: Hydrochloric Acid
kb	: Kilobase
L	: Liter
M	: Molar
ml	: Mililiter
rpm	: Round per minute



LIST OF TABLES

	<u>Page</u>
Table 2.1: Used equipments in this study.	15
Table 2.2: Used chemicals in this study.....	16
Table 2.3: Used molecular biology kits in this study.	17
Table 2.4: Used antibodies in this study.	17
Table 2.5: 12% Stacking and separating gel contents.....	20
Table 2.6: Content of cell lysis buffer, IMAC purification buffers.	20
Table 2.7: AEX purification binding and elution buffer content.	20
Table 2.8: The PCR reaction composition.	22
Table 2.9: PCR cycling conditions.....	22
Table 2.10: Reaction mixture of double digested plasmid.....	23
Table 2.11: Reaction mixture of DNA ligation.....	23
Table 2.12: Colony PCR reaction mixture composition.	25
Table 2.13: Colony PCR cycling parameters.....	25



LIST OF FIGURES

	<u>Page</u>
Figure 1.1: Mechanism of TNF- α /TNFR1 signaling pathway (Adapted from G. Chen & Goeddel, 2002).	4
Figure 1.2: Disulfide bond formation.	6
Figure 1.3: Protein thiol-disulphide exchange reaction (Adapted from Sevier & Kaiser, 2002).	7
Figure 1.4: Disulfide bond reduction mechanisms in <i>E. coli</i> cytoplasm (Adapted from Hatahet et al., 2010).....	8
Figure 1.5: Periplasmic disulfide bond formation. The sec route is used to transport the protein to be folded to the periplasm. DsbA creates a disulfide linked complex with its reduced substrate, causing a disulfide bond to form in the substrate protein and cysteine reduction in DsbA's active region. The inner membrane protein DsbB reoxidizes DsbA via giving electrons. If DsbA misoxidizes the substrate protein, DsbC recognizes the misfolded protein and either isomerizes it to its native state or reduces it. The inner membrane protein DsbD keeps DsbC in an active decreased state. (Generated with Biorender and adapted from Berkmen, 2012).	9
Figure 3.1: The agarose gel results. A) Vector digestion and B) DsbC amplification. 0.8 % Agarose / TAE.....	33
Figure 3.2: The agarose gel results. A) colony PCR result of ligation of DsbC and TNFR1 gene B) Restriction enzyme digestion control.....	34
Figure 3.3: Design of the expression plasmid structure.....	35
Figure 3.4: Control of the TNFR1 plasmid expression in <i>E. coli</i> SHuffle T7 strain with and without DsbC. The SDS-PAGE (12%) result is shown on the left, while the Immunoblotting result is shown on the right. TNFR1 with DsbC has a molecular weight of 43 kDa, whereas TNFR1 without DsbC has a molecular weight of 25 kDa. 1 mM IPTG was used for induction. S is supernatant and P is pellet.	36
Figure 3.5: Control of the TNFR1 plasmid expression in <i>E. coli</i> BL21(DE3) strain with and without DsbC. The SDS-PAGE (12%) result is shown on the left, while the Immunoblotting result is shown on the right. TNFR1 with DsbC has a molecular weight of 43 kDa, whereas TNFR1 without DsbC has a molecular weight of 25 kDa. 1 mM IPTG was used for induction. S is supernatant and P is pellet.	37
Figure 3.6: The SDS PAGE and immunoblotting results of the target protein production from <i>E. coli</i> BL21 (DE3) and Rosetta (DE3) cells at 37°C for 4 hours. 1 mM IPTG was used for induction. S is supernatant and P is pellet.	37
Figure 3.7: The SDS PAGE (12%) and immunoblotting results of the target protein production from <i>E. coli</i> BL21 (DE3) and Rosetta (DE3) cells at different temperature. Cultures were incubated 30°C for 8 hours, 25°C for 12 hours and 18°C 20 hours. 1 mM IPTG was used for induction. S is supernatant and P is pellet.....	38

Figure 3.8: The SDS PAGE (12%) and immunoblotting results of autoinduction media (ZYP-5052 medium) effect on the target protein production. Cultures were incubated at different temperatures. TNFR1 expression without DsbC on the left of the marker and with DsbC on the right of the marker.....	39
Figure 3.9: Ni-NTA affinity chromatography result. The collected elution fractions were shown in the red dashed circle. FT is Flow-through, CW is Column Wash.....	40
Figure 3.10: SDS-PAGE (12%) and immunoblotting results of the IMAC purification of DsbC-TNFR1. The elution fractions are indicated numerically. The collected fractions were shown in the orange box. FT is Flow-through, CW is Column Wash.	40
Figure 3.11: Anion exchange chromatography result. The collected elution fractions were shown in the red dashed circle. FT is Flow-through, CW is Column Wash.....	41
Figure 3.12: SDS-PAGE (12%) and immunoblotting results of the AEX purification of DsbC-TNFR1. The elution fractions are indicated numerically. The collected fractions were shown in the orange box. FT is Flow-through, CW is Column Wash.	41
Figure 3.13: The circular dichroism spectra of DsbC-TNFR1 without urea (green), with urea (pink) and comparison.	42
Figure 3.14: Electropherograms of the purity analysis of fusion protein. The results of the purity analysis electropherograms A. belongs to affinity purification and B. belongs to the AEX purification.	43
Figure 3.15: The results of the cIEF analysis of fusion protein.	44
Figure 3.16: LC/MS spectrum of DsbC-TNFR1 (left) and the deconvoluted mass spectra of peaks corresponds to DsbC-TNFR1 peak (right).....	44
Figure 3.17: BN-PAGE result of the native DsbC TNFR1 fusion protein.....	45
Figure 3.18: The immunoblotting results of a pull-down assay using purified DsbC-TNFR1 (bait) and commercial TNF- α (prey) protein. Nickel bound agarose beads were incubated with pure DsbC-TNFR1 (load lane). Following bead washing, pure TNF- α was loaded onto the bead and incubated. After incubation, unbound ligand was discarded via centrifugation (flow-through). After washing, bead-bound proteins were eluted with elution buffer containing imidazole (elution lane). In addition, ligand protein was applied to the unbound blank bead (ligand only), and it was checked whether the ligand was bound non-specifically.	46
Figure A.1: The DsbC was adopted from the crystal structure with PDB id 1EXT.	58
Figure B.1: The extracellular conformation of TNFR1 was adopted from the crystal structure with PDB id 1EXT.....	59
Figure C.1: Replicate of purification of DsbC-TNFR1. SDS-PAGE (12%) and immunoblotting results of the AEX purification of DsbC-TNFR1. The elution fractions are indicated numerically. The collected fractions were shown. FT is Flow-through, CW is Column Wash.	59
Figure C.2: Replicate of purification of DsbC-TNFR1. SDS-PAGE (12%) and immunoblotting results of the AEX purification of DsbC-TNFR1. The elution fractions are indicated numerically. The collected fractions were shown. FT is Flow-through, CW is Column Wash.	60
Figure C.3: Replicate of capillary electrophoresis purity analysis of DsbC-TNFR1. Electropherograms belongs to two step purification.	61

Figure C.4: Replicate of intact mass analysis of DsbC-TNFR1 protein. LC/MS spectrum of DsbC-TNFR1 (left) and the deconvoluted mass spectra of peaks corresponds to DsbC-TNFR1 peak (right). 61

Figure C.5: Replicate of *in-vitro* pull down assay. The immunoblotting outcome of a pull-down assay using purified DsbC-TNFR1 (bait) and commercial TNF- α (prey) protein. Nickel bound agarose beads were incubated with pure DsbC-TNFR1 (load lane). Then unbound proteins were removed (flow-through 1 lane) and the agarose beads were washed with binding buffer. Pure TNF- α were loaded onto the bead and incubated. After incubation, unbound ligand was discarded via centrifugation (flow-through 2 lane) and the bead washed with binding buffer (wash lane). After washing, bead-bound proteins were eluted with elution buffer containing imidazole (elution lane). 62





EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF SOLUBLE RECOMBINANT TNFR1

SUMMARY

Tumor Necrosis Factor Alpha (TNF- α) is a trimeric cytokine secreted by macrophages and monocytes. It belongs to type II transmembrane protein family and is involved in both innate and adaptive immune system. TNF- α exists in two forms: transmembrane TNF (tmTNF), which is synthesized as a precursor form, and solubilized TNF (sTNF), which is created after further processing. TNF- α functions in a variety of biological processes by interacting with specific receptors namely as TNFR1 and TNFR2.

TNFR1 consists of intracellular, transmembrane, and extracellular domains. The extracellular part consists of four cysteine-rich parts. When interacting to TNFR1, TNF- α can activate various signaling pathways for instance inflammation and apoptosis.

TNF- α level is undetectable in normal individuals, yet under inflammation conditions the protein concentration in serum increases proportionally to the inflammation level in the body, thus making the protein detectable. Controlled production of TNF- α is crucial for tissue healing and fight against infection, but a constantly high level of TNF- α can cause various diseases including rheumatoid arthritis (RA), ankylosing spondylitis (AS), psoriasis/psoriatic arthritis, and Chron's disease.

TNF- α inhibitors are designed and used in treatment of diseases caused by overexpression of TNF- α . An alternative pathway includes employment of TNFR1 extracellular domain as a biotherapeutic tool to inhibit the effects of overexpressed TNF- α . The latter approach seems to be way more preferred than other therapeutics due to its targetselectivity and high affinity.

Production of proteins like TNFR1 are characterized by cysteine-rich domains by using bacterial systems is quite advantageous in terms of cost, yield, and time. However, lack of an effective translation system in *Escherichia coli* (*E. coli*) may hinder production of such disulfide bond containing proteins mainly in terms of generating mismatched cysteine residues or failing in acquiring a sulfide bridge formation thus leading to protein aggregation and inclusion body formation. Recovering proteins from inclusion bodies is time-consuming and expensive while considering the protein loss and incorrectly folded proteins due to the wrongly formed disulfide bonds.

Dsb protein family including DsbA, DsbB, DsbC, DsbD, and DsbG, is responsible for the formation of disulfide bonds in bacteria. The DsbA-DsbB complex plays role in the oxidative pathways, while DsbC-DsbD complex functions in the isomerization pathway. Among all Dsb proteins, DsbA and DsbC are the most extensively studied and widely used in the field of biotechnology.

Our previous studies showed that the extracellular portion of TNFR1 forms inclusion bodies in *E. coli*, and *in vitro* refolding steps are needed to obtain the correct

conformation. Protein aggregations during the refolding process also caused a decrease in yield.

The purpose of this study is to obtain the extracellular part of TNFR1 as a soluble protein in *E. coli*. Initially, a SHuffle T7 strain with a cytoplasmic DsbC copy was selected as the host cell and expression experiments were carried out accordingly. However, the target protein formed an inclusion body.

To assess the effect of DsbC in the correctly folded-soluble TNFR1 production, a expression vector containing fusion protein DsbC-TNFR1 was constructed. Formation of DsbC-TNFR1 fusion protein was held on the basis of plasmid design conferring the order of a 6x histidine tag, DsbC, TEV cleavage site, and TNFR1 from N to C terminus respectively. It was assured that periplasm target sequence was removed from DsbC gene, allowing its production within the bacterial cytoplasm.

DsbC-TNFR1 production was verified by SDS-PAGE and immunoblotting analyses, which revealed that some conditions led to the production of the fusion protein in soluble form. In order to detect the optimum conditions to produce DsbC-TNFR1, four different *E. coli* strains as host cells were employed, namely as Rosetta (DE3), Rosetta-gami 2, SHuffle T7, and BL21 (DE3). However, since production of the Rosetta (DE3) strain was higher than others, the research was conducted on that particular strain.

Induction conditions including IPTG induction and autoinduction medium were also assessed to get higher yield from the selected cells. As a result of induction trials, the target protein was obtained in soluble form when autoinduction was utilized. Following sonication and centrifugation of the cells, the affinity chromatography was performed for separation of the recombinant protein from other host cell proteins. Anion exchange chromatography (AEX) was used as a second purification step to remove remaining impurities. The efficiency of purification was evaluated by using SDS-PAGE and immunoblotting analyses. Then, protein characterization studies were performed.

The isoelectric point of the pure protein was calculated by isoelectric focusing via capillary electrophoresis device, and the purity of the protein was checked using the purity determination method. The secondary structure of the fusion protein was analyzed by circular dichroism spectroscopy and it was determined to be in the α -helix structure. Intact mass analysis through LC/MS was also performed to calculate the molecular weight of the protein. In addition, the peptide mapping was used to identify the amino acid sequence. The native structure of the pure protein was investigated by using blue native polyacrylamide gel electrophoresis (BN-PAGE), which revealed that the protein exists in both dimers and different oligomer structures. The functionality of the fusion protein was assessed by performing a pull-down assay, which showed that DsbC-TNFR1 is functional as it was capable of binding to TNFR1 ligand TNF- α .

As a consequence of the research, it was revealed that the implementation of such a fusion protein is a successful tool for expressing TNFR1 in its soluble form in bacterial cells. After analysis of the pure protein generated as the outcome of downstream steps, it was shown that the TNFR1 produced by using the implemented fusion method is functional.

ÇÖZÜNÜR REKOMBİNANT TNFR1'İN ÜRETİMİ, SAFLAŞTIRILMASI VE KARAKTERİZASYONU

ÖZET

Tümör Nekroz Faktör Alfa (TNF- α), makrofaj ve monositler tarafından salınan trimerik bir sitokindir. Tip II transmembran protein ailesinden olup doğuştan ve adaptif bağışıklıkta görev alır. TNF- α 'nın çözünür ve transmembran olmak üzere iki formu bulunmaktadır. Transmembran TNF (tmTNF) öncül form olarak sentezlenmektedir ve tmTNF işlenerek çözünür (sTNF) hale getirilir. TNF- α , biyolojik işlevlerinin birçoğunu spesifik reseptörlerle (TNFR1, TNFR2) etkileşerek gerçekleştirir.

TNFR1, intraselüler, transmembran ve ekstraselüler olmak üzere üç bölümden oluşmaktadır. Ekstraselüler kısmı ise sistince zengin dört bölümden oluşur. TNF- α , TNFR1'in ekstraselüler kısmına bağlanarak inflamasyon, apoptoz gibi çeşitli sinyal yollarını aktive edebilmektedir.

Normalde sağlıklı bireylerde TNF- α seviyesi tespit edilemezken, inflamasyon koşullarında serum ve dokuda artan TNF- α , tespit edilebilir duruma gelmektedir. Bununla birlikte TNF- α seviyesindeki artışla inflamasyon arasında korelasyon bulunmaktadır. TNF- α 'nın kontrollü üretimi doku iyileşmesi ve enfeksiyon ile mücadele için gerekli olmasına karşın TNF- α seviyesinin sürekli yüksek olması Romatoid artrit (RA), ankilozan spondilit (AS), psoriasis/psoriatik artrit, Chron hastalığı gibi çeşitli hastalıklara sebep olabilmektedir.

Son zamanlarda hedefe yönelik seçicilikleri ve yüksek afinitelerinden dolayı biyoterapötikler diğer terapötiklere nazaran daha çok tercih edilmektedir. Hücre içerisinde hayati rollere sahip olan TNF- α 'nın aşırı ekspresyonu sonucu oluşan hastalıkların tedavisi için TNF- α inhibitörleri tasarlanmakta ve tedavide kullanılmaktadır. Ayrıca, aşırı eksprese edilen TNF- α 'nın etkilerinin engellenmesi için TNFR1'in ekstraselüler domaini biyoterapötik bir araç olarak kullanılmaktadır.

TNFR1'in ekstraselüler kısmı gibi biyoteknolojik değere sahip bu sistince zengin proteinlerin üretiminde bakteriyel sistemlerin kullanılması maliyet, üretim hacmi ve zaman açısından avantajlıdır. Ancak *E. coli*'de etkili bir translasyon sisteminin olmaması disülfid bağı içeren proteinlerin üretiminde engel teşkil etmektedir. Sistein kalıntılarının yanlış eşleşmesi ya da eşleşmemesinden dolayı proteinler agregre olarak inklüzyon cisimcikleri oluşturmaktadır. Inklüzyon cisimciklerinden protein geri kazanılması sırasında kayıplar yaşanmakta ve yeniden katlama sürecinde proteinin yapısında olmayan ya da yanlış oluşturulmuş disülfid bağ görülebilmektedir. Bu nedenle inklüzyon cisimciklerinden proteinin geri kazanım stratejileri zahmetli, zaman alıcı ve pahalı bir süreçtir.

Bakterilerde disülfid bağlarının oluşmasından Dsb protein ailesi sorumludur. Başlıca Dsb proteinleri: DsbA, DsbB, DsbC, DsbD ve DsbG'dir. Bakterilerde DsbA-DsbB oksidatif yolağı ve DsbC-DsbD izomerizasyon yolağı bir arada bulunur.

DsbA ve DsbC periplazmik proteinler olup DsbB ve DsbD ise transmembran proteinlerdir. Disülfid bağlarının oluşturulmasında DsbA görev alır. Çözünür ve yüksek oranda oksitleyici bir protein olan DsbA hızlı ama spesifik olmayan bir şekilde bağ kurduğu için yanlış katlanmış disülfid bağları oluşturabilmektedir. DsbB ise DsbA'nın uygun formda (oksitlenmiş formda) bulunmasını sağlamaktadır. DsbA tarafından kurulan yanlış disülfid bağlarının düzeltilmesinde DsbC sorumludur. DsbC bir izomeraz olup aynı zamanda şaperon aktivitesine sahiptir. DsbD ise DsbC'nin indirgenmiş formda tutulmasını sağlamaktadır. Dsb proteinleri arasında DsbA ve DsbC en kapsamlı şekilde incelenmekte ve biyoteknoloji alanında yaygın olarak kullanılmaktadır.

Önceki çalışmalarımız, TNFR1'in ekstraselüler kısmının *E. coli*'de inklüzyon cisimciği oluşturduğunu ve doğru konformasyonun elde edilmesi için *in vitro* olarak yeniden katlama adımlarına ihtiyaç olduğunu gösterdi. Yeniden katlama sürecinde gerçekleşen protein agregasyonları verimin düşmesine de neden olmuştur.

Bu çalışmada, TNFR1'in ekstraselüler kısmının *E. coli*'de çözünür formda elde edilmesi hedeflenmiştir. Bu amaç doğrultusunda öncelikle konak hücre olarak genomunda sitoplazmik DsbC kopyasına sahip SHuffle T7 suşu seçilmiş ve ekspresyon denemeleri yapılmıştır. Ancak hedef proteinin inklüzyon cisimciği oluşturduğu tespit edilmiştir.

Daha sonra TNFR1 ile DsbC füzyon proteini (DsbC-TNFR1) oluşturulmuştur. Füzyon proteini oluşturmak için tasarlanan plazmitte N ucundan C ucuna doğru sırasıyla 6X Histidin etiketi, DsbC TEV kesim bölgesi ve TNFR1 bulunmaktadır. Hedef proteini bakteri sitoplazmasında üretebilmek için DsbC geninden periplazma hedef sekansı çıkarılmıştır. Öncelikle DsbC dizisi içermeyen TNFR1 plazmitinin ve DsbC dizisi içeren TNFR1 plazmitinin ekspresyonu sağlanarak oluşturulan füzyonun işlevselliği kontrol edilmiştir. Ekspresyon seviyeleri SDS-PAGE ve immüno blotlama teknikleriyle kontrol edilmiş ve üretilen DsbC-TNFR1'in bir kısmının çözünür formda olduğu tespit edilmiştir. Daha sonra DsbC-TNFR1'in uygun formda üretilmesi için konak hücre seçimi yapılmıştır. Rosetta (DE3), Rosetta-gami 2, SHuffle T7, BL21 (DE3) ve C43 olmak üzere 5 farklı *E. coli* suşları kullanılarak proteinin ekspresyonu denenmiştir. Rosetta-gami ve C43 suşlarında yeterli miktarda ekspresyon görülebilmiştir. Rosetta (DE3) suşundaki üretim diğerlerine göre daha yüksek olduğu için Rosetta (DE3) ile çalışmalara devam edilmiştir. Protein ekspresyonunu kontrol etmek için başlangıçta IPTG kullanılmıştır. Ancak sonrasında sürecin optimizasyonu için farklı inkübasyon sıcaklıkları, inkübasyon süreleri farklı indüklenme yöntemleri (IPTG ya da oto indüksiyon) gibi parametreler denenerek en iyi ortam koşulu belirlenmiştir. Denemeler sonucunda otomatik indüksiyon metodu kullanılarak hedef protein çözünür formda elde edilmiştir. Sonikasyon ile hücreler patlatıldıktan sonra santrifüj yardımıyla supernatant kısım elde edilmiş ve konak hücrenin proteinlerinden rekombinant proteini ayırmak için afinite kromatografi yöntemi kullanılmıştır. Safsızlıkların giderilmesi için ikinci bir saflaştırma adımı olarak anyon değişim kromatografisi (AEX) yöntemi kullanılmıştır. Yapılan saflaştırmaların etkinliğini değerlendirmek için SDS-PAGE ve immüno blotlama metotları kullanılmıştır. Daha sonra kapiler elektroforez cihazı ile izoelektrik odaklama metodu kullanılarak saf proteinin izoelektrik noktası hesaplanmış ve saflık tayini metodu kullanılarak proteinin saflığı kontrol edilmiştir.

DsbC- TNFR1 füzyon proteinin ikincil yapısı, dairesel dikroizm spektroskopisi ile analiz edilmiştir.

Mavi doğal poliakrilamit jel elektroforezi (BN-PAGE) kullanılarak elde edilen saf proteinin doğal (nativ) yapısı incelenmiştir. Proteinin dimer ve farklı oligomer yapılarda bulunduğu görülmüştür.

LC/MS aracılığıyla intak kütle analizi yöntemi kullanılarak üretilen proteinin moleküler ağırlığı, peptit haritalama yöntemi kullanılarak amino asit sekansı belirlenmiştir.

Proteinin fonksiyonel olduğunu göstermek için aşağı çekme deneyi (pull-down assay) yapılmıştır. Saf olarak elde edilen protein, sahip olduğu polihistidin etiketi aracılığıyla nikel bağlı agaroz rezine bağlanmış ve reseptörün ligandı olan TNF- α rezine bağlı reseptörün üzerine eklenerek inkübe edilmiştir. İnkübasyon sonrasında yıkama ve elüsyon yapılarak fraksiyonlar toplanmıştır. Deneyin sonunda elde edilen fraksiyonlar immünoablota yöntemiyle analiz edilmiştir. Reseptör için Histidin antikoru, ligand için ise TNF- α antikoru kullanılmıştır. Bağlanma deneylerinin sonucunda reseptörün TNF- α 'ya bağlandığı görülmüştür.

Yapılan deneylerin sonucunda, oluşturulan füzyon proteinin bakteride çözünür formda TNFR1 ekspresyonunda başarılı olduğu görülmüştür. Gerçekleştirilen alt akım prosesleri sonucunda elde edilen saf proteinin karakterizasyonu yapılmış ve TNFR1'in fonksiyonel olduğu belirlenmiştir.



1. INTRODUCTION

1.1 Tumor Necrosis Factor-Alpha

Tumor necrosis factor-alpha (TNF- α) is a member of the type II transmembrane protein family and it has an important role in innate and adaptive immunity (Roman Fischer). TNF- α is one of the most important and well-known cytokines in the TNF superfamily (Fischer et al., 2015).

The TNF- α protein exists in two different forms such as soluble and transmembrane forms (Black et al., 1997). The transmembrane TNF, abbreviated as tmTNF, is initially synthesized in a precursor form and has a size of 26 kDa (Black et al., 1997; Kriegler et al., 1988). The ectodomain part of the tmTNF is cut by TNF- α converting enzyme (TACE or ADAM17) in order to dissociate it from the membrane, which leads the formation of 17 kDa soluble TNF (sTNF). In trimeric structure, both tmTNF and sTNF exhibit biologically active properties (Luettig et al., 1989). Additionally, the similar aromatic components of TNF- α monomers being responsible for trimer structure formation (Locksley et al., 2001).

TNF- α regulates the immune function via its interaction with TNFR1 play a role in the inflammatory response (G. Chen & Goeddel, 2002). The expression of TNF- α is induced by immune cells called macrophages, T-lymphocytes and natural killer cells (NK cells) that are activated in response to tissue damage and infection (Xu et al., 2007). Moreover, TNF- α is a potential inflammation promoter as it triggers various inflammatory molecules including cytokines and chemokines after they are induced by immune cells (Wajant et al., 2003). Recruitment of immune cells into the inflamed tissues is facilitated by TNF- α -induced chemokines. TNF- α has the ability of activating NF- κ B pathway, and thus, it causes the transformation of immature dendritic cells into mature antigen presenting cells. Therefore, TNF- α plays a crucial role in the initiation of antigen-specific B and T cell responses and, moreover, it plays a role in differentiation of macrophages and trigger tumor cells apoptosis (G. Chen &

Goeddel, 2002). Whenever immune reaction must get terminated, TNF- α increases secretion of anti-inflammatories, and thus reduces the inflammation (Boissier et al., 2012).

The controlled-production of TNF is mandatory both for tissue healing processes and for fighting against the infection. Constant high levels of TNF- α may cause multi-organ failure and various diseases including sepsis and fever syndromes, autoimmune diseases as well as chronic inflammation (Hehlhans & Pfeffer, 2005). For instance, in autoimmune patients TNF- α was found to be overproduced by their mast cells, macrophages, monocytes, and fibroblasts (Grivennikov et al., 2005).

TNF- α provides the signal transduction by interacting with two different cell surface receptors named TNFR1 and TNFR2. The role of TNFR1 in the initiation of most of the biological activities was revealed previously (Guoqing Chen-2002). New therapies are being developed for the treatment of autoimmune diseases by elucidating the pathway of TNF (Guoqing Chen-2002).

1.2 Tumor Necrosis Factor Receptor Superfamily: TNFR1-TNFR2

TNF-TNFR pathway is responsible for numerous biological processes including cell proliferation, cell differentiation, cell survival and apoptosis. Thus it is crucial for generating a host defence mechanism by triggering immune responses in the case of the inflammation (G. Chen & Goeddel, 2002; Hehlhans & Pfeffer, 2005).

Belonging to type I transmembrane protein family, TNFRs are divided into three groups according to their cytoplasmic signal properties and sequence motifs. The first group of TNFRs consists of the proteins that contain death domain, including TNFR1. The second group of TNFRs includes TRAF binding sites in protein's cytoplasmic ends. The last group of TNFRs consists of receptors that do not have neither transmembrane nor cytoplasmic domains (Cheung & Ware, 2013).

The extracellular domains of TNFRs can also be divided into four cysteine rich domains (CRD) groups as CRD1, CRD2, CRD3, and CRD4 (Naismith et al., 1996). CRD1, also known as the pre-ligand assembly domain (PLAD), has a significant role in the initiation of TNF-TNFR signaling pathways due to PLAD portion's help into forming of the receptor complex (trimerization) for both TNFR1 and TNFR2;

characterized by a cysteine-rich extracellular region (Loetscher et al., 1990). These two also exhibit a cysteine-rich extracellular regions showing homologies between each other (Idriss & Naismith, 2000). The CRD2 and CRD3 domains are confiners of the ligand binding region of TNFR proteins (Chan et al., 2000). The extracellular portions of TNFRs are shown to be prone to aggregation. Although it has not been observed in dilute solution yet, TNFR1 has the ability of forming aggregates (Naismith et al., 1995).

The intracellular regions of TNFR1 and TNFR2 do not share homology indicating that they would be activating strikingly different pathways in cells (Idriss & Naismith, 2000; Puimège et al., 2014). TNFR2 also known as TNFRSF1B, CD120b or p75, is induced by tmTNF and expressed in immune cells such as endothelial, lymphocytes and mesenchymal stem cells (Locksley et al., 2001; Vandenabeele et al., 1995).

TNFR1, also denoted as TNFRSF1A, CD120a or p55, is found to be expressed in almost all cells (Black et al., 1997). TNFR1 is essential for the induction of proinflammatory and cytotoxic TNF responses (Jang et al., 2021). Regulation of TNFR1's cytokines and chemokines are induced by both tmTNF and sTNF, and this regulation as a whole can activate further signaling pathways where the most prominent would be the migration of neutrophils. Another feature is related to TNFR1 is having a death domain which allows it to trigger cytotoxic signaling (Locksley et al., 2001).

Remarkable properties of TNFR2 include that it does not contain a death domain, so it cannot directly trigger programmed cell death (apoptosis) yet, it can directly interact with TRAF1 (Wajant et al., 2003). TNFR2 recruits TRAF2, which is followed by the recruitments of TRAF1, cIAP1 and cAIP2 to form a complex providing the activation of NF- κ B, MAPKs and AKT. Additionally, TNFR2 is involved in processes including cell survival, proliferation, regeneration, and host defense (Brenner et al., 2015).

TNF can bind to TNFR1 and TNFR2 receptors with high affinity which may caused by being type 1 transmembrane receptors, nonetheless, most biological roles of TNF are mediated through TNFR1 (G. Chen & Goeddel, 2002; Puimège et al., 2014). The interaction of TNF α and TNFR1 enhances the inflammation, while TNF α -TNFR2 interaction maintains the homeostasis via regulating inflammation (David J. MacEwan, 2002).

Intracellular molecules involved in the signaling of TNF-TNFR1 pathway include SODD, TRAF2 (TNF receptor associated factor 2), FADD (Fas associated death domain), TRADD (tumor necrosis factor receptor associated death domain) and RIP (receptor interacting protein)(Cheung & Ware, 2013) (Figure 1.1).

Death domain (DD) or TRAF molecules are associated with TNFR signaling, whereas SODD was shown to bind to the intracytoplasmic part and function as an inhibitor of TNFR signals (Cheung & Ware, 2013; Hawari et al., 2004).

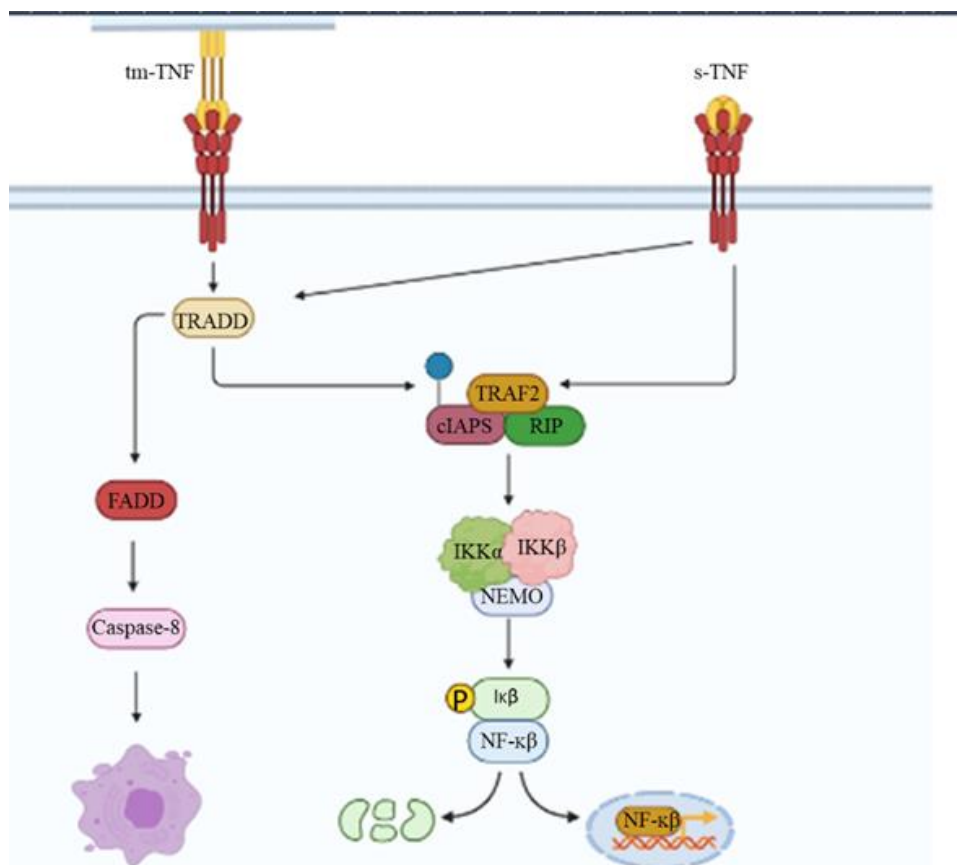


Figure 1.1: Mechanism of TNF- α /TNFR1 signaling pathway (Adapted from G. Chen & Goeddel, 2002).

The signaling process is initiated by association of TNF trimer with the extracellular portion of two or three TNFR receptors (Naismith et al., 1996). Figure 1.1 demonstrates the mechanism of TNF- α /TNFR1 signaling pathway. Firstly, SODD is released from the intracellular domain (ICD) of TNF-R1 (Hawari et al., 2004), and afterwards TNF-R1 ICD gets recognized by TRADD, so it recruits the binding of various adapter proteins like RIP, TRAF2, FADD according to the signal (G. Chen & Goeddel, 2002).

Thus, signal transduction occurs by the action of cIAPs, TRAF2, RIP1, I κ B α kinase (IKK) complexes (Walczak, 2011). TRAF2 which mediates TNFR signal transduction pathways, recruits cIAP-1 and cIAP-2 which are two antiapoptotic proteins that having activity of ubiquitin protein ligases (G. Chen & Goeddel, 2002).

Two important pathways are activated when TNF binds to TNFR. One of which activates NF- κ B transcription factor that induces the expression of anti-apoptotic genes. RIP protein kinase is critical for the activation of the NF- κ B (G. Chen & Goeddel, 2002). This pathway plays a prominent role in the initiation of antigen-specific B and T cell responses via transformation of the immature dendritic cells into mature antigen presenting cells (X. Chen et al., 2007).

Programmed cell death exhibited in the form of apoptosis and necrosis is the second important outcome of TNF to TNFR binding (G. Chen & Goeddel, 2002; Wajant et al., 2003). It is mandatory to block NF- κ B pathway for TNF dependent induction of the cell death, which is maintained by activating the anti-apoptotic pathway by inducing the expression of anti-apoptotic proteins including cFLIP, cIAP1, cIAP2, XIAP, A20 and TRAF(Wajant et al., 2003).

The IKK complex consisting of an NF- κ B key modulator (NEMO or IKKg) and two catalytic subunits denoted as IKK α and IKK β is a fundamental element within the NF- κ B pathway. The activation of NF- κ B itself, activates the synthesis of I κ B and other inhibitory molecules like cIAPs. Furthermore, NF- κ B activation influences the duration of TNF signals (G. Chen & Goeddel, 2002).

The TRADD-FADD mechanism trigger apoptosis by activating the caspase-dependent pathway (G. Chen & Goeddel, 2002; David J. MacEwan, 2002). It is important to highlight that such signaling pathways have become attractive targets for the treatment of many diseases owed to significant roles in cell signaling (Bremer, 2013).

On the other hand, the soluble versions of TNFR1 and TNFR2, which have been identified as the result of shedding or alternative splicing, inhibit TNF (Wajant & Siegmund, 2019).

1.3 Disulfide Bond Formation in *E. coli*

The most fundamental feature related to proper protein folding and stability of multimeric proteins is attributed to covalent disulfide bridge formation between

cysteine amino acids (Wiedemann et al., 2020). Disulfide bonds providing a vast variety of biological roles related to protein's stability, folding, signaling, and activity are considered as the most prevalent protein post-translational modifications (Bardwell et al., 1991; Berkmen, 2012). Covalent disulfide bonds can modify the structure of the proteins depending on the environment due to their ability of quickly reduced and/or oxidized. Yet, the role of disulfide bonds differs depending on whether they have structural, catalytic, or signaling functions (Berkmen, 2012).

Disulfide bridges provides proteins strength in their stability upon some external stimuli. Formation of catalytic disulfide bonds includes redox reactions (Berkmen, 2012) (Figure 1.2). Formation of a disulfide bridge leads the releasing of two electrons from the protein (oxidation), while two electrons are gained during bond breakdown (reduction) (Bardwell et al., 1991). By limiting the number of conformations, structural disulfide linkages improve the thermostability of the proteins.



Figure 1.2: Disulfide bond formation.

Thus, it can be stated that presence of correctly assigned disulfide bonds are essential for proper functioning of proteins (Guo et al., 2004). Aggregation, misfolding, and dysfunction are caused by incorrectly created disulfide bonds, which should be repaired (Zhang et al., 2011). Hence, the identification and repair of malformed S-S bonds is a crucial quality control mechanism for oxidative protein folding (Merdanovic et al., 2011).

To create a bond between two thiols, an electron acceptor or oxidation is necessary. In the presence of a suitable electron acceptor, the disulfide bond can be formed spontaneously *in vitro* by donating electrons from two cysteine-thiols because the thiol-disulfide exchange process is the most prevalent mechanism for disulfide bond production *in vivo*. The enzymes responsible for catalyzing such reaction are of thiol

disulfide oxidoreductases family, which can create and breakdown the bonds by replacing the sulfur of the disulfide bond with the thiolate anion (S^-) produced by deprotonation of the free thiol (Figure 1.3) (Sevier & Kaiser, 2002). Moreover, temporary mixed bonds can occur between two proteins or between a protein and a redox molecule. The residual thiolate anion assaults the mixed bond and breaks it down. As one gets reduced, the other would get concurrently oxidized within the process (Sevier & Kaiser, 2002).



Figure 1.3: Protein thiol-disulfide exchange reaction (Adapted from Sevier & Kaiser, 2002).

The C-X-X-C motif found in thioredoxin loops of all oxidoreductases' active sites is critical to keep cysteines in this area in the right redox state and thus allow them to operate properly (Rietsch & Beckwith, 1998).

In eukaryotes, the natural disulfide bonds are formed in the endoplasmic reticulum and mitochondria, whereas in prokaryotes, they are formed in the periplasm (Missiakas et al., 1994; Rietsch & Beckwith, 1998). In the case of gram-negative bacteria for instance *E. coli*, apart from the fact that the peptidoglycan rich periplasm would be maintaining the cell structure and protecting the cell from osmotic structure, it would also aid in the production of disulfide bonds by thus providing an oxidizing environment (Merdanovic et al., 2011).

On the other hand, due to the reducing environment, bacterial cytoplasm is not suitable for disulfide bond formation. In cytoplasm of *E. coli*, which has two NADPH-dependent disulfide reduction pathways: glutathione reductase and thioredoxin reductase (Figure 1.4) (Gleason & Holmgren, 1988; Hatahet et al., 2010). Bond reduction is inhibited when these pathways are blocked. Mutations in these pathways have been carried out in order to enable suitable disulfide bond formation in bacteria and have developed new strains (Hatahet, 2010; Kurokawa, 2000).

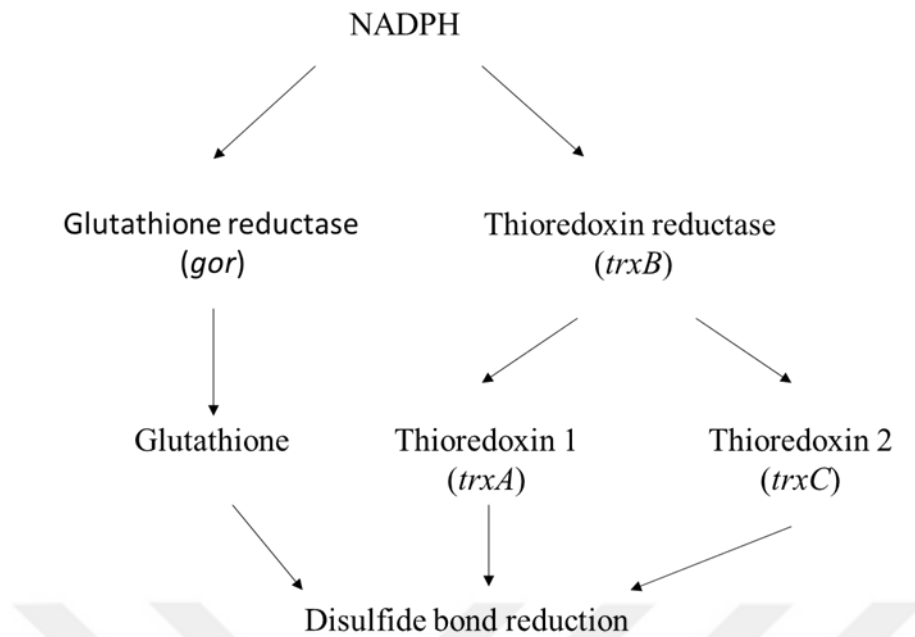


Figure 1.4: Disulfide bond reduction mechanisms in *E. coli* cytoplasm (Adapted from Hatahet et al., 2010).

There are approximately 300 proteins in bacteria that have disulfide bonds such as MepA, RNase1, AppA, and End1, that are being transported to the periplasm, which acquire the potential to become oxidoreductase substrates. (Hatahet et al., 2010; Kadokura & Beckwith, 2010).

Bond formation and isomerization are the two mechanisms for disulfide bond catalysis in *E. coli*. This process is aided by Dsb proteins (Figure 1.5). Isomerization activity may be necessary since the bond formation is fast and non-specific (Haebel et al., 2002; Zhuo et al., 2014).

In proteins having more than one disulfide link or more than two cysteines, the disulfide bond can be produced in a variety of ways (Berkmen, 2012; Kurokawa et al., 2000). DsbA can produce aberrant disulfide bonds during the folding of proteins containing a lot of cysteines in the periplasm. Therefore, proteins with complicated disulfide bond patterns are more likely to generate abnormal disulfide bonds, necessitating DsbC-dependent isomerization for proper folding (Kurokawa et al., 2000).

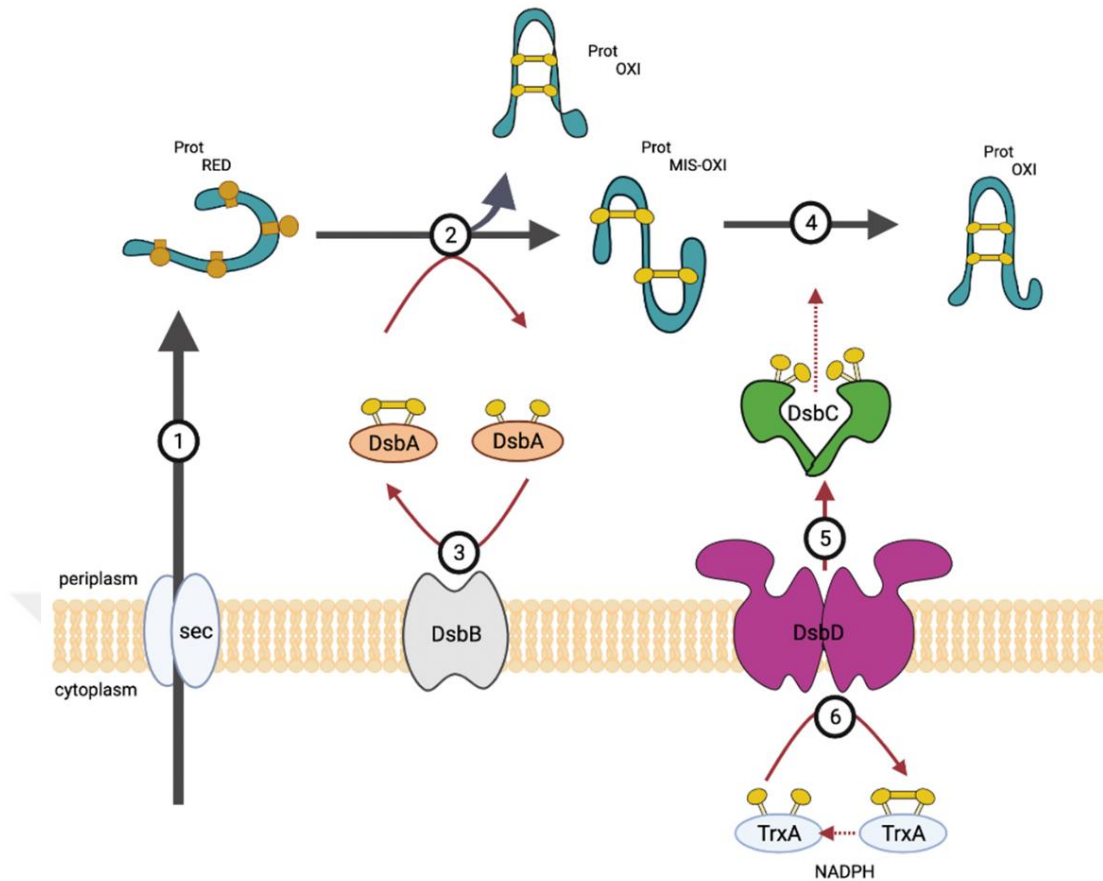


Figure 1.5: Periplasmic disulfide bond formation. The sec route is used to transport the protein to be folded to the periplasm. DsbA creates a disulfide linked complex with its reduced substrate, causing a disulfide bond to form in the substrate protein and cysteine reduction in DsbA's active region. The inner membrane protein DsbB reoxidizes DsbA via giving electrons. If DsbA misoxidizes the substrate protein, DsbC recognizes the misfolded protein and either isomerizes it to its native state or reduces it. The inner membrane protein DsbD keeps DsbC in an active decreased state. (Generated with Biorender and adapted from Berkmen, 2012).

On the other hand, false bond formation is common, especially when the disulfide bonds in the protein are non-consecutive. DsbC activity is not required for proteins with consecutive disulfide bonds, but it is required for proteins with non-consecutive links. This is what makes disulfide bond isomerization critical for non-consecutive bonds (Berkmen, 2012).

All known Dsb proteins involve in the Cys-X-X-Cys sequence characteristic of the thioredoxin superfamily (Bessette et al., 1999).

1.4 Dsb Proteins

1.4.1 DsbA

DsbA refers to a strong oxidant monomeric periplasmic protein with a C-X-X-C catalytic site at its N terminus (Bessette et al., 1999; Martin et al., 1993). The protein catalyzes the production of new disulfide bonds by transferring its disulfide link to proteins being released into the periplasm (Bessette et al., 1999; Kadokura & Beckwith, 2010). The substrate protein's deprotonated cysteine attacks DsbA's bound cysteine (Cys 30) to produce an unstable complex. The substrate's other cysteine attacks the formed DsbA-substrate protein complex and reduces DsbA by forming a disulfide bond inside of the substrate (Kadokura & Beckwith, 2010).

DsbA can undergo self-reduction as it would be simultaneously oxidizing its substrate protein. Then, the protein restores its active, oxidized state through the action of DsbB (Bessette et al., 1999). Electron flow starts from DsbA to DsbB, and then the electrons can be received by ubiquinone/menaquinone (Kadokura et al., 2003).

DsbA has a propensity to oxidize cysteines sequentially because it can catalyze disulfide bond formation quickly. However, if a protein's structure has non-consecutive disulfide links, it is more likely to misfold or undergo destruction by periplasmic proteases (Berkmen, 2012).

It has been shown that DsbA has the potential of naturally catalyzing production of disulfide bonds even in proteins that do not have any disulfide bonds in their structure. Hence, when the normally reduced β -lactamase CcrA from *Bacteroides fragilis* was expressed in the *E. coli*'s periplasm, the β -lactamase cysteine amino acids were oxidized under the presence of DsbA and eventually inactivate it (Alksne et al., 1995).

Cysteine oxidation via DsbA occurs really quickly making the process error prone and subjected to incorrect disulfide linkages that require further adjustments (Kadokura et al., 2003). Such feature is not considered as problematic when the number of disulfide bonds is minimal, yet, as the number of bonds grows, presence and activity of DsbC becomes critical for correct disulfide bond formation (Zhuo et al., 2014).

1.4.2 DsbB

DsbB is a transmembrane protein with a molecular weight of 20 kDa having four transmembrane and two periplasmic regions (Jander et al., 1994; Rietsch & Beckwith,

1998). The protein is involved in the reactivation of DsbA by oxidizing the reduced form of DsbA. It was reported that accumulation of reduced DsbA is possible in the presence of DsbB nonfunctional mutants (Debarbieux et al., 2021).

Cys130 of reduced DsbA interacts with Cys104 of DsbB, leading to a complex formation that is maintained by oxidation of the Cys40-Cys41 disulfide link by preventing DsbA from being re-reduced. Electrons flow from DsbA to the Cys130-Cys104 pair, to Cys40-Cys41 pair, and finally to quinone. When both Cys40-Cys41 and Cys104-Cys130 are oxidized, a disulfide bond formation would be observed (Kadokura & Beckwith, 2002).

1.4.3 DsbC

This protein is characterized by a thioredoxin site located at the C terminus on each of its two monomers. A dimerization area located at the N-termini of each subunit, is responsible for the V-shaped structure of DsbC where monomers are shown to be joined via an α -helix structure (McCarthy et al., 2000). Each V-arm has a thioredoxin solid dimerization area with the inside of such structure having hydrophobic characteristics (J. Chen et al., 1999; Heras et al., 2007).

There are four cysteines located on the thioredoxin domain of DsbC, two of which found in the active site of the protein (Cys98-Cys101), whereas the other two are found on Cys141 and Cys163 loci. Also, the disulfide linkage between cysteines on C-termini is crucial for the overall stability of DsbC (J. Chen et al., 1999; Rietsch et al., 1997). This protein retains its reduced state by the action of DsbD that can keep the cysteines reduced as them being part of CxxC motif (Missiakas et al., 1995).

DsbC is required for the proper folding of several disulfide-containing proteins (Rietsch et al., 1996). The protein has been identified as a counterpart of eukaryotic PDI, and it can catalyze the rearrangement of natural disulfide bonds that have been initially produced by the action of DsbA (Joly & Swartz, 1997). Its reductase functionality aids in the proper folding, stability, and activity of proteins by repairing wrongly generated disulfide bonds. Additionally, it allows isomerization of non-consecutive disulfide bonds (Berkmen et al., 2005).

The dimeric structure of DsbC not only confers for the protein's functionality but it is as well related to the ability of DsbD for reducing it. The same V-shaped dimeric structure is what shields DsbC from the DsbB oxidizing activity. Hence, only a mutant

form of DsbC that prevent dimer formation has shown to be oxidized by DsbB (Kadokura et al., 2003).

As a mode of action, a misfolded disulfide link is attacked by Cys98 of DsbC, which then forms a transient complex with the substrate. It is not well known whether DsbC restores the substrate to the correct form by isomerization or by a redox reaction, however, the active site of DsbC must be reduced in both circumstances (Kadokura et al., 2003).

DsbD must reduce oxidized DsbC before it gets revived, and the whole process is aided by the presence of thioredoxin and thioredoxin reductase. As in DsbD null mutants, *trxA* null mutants will also exhibit an increase in the amount of oxidized DsbC resulting in protein folding problems. In DsbC null mutants, the quantity of misfolded protein increases (Rietsch et al., 1997).

DsbC also serves as a chaperone (J. Chen et al., 1999). The hydrophobic area in the V region is hypothesized to interact with unfolded proteins. Yet, chaperone activity may be inhibited by mutations in the dimerization area, however, even as a monomer DsbC can still preserve its isomerase activity. (Bader et al., 2001).

In vivo experiments indicate that DsbC overexpression can significantly increase the amount of properly folded proteins located on the cytoplasm and periplasm. This is because DsbC acting as an oxido-reductase chaperone with the ability of accelerating the oxidative folding of proteins (Lobstein-2012). Also, when used for co-expression isomerase DsbC can indeed preserve its chaperonening activity (Bader et al., 2001; Joly et al., 1998; Nozach et al., 2013).

1.4.4 DsbD

DsbD (59 kDa) comprises three domains: an immunoglobulin-like N-terminal periplasmic domain (DsbD α), an eight-transmembrane-segmented hydrophobic core domain (DsbD β), and a thioredoxin-like C-terminal periplasmic domain (DsbD γ). Each domain contains a pair of cysteines that are required for the activity of the protein (Stewart et al., 1999). Electrons are transferred from NADPH in the cytoplasm to periplasmic dithiol/disulfide-containing proteins in the *E. coli* DsbD system via an electron transfer chain that includes NADPH, thioredoxin reductase (TrxB), thioredoxin (TrxA), DsbD, and the periplasmic electron acceptors in the *E. coli* DsbD system. DsbD transfers electrons via reducing and oxidizing its three structural

domains sequentially and so reduces the oxidized DsbC (Collet et al., 2002; Stewart et al., 1999).

1.5 Aim of The Study

The Tumor Necrosis Factor Receptor Superfamily (TNFRs) is a family of transmembrane glycoproteins that plays a key role in signalling pathways such as host defense, inflammation, apoptosis, and autoimmunity. When TNF molecules, which are cytokines that bind to TNFR1, the signalling process is activated, demonstrating the necessity of the TNF-TNFR interaction for cell homeostasis. Increased TNF levels are correlated to a variety of illnesses. For that reason, TNF receptors and antibody-based drugs have been developed to neutralize TNF. Soluble TNFRs, in particular, are important regulators of TNF bioactivity. Due to the extracellular domain of TNFR1 has a cysteine-rich region, generating a soluble form of the protein in bacterial cells is challenging.

Previous research has shown that the extracellular region of TNFR1 generates inclusion bodies in *E. coli*, are required *in vitro* refolding processes to achieve the proper conformation. The yield after the refolding was also reduced due to protein aggregation.

The aim of this study is to produce the extracellular domain of TNFR 1 (hereafter TNFR1) in bacteria-soluble form as it is potential biotechnological product.

To achieve this goal, a vector containing fusion protein DsbC- TNFR1 was constructed to be produced in bacterial systems. The target protein was purified using the immobilized metal affinity chromatography (IMAC) method, and the anion exchange chromatography technique. The purified protein was characterized by analysis of secondary structure, purity, isoelectric point and functionality.



2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Table 2.1 indicates all the laboratory equipments used in this study.

Table 2.1: 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
Capillary Electrophoresis	Beckman Coulter PA 800 Plus
Centrifuge	NUVE NF 1200R
Centrifuge	VWR Mega Star 3.0R
Chromatographic System	GE Healthcare Äkta Avant25
Circular Dichroism	Jasco J-1500
Deepfreeze (-20°C)	Bosch
Electrophoresis Power Supply	Bio-Rad
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+
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
Minispin	Weightlab WN-CMV6000
Orbital Shaker	Lab Companion™ ISF-7200
pH Meter	Mettler Toledo
Platform Shaker	DLAB SK-R1807-E
Refrigerator (+4°C)	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

2.1.2 Chemicals

Table 2.2 indicates all the chemicals used in this study.

Table 2.2: Used chemicals in this study.

Chemical	Supplier Company
A-lactose	Biolife
Acetic Acid	Sigma-Aldrich
Acetonitrile	Merck
Acrylamide/Bis-Acrylamide (40%)	Bio-Rad
Agar Bacteriological Grade	MultiCell
Agarose Molecular Biology Grade	MultiCell
Ammonium Bicarbonate (AMBIC)	Merck
Ammonium Persulfate (APS)	Sigma-Aldrich
Ammonium Sulfate	Sigma-Aldrich
Blotto, Non-Fat Dry Milk	Santa Cruz
Bromophenol Blue	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Calcium Chloride Anhydrous	Merck
Dimethyl Sulfoxide (DMSO)	BioMatik
Disodium Phosphate	Merck
Dithiothreitol (DTT)	Fermentas
Ethanol Absolute	Merck
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich
Formic Acid	Merck
Glucose	Sigma-Aldrich
Glycine	Chem Cruz
Glycerol	MultiCell
Hydrochloric Acid (HCl)	Sigma-Aldrich
Imidazole	Sigma-Aldrich
Iodoacetamide	Bio-Rad
Isopropanol	Merck
Kanamycin	Sigma-Aldrich
Methanol	Merck
Magnesium Chloride Hexahydrate	Merck
Magnesium Sulfate	Merck
NcoI	NEB
NdeI	NEB
Nitrocellulose Membrane (0.45µm)	GVS
Peptone from Casein Pancreatically Digested	Merck
Potassium Chloride	Merck
Potassium Dihydrogen Phosphate	Merck
Prestained Protein Ladder (PageRuler)	Thermo Fisher
Pipes	Biolife
PVDF Membrane	GVS
Q5 High-Fidelity 2X Master Mix	NEB
EzView	Biomatik
Sodium Chloride (NaCl)	Chem Cruz
Sodium Dihydrogen Phosphate Dihydrate	Merck

Table 2.2 (continued): Used chemicals in this study

Chemicals	Supplier Company
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich
Sodium Hydroxide (NaOH)	MultiCell
Taq 2X Master Mix	NEB
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Triton X-100	Biomatik
Tris Base	MultiCell
Trypsin-EDTA (1X)	Thermo Fisher
Trypsin from Porcine Pancreas	Sigma-Aldrich
Tween-20	BioShop
Urea	Sigma-Aldrich
Yeast Extract	Pronadisa
2-Mercaptoethanol	Sigma-Aldrich
10X CutSmart Buffer	NEB
6X Loading Dye Solution	Intron Biotech.
100 bp DNA Ladder	Invitrogen
1 kb DNA Ladder	NEB

2.1.3 Molecular biology kits

Table 2.3 indicates all the molecular biology kits used in this study.

Table 2.3: Used molecular biology kits in this study.

Name of the Kit	Supplier Company
CE IgG Purity Kit	Sciex
Clarity Western ECL Substrate	Bio-Rad
DNA-midi Plasmid DNA Purification Kit	Intron Biotech.
DNA-mini Plasmid DNA Purification Kit	Intron Biotech.
NucleoSpin Gel and PCR Clean-up Kit	Macherey-Nagel
NucleoSpin Plasmid Kit	Macherey-Nagel

2.1.4 Antibodies

Table 2.4 indicates all the antibodies used in this study.

Table 2.4: Used antibodies in this study.

Antibody	Type	Dilution
Anti-His (H-3) (SC-8036)	Monoclonal Mouse	1:2000 (5% BSA)
Anti-Mouse IgG (CST, 7076S)	Polyclonal HRP-linked	1:3000 (5% Milk)
Anti- TNF α (C4) (SC-133192)	Monoclonal Mouse	1:2000 (5% BSA)

2.1.5 Buffers and solutions

2.1.5.1 Luria bertani (LB) medium

For the Luria Bertani (LB) media, 10 g Bacterial Peptone, 5 g Yeast Extract, and 10 g NaCl were dissolved in 1 L of dH₂O.

2.1.5.2 Luria bertani (LB) agar medium

For the Luria Bertani (LB) media, 10 g Bacterial Peptone, 5 g Yeast Extract, 15 g Agar, and 10 g NaCl were dissolved in 1 L of dH₂O.

2.1.5.3 ZY medium solution

For the ZY medium solution, 10 g Bacterial Peptone, and 5 g Yeast Extract were dissolved in 930 mL of dH₂O.

2.1.5.4 50X 5052 solution

For the 50X 5052 solution, 1.25 g Glucose, 5 g α-lactose, and 12.5 g Glycerol were dissolved in 50 mL of dH₂O.

2.1.5.5 20X NPS solution

For the 20X NPS solution, 6.6 g Ammonium Sulfate, 13.6 g Potassium Dihydrogen Phosphate, and 14.2 g Disodium Phosphate were dissolved in 100 mL of dH₂O.

2.1.5.6 ZYP-5052 autoinduction medium

For the ZYP-5052 medium, 50 mL 20X NPS, 20 mL 50X 5052, 930 mL ZY, and 1 mL MgSO₄ solutions were mixed.

2.1.5.7 10X tris-buffered saline (TBS) buffer

For the 10X TBS stock solution, 87.66 g NaCl and 24.22 g Tris Base were dissolved in a total volume of 1 L dH₂O, and the pH was adjusted to 7.6 using HCl.

2.1.5.8 50X TAE buffer

For the 50X stock TAE buffer, 242 g Tris Base, 57.1 mL Acetic Acid, and 100 mL EDTA (pH 8.0) were dissolved in a total volume of 1 L dH₂O.

2.1.5.9 10X running buffer

For the 10X stock Running Buffer, 10 g SDS, 144 g Glycine, and 30.3 g Tris Base were dissolved in a total volume of 1 L dH₂O.

2.1.5.10 10X transfer buffer

144 g Glycine and 30.3 g Tris were dissolved in total volume of 1 L of dH₂O for the 10X stock Transfer Buffer.

2.1.5.11 4X laemmli sample buffer

4 mg Bromophenol Blue, 4 ml Glycerol, 0.8 g SDS, 0.5 ml 2-mercaptoethanol and 2.4 ml 1 M Tris-HCl (pH 6.8) were dissolved in total volume of 10 ml dH₂O for the buffer.

2.1.5.12 Coomassie brilliant blue stain solution

For the stock Coomassie Brilliant Blue solution, 1 g Coomassie Brilliant Blue, 400 mL Methanol, and 100 mL Acetic Acid were dissolved in a total volume of 1 L of dH₂O.

2.1.5.13 Destaining solution

For the stock Destaining solution, 400 mL Methanol, 100 mL Acetic Acid, and 500 mL dH₂O were mixed.

2.1.5.14 1X mild strip buffer

For the 1X Mild Strip Buffer, 15 g Glycine, 1 g SDS, and 10 ml Tween-20 were dissolved in a total volume of 1 L of dH₂O, and the pH was adjusted to 2.2 using HCl.

2.1.5.15 10% Ammonium persulfate (APS) solution

For the 10% Ammonium Persulfate (APS) solution, 1 g APS were dissolved in a total volume of 10 mL of dH₂O.

2.1.5.16 5% non-fat dry milk solution

For the 5% non-fat dry milk solution, 2.5 g non-fat dry milk were dissolved in a total volume of 50 mL of TBS-T.

2.1.5.17 SDS polyacrylamide stacking and separating gels

SDS polyacrylamide stacking (12%) and separating (12%) gels were prepared as listed in Table 2.4.

Table 2.5: 12% Stacking and separating gel contents.

Content	Amount	
	Stacking Gel	Separating Gel
dH ₂ O	1.46 ml	1.93 ml
40% Acrylamide/Bis-acrylamide	270 µl	1.52 ml
1.5 M Tris-HCl	250 µl	1.5 ml
10% SDS	20 µl	60 µl
10% APS	20 µl	60 µl
100% TEMED	2 µl	6 µl

2.1.5.18 5% bovine serum albumin (BSA) solution

For the 5% BSA solution, 1.5 g non-fat dry milk were dissolved in a total volume of 30 mL of TBS-T.

2.1.5.19 Lysis and purification buffers

Cell lysis, binding, wash and elution buffers were prepared as listed in Table 2.5 and Table 2.7.

Table 2.6: Content of cell lysis buffer, IMAC purification buffers.

Lysis Buffer (pH 8.0)	Binding Buffer (pH 8.0)	Wash Buffer (pH 8.0)	Elution Buffer (pH 8.0)
50 mM Tris	100 mM NA ₂ HPO ₄	100 mM NA ₂ HPO ₄	100 mM NA ₂ HPO ₄
300 mM NaCl	10 mM Tris	10 mM Tris	10 mM Tris
5% Glycerol	8 M Urea	300 mM NaCl	300 mM Imidazole
Triton X-100		40 mM Imidazole	5% Glycerol

Table 2.7: AEX purification binding and elution buffer content.

Binding Buffer	Elution Buffer
20 mM Tris (pH 7.8)	20 mM Tris (pH 7.8)
	1 M NaCl

2.1.5.20 Storage buffer

For the storage buffer, 100 mM NaCl and 25 mM Na₂HPO₄ pH (8.0) were dissolved in ddH₂O.

2.1.5.21 Bacterial strains

Several bacterial strains were used in this study. *Escherichia coli* Rosetta (DE3) *F⁻ ompT hsdSB(rB⁻ mB⁻) 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)*], Rosetta-gami 2 (DE3) *Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL (DE3) F'[lac⁺ lacIq pro] gor522::Tn10 trxB pRARE2 (Cam^R, Str^R, Tet^R)*, Shuffle T7 *F' lac, pro, lacIq / Δ(ara-leu)7697 araD139 fhuA2 lacZ::T7 gene1 Δ(phoA)PvuII phoR ahpC* galE (or U) galK λatt::pNEB3-r1-cDsbC (Spec^R, lacIq) ΔtrxB rpsL150(Str^R) Δgor Δ(malF)3*, C43 (DE3) *F⁻ ompT hsdSB (rB⁻ mB⁻) gal dcm (DE3)*, NEB 5-alpha *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*, 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-*].

2.2 Methods

2.2.1 Vector and primer design

For cloning, the vector containing the TNFR1 gene found in our lab was chosen. The DsbC-TEV gene was cloned into the vector containing TNRF1. In addition, the 6xHis tag sequence was added to the N terminus of the vector for ease of purification.

2.2.2 Molecular cloning

2.2.2.1 Subcloning and plasmid DNA isolation

A single colony of bacteria containing DsbC plasmid was grown into LB medium containing 15 μL of kanamycin and incubated at 37°C overnight (16 h). After incubation, the culture was centrifuged at 4000 xg for 15 minutes at 4°C. The NucleoSpin Mini Kit was used to isolate plasmid DNA according to the manufacturer's

instructions. Then, concentration of the DsbC plasmid was measured using the Thermo Scientific NanoDrop 2000 spectrophotometer at 280 nm.

2.2.2.2 DsbC amplification with polymerase chain reaction (PCR)

The Q5 High-Fidelity 2X Master Mix was used for cloning DsbC gene by polymerase chain reaction. Annealing temperatures for primers were calculated using the Oligo Analyzer tool. The reaction composition and number of cycles are listed in Table 2.8 and Table 2.9 below.

Table 2.8: The PCR reaction composition.

Reagent	Final Concentration	Volume
Q5 High-Fidelity 2X Master Mix	1X	10 μ L
10 μ M Forward Primer	0.5 μ M	1 μ L
10 μ M Reverse Primer	0.5 μ M	1 μ L
DNA Template	30 ng	
Nuclease-free water	-	up to 20 μ L

Table 2.9: PCR cycling conditions.

PCR Cycle	Temperature ($^{\circ}$ C)	Duration	Cycle Number
Initial Denaturation	98	30 seconds	1
Denaturation	98	10 seconds	
Annealing	52	30 seconds	33
Extension	72	20 seconds	
Final Extension	72	2 minutes	1
Hold	10	∞	1

2.2.2.3 Agarose gel electrophoresis and DNA extraction from the gel

The agarose gel electrophoresis was used to control the PCR reaction, which allows the separation, extraction, and visualization of nucleic acids. Agarose gels containing 0.8 % agarose were prepared in 1X TAE Buffer and run for 45 minutes at 100 V. UV light or the GelDocTM XR+ System (Bio-Rad, 1708195) are used to visualize the gel.

The PCR product of amplified DsbC-TEV genes were cut from the agarose gel under UV light. Using the Gel and PCR Clean-up Kit (MACHEREY-NAGEL), desired genes were isolated and their concentrations were measured by using Nanodrop UV

spectrophotometer. The procedure followed for this method was taken from the manufacturer' guidelines.

2.2.2.4 Restriction enzyme digestion and DNA ligation

The vector and insert DNA fragment were cut using restriction endonucleases for 3 hours at 37°C to create sticky ends (Table 2.10). Ligation insert DNA and vector was performed using the T4 DNA Ligase enzyme at 22°C for 1 hour. The reaction mixture was set up according to the Table 2.11 instructions.

Table 2.10: Reaction mixture of double digested plasmid.

Content	Volume for digestion reaction	
	Insert	Vector
Cut Smart Buffer (10X)	2.5 µl	2.5 µl
NcoI	1.25 µl	1.25 µl
NdeI	1.25 µl	1.25 µl
DNA	1500 ng	1500 ng
Nuclease Free Water	up to 20 µL	up to 20 µL

Table 2.11: Reaction mixture of DNA ligation.

Component	Amount
T4 DNA Ligase Buffer (10X)	2 µl
T4 DNA Ligase	1 µl
Vector	100 ng
Insert DNA Fragment	240 ng
Nuclease-free water	up to 20 µl

The TNFR1 containing vector that was cut using restriction enzymes was extracted from an agarose gel using a Gel and PCR Clean-up Kit and insert DsbC gene was cleaned up using a Gel and PCR Clean-up Kit. In addition, control digestion of plasmid was also achieved by using the same enzymes.

2.2.3 Competent cell preparation

To create competent cells for chemical transformation, the Inoue technique was applied (Inoue et al., 1990). Cells were streaked onto Luria Broth (LB) Agar plate and incubated at 37°C overnight. Subsequently, a single colony was picked and grown in 5 mL LB medium overnight on an orbital shaker at 180 rpm at 37°C. After the

overnight growth, bacterial culture was transferred into 60 mL LB and incubated at 37°C on an orbital shaker at 180 rpm until the optical density at 600nm (OD₆₀₀) was reached to 0.55. Later on, bacterial culture was incubated on ice for 10 minutes. After the incubation, cell suspensions were centrifuged at 2500 g for 10 minutes at 4°C. Supernatant was discarded and pellets were resuspended in 20 mL inoue solution containing 0.25 M KCl, 15 mM CaCl₂ 2H₂O, 55 mM MnCl₂ 4H₂O, and 10 mM Pipes (pH 6.7). After cell suspensions were centrifuged at same conditions, the cells were resuspended in 5 mL of the same solution that supplemented with DMSO, and then incubated on ice for 10 minutes. Competent cells were stored at -80°C as 50 µl aliquots immediately.

2.2.4 Plasmid transformation

For transformation, the competent cells (NEB5 α , BL21 (DE3), Rosetta (DE3), C43 (DE3), SHuffle T7, and Rosetta-gami 2 (DE3)) were thawed on ice after being retrieved from a freezer at -80°C. 100 ng plasmid or 5 uL of ligation product were introduced to the 50 uL competent cell and the tube was flipped 4–5 times. After that, the tube was placed on ice for 30 minutes to incubate. The competent cells were given a heat shock by placing the tube in a heat block at 42°C for 90 seconds, after which the cells were shocked on ice for 5 minutes. Later on, 900 µL LB medium was added to the cell suspension and cells were grown for 90 minutes on an orbital shaker at 180 rpm at 37°C. Finally, transformed cells were spread on an LB Agar plate containing antibiotics (with 30 µg/mL Kanamycin) and incubated at 37°C overnight.

2.2.5 Colony PCR

Colony PCR was performed to check the whether the colonies contain the vector or not. The selected colony from the Kanamycin agar plate was dissolved in 20 µL of nuclease-free water and used as a PCR template. Tables 2.12 and 2.12 shows the reaction mixture as well as the process. The presence of the target gene of interest was confirmed by sequencing a chosen clony. DNA sequence were verified via sanger sequencing (BMLabosis, Ankara, Turkey).

Table 2.12: Colony PCR reaction mixture composition.

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

Table 2.13: Colony PCR cycling parameters.

Step	Temperature	Time	Number of Cycle
Initial Denaturation	95 °C	4 min	1
Denaturation	95 °C	30 sec	30
Annealing	46 °C	30 sec	
Extension	68 °C	1.5 min	
Final Extension	68 °C	5 min	1
Incubation	10 °C	∞	

2.2.6 Bacterial growth

From the overnight growing plate, a single colony was selected and transferred into LB medium containing the appropriate antibiotic (30 mg/ml kanamycin). It was then shaken at 180 rpm in an orbital shaker overnight at 37°C. The culture was inoculated in 1% ratio into new Luria Bertani (LB) media the next day after incubation at 37°C and 180 rpm. The cells were induced with a suitable quantity of IPTG when OD600 was reached the value between 0.6 and 0.7. Different growth temperatures (37°C, 30°C, 24°C, and 17°C), *E. coli* host strains, IPTG induction and autoinduction were all tried to determine the best culture conditions.

2.2.7 Expression of DsbC-TNFR1

E. coli Rosetta strain was transformed with the recombinant plasmid: DsbC-TNFR1 (DE3). The bacteria were grown in a ZYP-5052 medium supplemented with kanamycin (30 g/ml) for 24 hours in a shaker incubator at 17 °C and 180 rpm. After the incubation, cells were harvested via centrifugation at 4000 rpm at 4°C for 20 min, and the cell pellet was stored at -20°C.

The cell pellet was resuspended in lysis buffer and sonication was performed at 50 W for 5 minutes (5 s working, 10 s free) on the ice. Later on, the burst cells were centrifuged at 23000 xg for 30 min at 4 °C. The supernatant was collected.

2.2.8 Purification of DsbC-TNFR1

The purification of DsbC-TNFR1 from total protein was performed by the use of immobilized metal affinity chromatography (IMAC) and the ÄKTA avant 25 system (Cytiva). IMAC binding buffer was used to equilibrate the 1 ml HisTrap FF column for 5 column volumes (CV). The protein sample was then loaded onto the column, which was subsequently washed with IMAC wash buffer for 10 CV. Finally, IMAC elution buffer was used to elute DsbC-TNFR1. The elution step was carried out as a linear gradient with 15 CV and then step with 15 CV. SDS-PAGE and immunoblotting assays were performed to examine the fractions. After the affinity purification, anion exchange chromatography (AEX) was also performed. For the AEX purification step, AEX binding buffer was used to equilibrate the 1 ml HiTrap Q-XL column for 5 column volumes (CV). The dialysed protein sample was then loaded onto the column, which was subsequently washed with AEX binding buffer for 10 CV. The elution step was carried out as a linear gradient with a CV of 30 and then step with 5 CV. Sample flow rate was set as 0.7 ml/min. SDS-PAGE and immunoblotting assays were used to examine the fractions.

2.2.9 Buffer exchange of DsbC-TNFR1 via ultrafiltration

Ultrafiltration is a membrane filtration method that is used for desalting, concentrating, and exchanging buffers of protein samples. After the purification, selected fractions were pooled and transferred into Amicon® Ultra-15 Centrifugal Filters with a membrane containing a 10 kDa molecular weight cut-off (10 kDa MWCO) and centrifuged at 4000 xg at 4 °C until the removal of the imidazole. Using centrifugal filters was also used to concentrate the purified DsbC-TNFR1.

2.2.10 SDS-PAGE and immunoblotting

SDS-PAGE denatures the proteins and coats them with a negative charge before separating them in an electrical field based on their size. All SDS-PAGE and immunoblotting analyses were performed using 12% SDS gels and Mini Protean Tetra System (Biorad, USA). Protein samples were boiled for 10 minutes at 95°C in 4X

laemmli buffer. Protein samples were loaded in the gel, and the voltage was raised to 120 V from 90 V after the marker went through the stacking gel. After the run, the gel was stained using Coomassie Brilliant Blue staining solution. The Coomassie Brilliant Blue staining on the gel was then removed using destaining solutions, revealing the proteins.

Immunoblotting was carried out at 4°C in a wet transfer system under a 100 V electric field for 90 minutes. After the transfer, membranes were blocked for 1 hour using 5% milk prepared in TBS-T. The Anti-Histidine antibody was used as a primary antibody and rotated for 16 hours at +4°C. After each of incubations with the primary and secondary antibodies, the membranes were washed with TBS-T three times for five-minutes. As a secondary antibody, a mouse IgG antibody was used for 2 hours at room temperature. ECL (Bio-Rad, CA, USA) solution was used to view gel screening in ChemiDoc Imaging System (Bio-Rad, CA, USA) by the Image Lab software.

2.2.11 Circular dichroism analysis

Circular dichroism (CD) is a sensitive and quick technique to detect the secondary structure of proteins and polypeptides. The secondary structures of purified proteins were analyzed by using J-1500 Circular Dichroism Spectrophotometer (Jasco, Easton, MD) and a 1 mm cuvette. Secondary structure experiments were carried out at wavelengths ranging from 195 to 250 nm. The CD scanning settings were set up as 1 nm step size, 1 nm bandwidth, 50 nm/min speed, and 2 seconds reaction time at 25°C.

2.2.12 Intact mass analysis

The measurement of a protein's entire molecular weight using mass spectrometry (MS) without any digestion is known as intact mass analysis. SYNAPT G2-Si HDMS was used to estimate the molecular mass of DsbC-TNFR1. A BEH C4 300, 1.7 μ m, 2.1 mm x 50 mm column (Waters, 186004495) was used in this experiment, with a flow rate of 0.3 ml/min.

2.2.13 Peptide mapping via liquid chromatography – mass spectrometry

For peptide mapping analysis, peptide mixtures were analyzed using MSE mode using LC-MS/MS (Waters Synapt G2 Si) with the Acquity UPLC BEH C-18 reverse phase column (Waters Corporation, Milford, Mass., USA). Before the sample injection, the system was equilibrated with a mobile phase of acetonitrile:water:formic acid (1 %) in

the ratio of 1:89:10. The samples were injected into the instrument at a flow rate of 0.2 mL /min with a volume of 10 μ L.

DsbC-TNFR1 samples for analysis were prepared from SDS-PAGE gels using the in-gel digestion process described below.

Each band of gel was sliced into little pieces and inserted into tubes.

- 100 μ L of double distilled water was added and vortexed it. Then the water was discarded.
- 100 μ L of 100 mM AMBIC/100% ACN solution was added into the tube and vortexed for 10 minutes. The AMBIC/100% ACN solution was then extracted and discarded from the gels. This process was continued until the gel fragments had lost their color.
- 100 μ L solution of 100% ACN was added to the tube and vortexed for 5 minutes. The ACN solution was then removed from the gels, and the gels were vacuum dried for around eight minutes.
- 100 μ L of 10 mM DTT in 100 mM AMBIC solution was added to the tube, and they were incubated at room temperature for 45 minutes. The supernatant was then thrown away.
- 100 μ L of 50 mM IAM in 100 mM AMBIC solution was added to the tube, and the reaction was carried out in the dark at room temperature for 45 minutes. The supernatant was then thrown away.
- The techniques outlined in steps 2-4 were repeated.
- 30-40 μ L trypsin (20 ng/ μ L) in 50 mM of AMBIC solution were added and incubated on ice.
- 50 μ L of 50 mM AMBIC was added and incubated on an orbital shaker at 180 rpm for 16 hours at 37°C.
- The supernatant was collected the next day and deposited in a new tube.
- 1 μ L of formic acid was added to the supernatant and vortexed for a few minutes. It was then maintained at 4°C.

- 100 μ L combination of 45% ddH₂O/50%ACN/5%formic acid was added to the gel fragments and vortexed for 5 minutes. The supernatant was then collected and stored at 4°C.
- The steps outlined in step 12 were repeated 3-4 times, after which the peptides were vacuum dried for around 2 hours.
- The dried peptides were then dissolved in a 40 μ L mixture containing 89%ddH₂O/1% ACN/10% formic acid.

2.2.14 Purity analysis by capillary electrophoresis

Capillary electrophoresis (CE) is an advantageous method due to its higher separation efficiency, high resolution, and shorter analysis times. In this method, proteins are denatured in the presence of SDS under reducing or nonreducing conditions and electrophoretically separated by molecular weight and hydrodynamic radius via a sieve polymer matrix.

The purity of purified proteins was analysed using PA800 Plus CE (Beckman Coulter) according to manufacturer's instructions.

The capillary was cleaned out with 0.1 N NaOH at 70.0 pressure for 3 minutes before being treated with 0.1 N HCl at 70.0 psi for 1 minute to neutralize the surface silanol group. After that, the capillary was filled with SDS gel buffer at a pressure of 70.0 psi for 10 minutes. The sample was injected for 40 seconds at 5.0 kV and separated for 30 minutes at 15 kV.

2.2.15 Isoelectric point determination by capillary electrophoresis

Capillary isoelectric focusing (cIEF) is a technique for separating protein/peptide combinations and other charge alterations according to their isoelectric point (pI). The ampholyte produces a pH gradient inside the gel to separate molecules according to their pI. The molecule of interest has a net charge of zero when it reaches the pH corresponding to its pI and stops traveling along the electrical field.

Isoelectric point determination was performed using a PA 800 plus CE (Beckman Coulter) with a neutral-coated capillary by using UV detector. The samples and needed buffers were prepared in accordance with the manual's instructions. Additionally, pI markers indicating 9.5, 7.0, and 5.5 values were used.

Prior to sample injection, the capillary was washed with urea solution at 50 pressure for 3 minutes, then rinsed with ultrapure water at 50 psi for 2 minutes. To fill the whole capillary with the sample solution, each sample was injected for 99.9 seconds at 25 psi. The capillary ends were dipped in ultrapure water before being immersed in the anolyte and catholyte and focussed at 25 kV under normal polarity for 15 minutes. By substituting the catholyte solution with a chemical mobilizer, the focussed peaks were then exposed to chemical mobilization at 30 kV for 30 minutes under normal polarity. The capillary was washed with ultrapure water at the conclusion of the run.

2.2.16 *In-vitro* pull down assay

The pull-down assay is an *in vitro* approach for determining protein physical interactions. It's a great method to confirm a protein–protein interaction or discover novel interacting partners. As affinity chromatography, this procedure also comprises many wash and elution phases. The experiments include adsorbing a protein complex onto beads and then isolating it. Beads with immobilized ligands attach to a specific component of the complex through an affinity tag or an antibody.

- Transfer 50 μ L Ni-NTA agarose beads to a microcentrifuge tube (1.5 mL).
- Add 500 μ L binding buffer to the tube and centrifuge the tube for 5 minutes at 4000 g. Discard the flow-through.
- Repeat step 2.
- Add 5 μ g His-tagged DsbC-TNFR1 protein with 300 μ L binding buffer and mix.
- Incubate for 3 hours with agitation at 4°C.
- Centrifuge the tube for 5 minutes at 4000 g and keep the flow-through for analysis.
- Wash beads with 500 μ L binding buffer, centrifuge the tube and discard the flow-through.
- Repeat step 7 three times.
- Mix the ligand protein with 300 μ L binding buffer and load it onto the tube.
- Incubate for 1 hour with agitation at RT.

- Centrifuge the tube for 5 minutes at 4000 g. Keep the flow-through for analysis.
- Wash beads with 500 μ L binding buffer, centrifuge the tube and discard the flow-through.
- Repeat step 12 three times.
- Wash beads with 300 μ L binding buffer, centrifuge the tube for 5 minutes at 4000 g. Keep flow-through for analysis.
- Add 300 μ L of elution buffer to the tube and incubate it for 10 minutes at RT.
- Centrifuge the tube for 5 minutes at 4000 g and keep an eluted fraction (DsbC-TNFR1/TNF- α complex) for analysis.
- Samples were analyzed by immunoblotting.

2.2.17 Blue-native PAGE

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) is a molecular weight based separation technique for multi-protein complexes in their native conditions. Coomassie G-250 dye was used to coat the proteins with a negative charge.

The dye binds to proteins without confining their denaturation. The protein complexes would be migrating across the polyacrylamide gel depending on the pore size. Coomassie Blue-G250 provides them not only the anionic character, but water solubility properties as well.

For BN-PAGE, a 4–16% gel was utilized to understand the protein's natural form and measure the molecular weight of the protein. Pure protein fractions were pooled and loaded in a 4–16% linear gradient gel after IMAC purification. At first, the gel was run at 150 V for 1 hour using a dark blue buffer, then the buffer was changed with light blue buffer, and gel was run for additional 1 hours at 250 V.

Afterwards, the gel was incubated with destaine solution. The results were then compared according to a molecular size marker.



3. RESULTS

3.1 Analysis of DsbC-TNFR1: PCR Amplification, Restriction Enzyme

Digestion, Ligation

The plasmid containing TNFR1 gene has already existed in our laboratory. The DsbC fusion partner (lacking the periplasmic signal sequence for cytoplasmic expression) is employed to improve solubility, correct oxidation, and help folding of the TNFR1. The fusion protein DsbC-TNFR1 is designed to contain an TEV (Tobacco Etch Virus) cleavage site at N terminal region of the TNFR1 that DsbC can be cut if needed. 6X His tag also added to the N terminus of the protein, which allows to perform nickel affinity purification.

DsbC DsbC was amplified using polymerase chain reaction (PCR) with DsbC-specific primers including the appropriate restriction sites. The PCR products were run on an agarose gel (Figure 3.1).

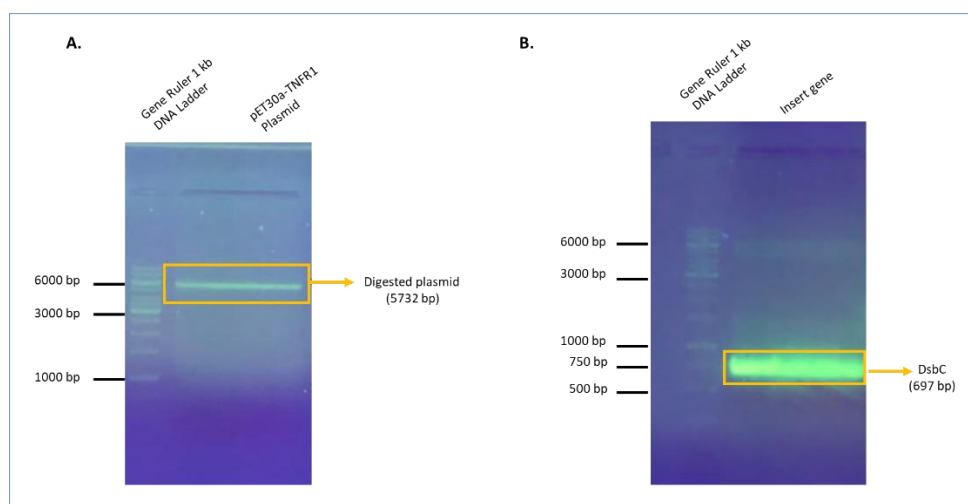


Figure 3.1: The agarose gel results. A) Vector digestion and B) DsbC amplification. 0.8 % Agarose / TAE.

Expected DNA fragment length of 697 base pair was observed in the agarose gel, which indicated that DsbC was amplified successfully using the designed primers (Figure 3.1 B). Both the PCR product and the plasmid were double digested with

restriction enzymes. The digested and linearized vector, as well as the digested insert, were ligated. The *E. coli* Neb5-alpha strain was then transformed with the ligated plasmid construct. Following that, four colonies were picked from the LB agar plate to check whether they contain ligated construct.

Colony PCR was performed to assess the success of the transformation. Plasmid isolation was performed from the colony with expected fragment length according to the colony PCR results. The isolated plasmid was run on agarose gel after performing single and double digestion controls (Figure 3.2).

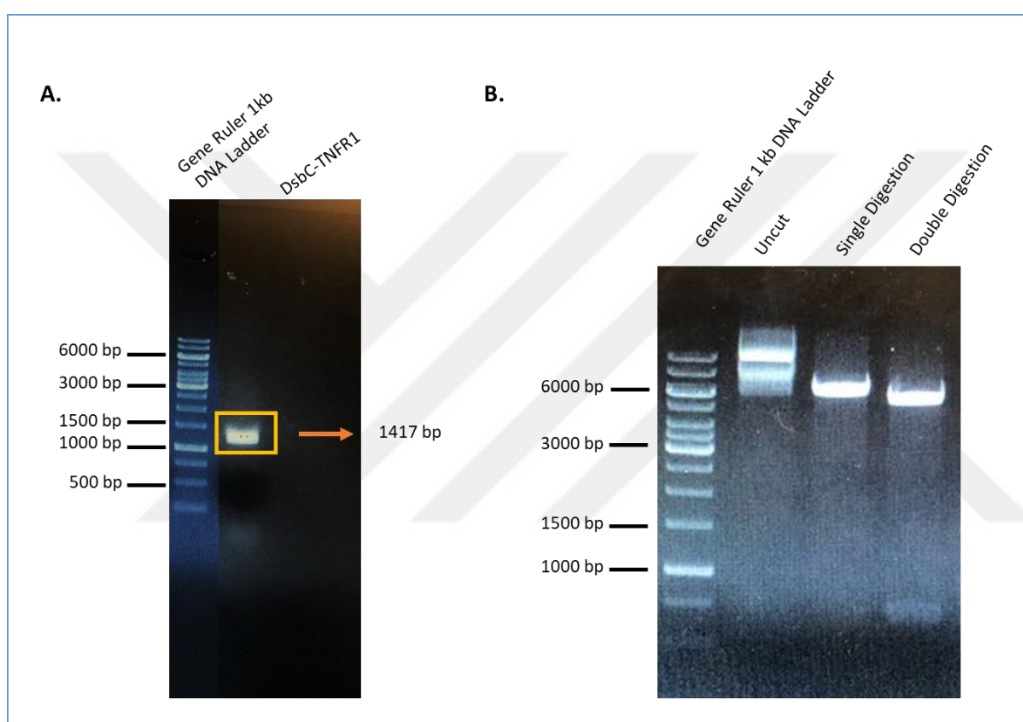


Figure 3.2: The agarose gel results. A) colony PCR result of ligation of DsbC and TNFR1 gene B) Restriction enzyme digestion control.

DNA sequencing was conducted via Sanger sequencing by BM Laboratory Systems (Ankara, Turkey). The plasmid map was created by BioRender.com (Figure 3.3).

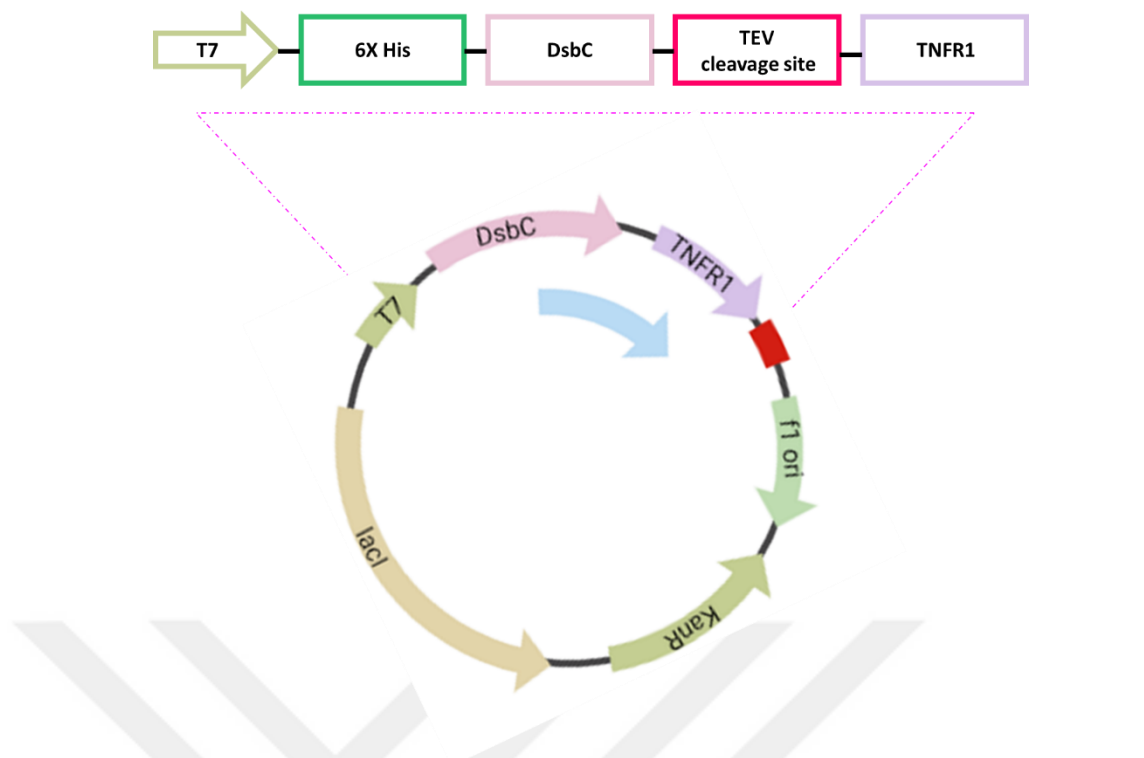


Figure 3.3: Design of the expression plasmid structure.

3.2 Protein Expression Optimization

In order to produce TNFR1 in soluble form, first, the SHuffle cell line was selected as the SHuffle was engineered to constitutively express disulfide bond isomerase (DsbC) in its cytoplasm, which improves the protein folding. However, TNFR1 could not be obtained in soluble form in the SHuffle strain (Figure 3.4). Therefore, an experiment was conducted by forming a protein fusion with DsbC and TNFR1.

The production of the DsbC-TNFR1 fusion protein in the SHuffle cell line resulted in the acquisition of insoluble TNFR1 (Figure 3.4).

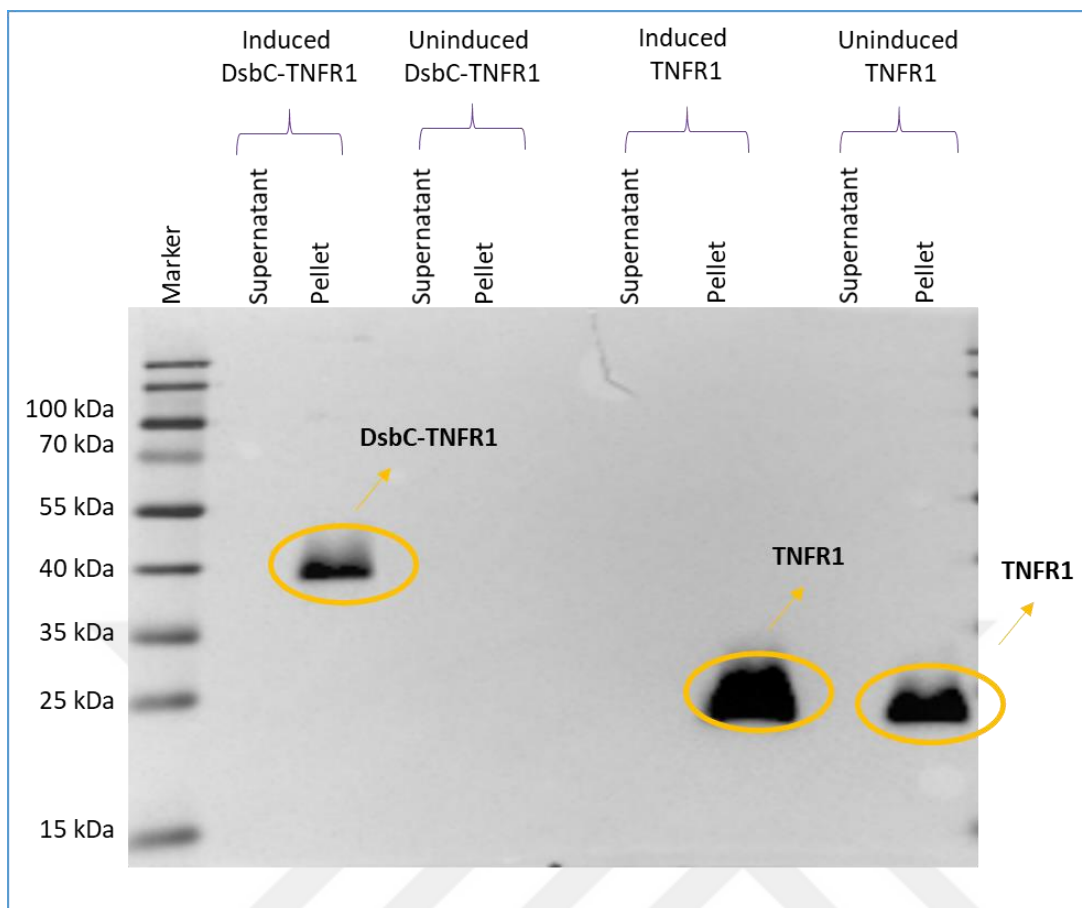


Figure 3.4: Control of the TNFR1 plasmid expression in *E. coli* SHuffle T7 strain with and without DsbC. The SDS-PAGE (12%) result is shown on the left, while the Immunoblotting result is shown on the right. TNFR1 with DsbC has a molecular weight of 43 kDa, whereas TNFR1 without DsbC has a molecular weight of 25 kDa. 1 mM IPTG was used for induction. S is supernatant and P is pellet.

Then *E. coli* strain BL21 (DE3) was used to examine the influence of DsbC fusion on the transition of the target protein to the supernatant. The cells containing TNFR1 vector with and without DsbC was cultured in LB medium at 37°C for 4 hours. The SDS-PAGE and the immunoblotting results of protein expression of IPTG-induced and non-induced cells are shown in the Figure 3.5.

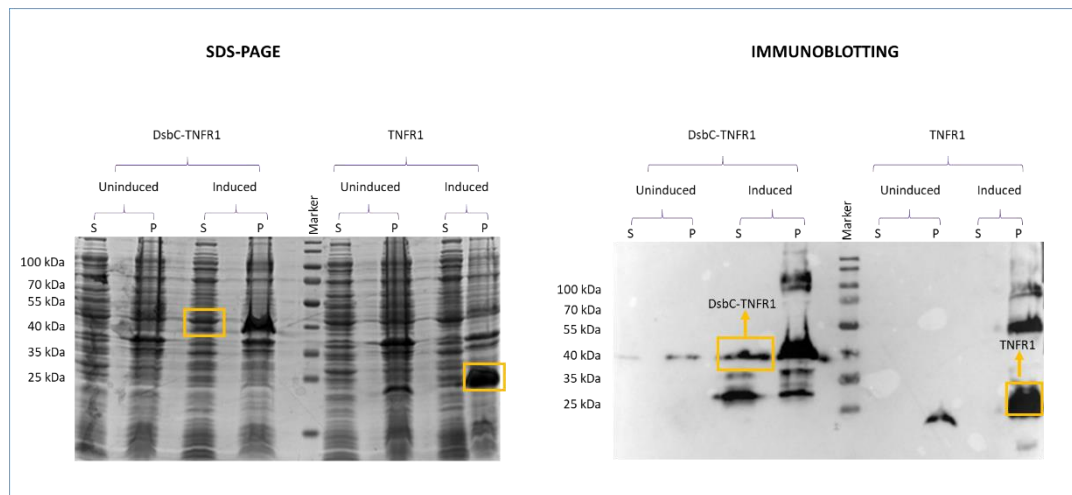


Figure 3.5: Control of the TNFR1 plasmid expression in *E. coli* BL21(DE3) strain with and without DsbC. The SDS-PAGE (12%) result is shown on the left, while the Immunoblotting result is shown on the right. TNFR1 with DsbC has a molecular weight of 43 kDa, whereas TNFR1 without DsbC has a molecular weight of 25 kDa. 1 mM IPTG was used for induction. S is supernatant and P is pellet.

The results of SDS-PAGE and immunoblotting assay revealed that, DsbC TNFR1 (43.8 kDa) was produced in soluble form, whereas TNFR1 without DsbC (25 kDa) remained in the pellet as inclusion bodies.

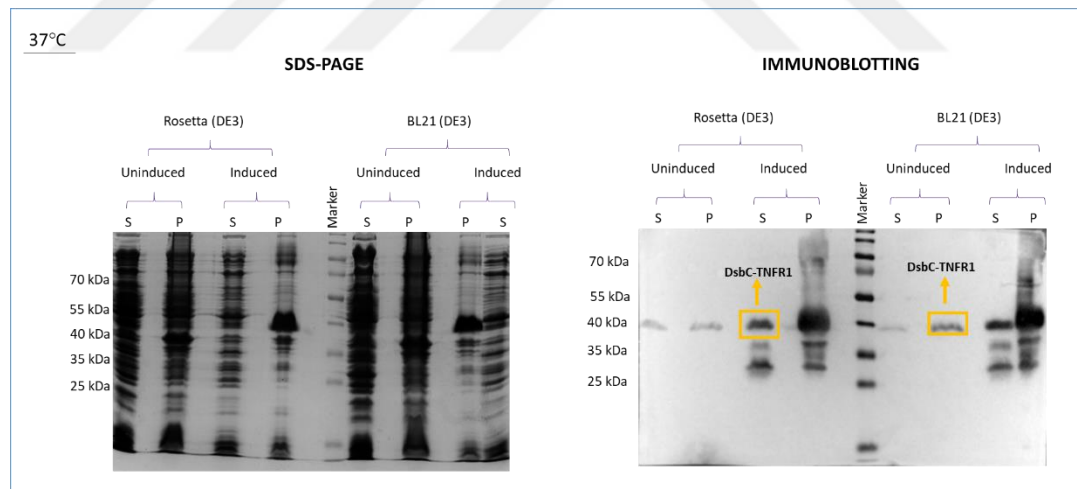


Figure 3.6: The SDS PAGE and immunoblotting results of the target protein production from *E. coli* BL21 (DE3) and Rosetta (DE3) cells at 37°C for 4 hours. 1 mM IPTG was used for induction. S is supernatant and P is pellet.

In order to determine the best conditions for production of DsbC-TNFR1 in soluble form, *E. coli* BL21 (DE3) and Rosetta (DE3) strains that transformed with DsbC-TNFR1 plasmid were induced with IPTG (1 mM) and grown at various temperatures. Effects of different growth conditions, including 37°C for 4 hours (Figure 3.6), 30°C for 6 hours, 25°C for 12 hours, and 18°C for 16 hours were assessed SDS-PAGE

analysis. Even if the opposite effect is expected, the SDS-PAGE results showed that lowering the temperature decreased the expression of the soluble protein. In addition, when *E. coli* strain BL21 (DE3) and Rosetta (DE3) were compared, Rosetta (DE3) was found to be more efficient, and thus it was decided to continue the experiments with Rosetta (DE3) (Figure 3.7).

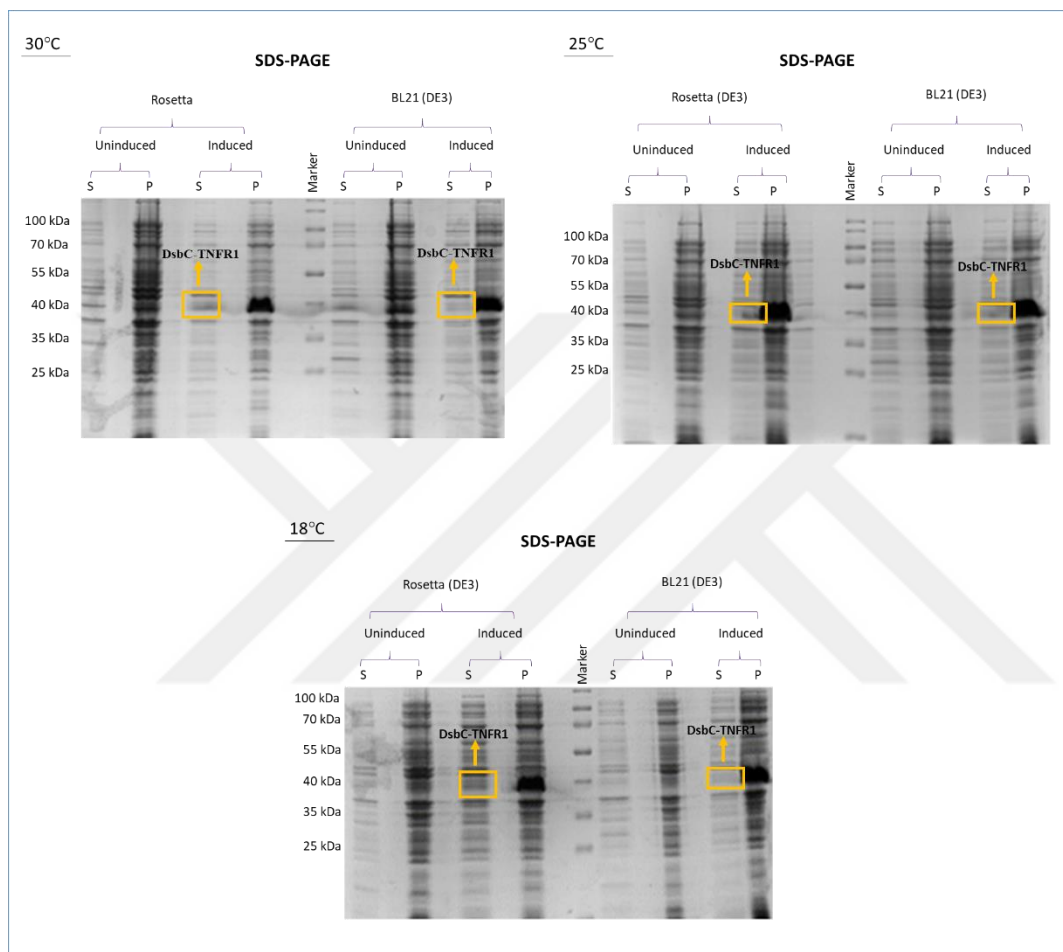


Figure 3.7: The SDS PAGE (12%) and immunoblotting results of the target protein production from *E. coli* BL21 (DE3) and Rosetta (DE3) cells at different temperature. Cultures were incubated 30°C for 8 hours, 25°C for 12 hours and 18°C 20 hours. 1 mM IPTG was used for induction. S is supernatant and P is pellet.

Additionally, instead of the IPTG induction, autoinduction medium ZYP-5052 was also tried at different temperatures. While looking at the effect of autoinduction media on protein solubility, a comparison was made using plasmids with and without DsbC (Figure 3.8).

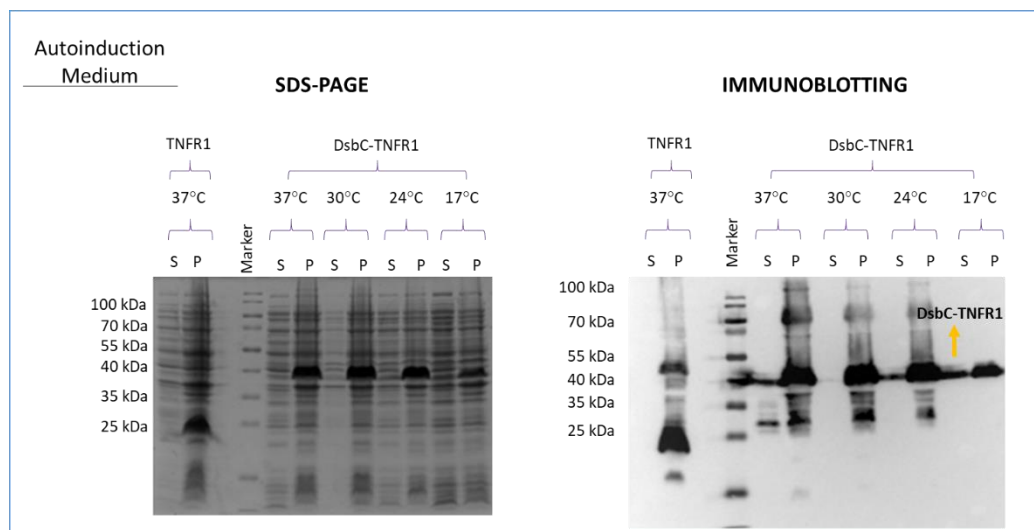


Figure 3.8: The SDS PAGE (12%) and immunoblotting results of autoinduction media (ZYP-5052 medium) effect on the target protein production. Cultures were incubated at different temperatures. TNFR1 expression without DsbC on the left of the marker and with DsbC on the right of the marker.

According to the results shown in the Figure 3.8, TNFR1 fused with DsbC was soluble when the cells were grown at 37°C, but TNFR1 was remained in the inclusion bodies in the absence of DsbC. In addition, various temperatures (37°C, 30°C, 24°C, and 17°C) were tested for DsbC-TNFR1 for autoinduction optimization. As Figure 3.8 showed, half of the total protein could be obtained in soluble form when the culture was grown at 17°C for 24 hours. Therefore, production in autoinduction medium at 17°C for 24 hours was selected for further studies.

3.3 Purification of DsbC-TNFR1 Fusion Protein

The cell lysate was diluted 5-fold with binding buffer before affinity purification. In this case, the sample diluted with the binding buffer ultimately contained approximately 6 M of urea to ensure better binding of the target protein to the column. Urea was removed after purification using urea-free wash and elution buffers.

Purification of DsbC-TNFR1 was carried out using immobilised metal affinity chromatography (IMAC) method by using the HisTrap FF Crude (Cytiva, Sweden AB) affinity column. As the purification results showed that one step purification was not sufficient (Figure 3.10) to remove host cell protein, the anion exchange chromatography (AEX) method was used as the second step by using the HiTrap® Q-XL (Cytiva, Sweden AB) anion exchange column. After the affinity chromatography,

the selected fractions according to the SDS-PAGE results (Figure 3.11) were dialyzed with binding buffer of AEX purification and then purified by ion exchange chromatography. Chromatograms were created using Unicorn 7.0 software. Fractions were collected and analysed with SDS-PAGE (12%) and immunoblotting analysis (Figure 3.11 and Figure 3.12). Figure 3.9 and Figure 3.10 display the affinity purification; Figure 3.11 and Figure 3.12 display the anion exchange chromatography results. The fractions that contain target protein were pooled and concentrated by using a 10 kDa cut-off filter.

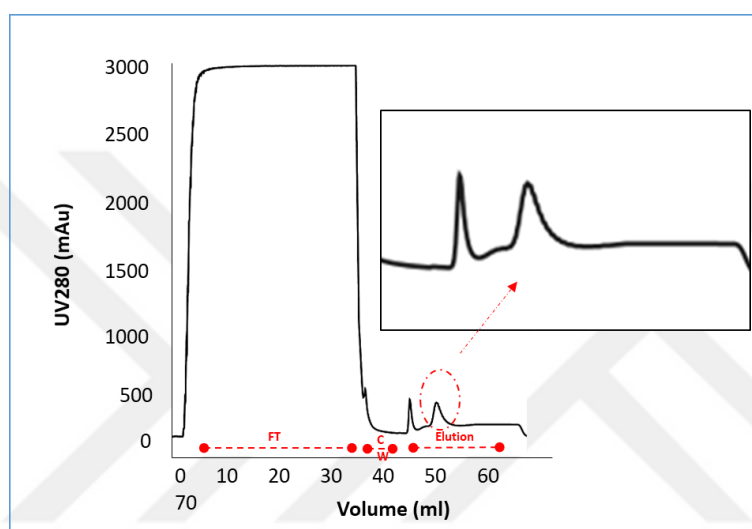


Figure 3.9: Ni-NTA affinity chromatography result. The collected elution fractions were shown in the red dashed circle. FT is Flow-through, CW is Column Wash.

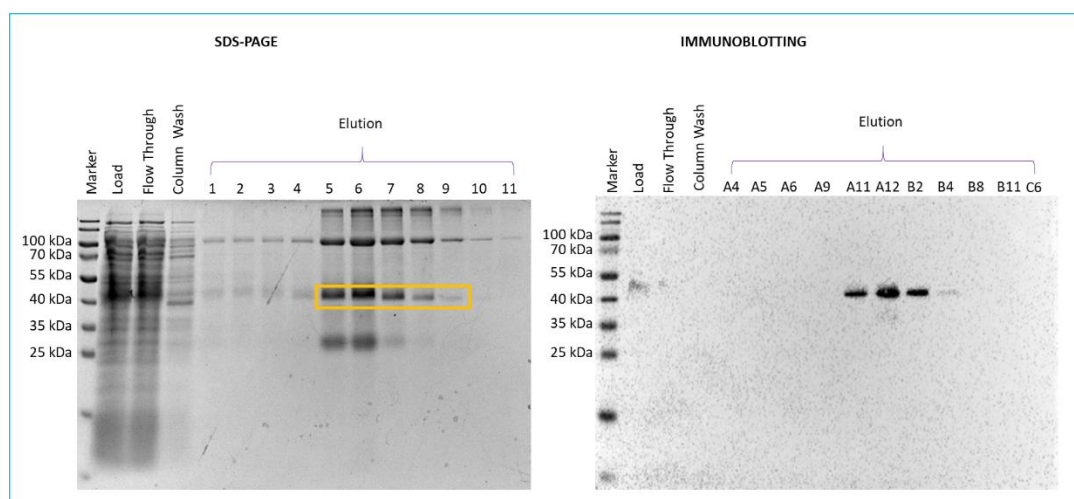


Figure 3.10: SDS-PAGE (12%) and immunoblotting results of the IMAC purification of DsbC-TNFR1. The elution fractions are indicated numerically. The collected fractions were shown in the orange box. FT is Flow-through, CW is Column Wash.

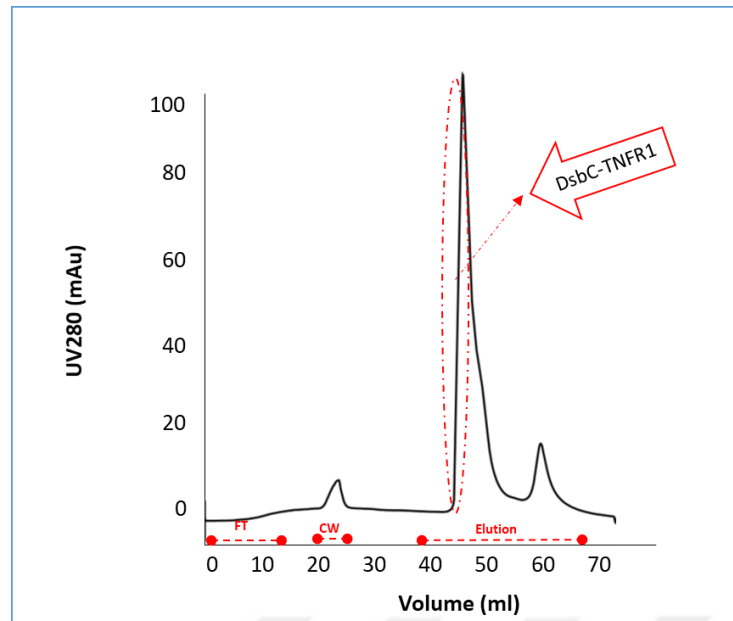


Figure 3.11: Anion exchange chromatography result. The collected elution fractions were shown in the red dashed circle. FT is Flow-through, CW is Column Wash.

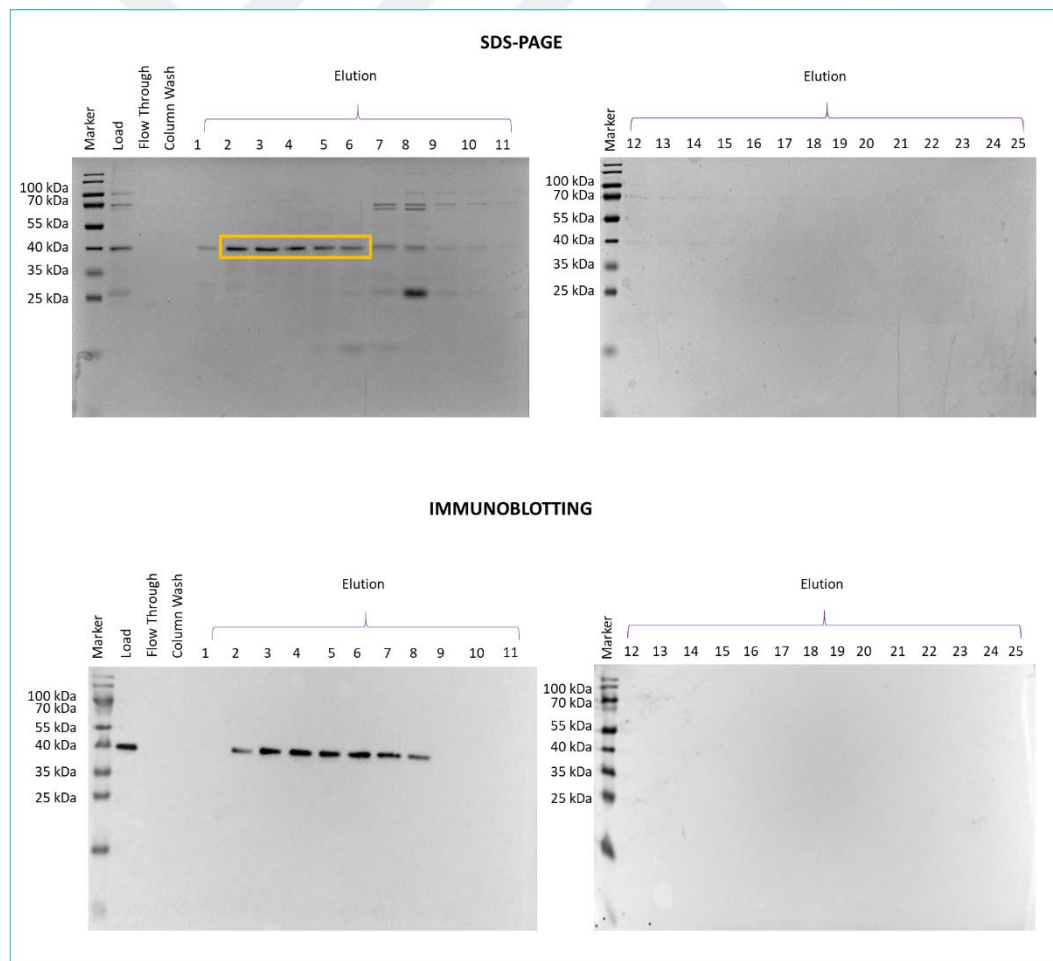


Figure 3.12: SDS-PAGE (12%) and immunoblotting results of the AEX purification of DsbC-TNFR1. The elution fractions are indicated numerically. The collected fractions were shown in the orange box. FT is Flow-through, CW is Column Wash.

As a result of performing AEX as a second purification step, the purity of the DsbC-TNFR1 fusion protein was increased and an approximate 90% purity were obtained according to the SDS PAGE analysis (Figure 3.12).

3.4 Secondary Structure Determination with Circular Dichroism

For a protein to be biologically active, it must be folded correctly. Circular dichroism (CD) is a powerful method for determining protein secondary structure and folding features quickly.

The secondary structure of the DsbC protein is mostly α -helix but it has also β -sheet structures (Zapun et al., 1995). On the other hand, the TNFR1 protein consists of β -sheet and loop structures (Naismith et al., 1996). The fusion protein was measured in the far-UV range, between 195 and 250 nm, and it was discovered that the fusion protein was dominantly α -helix but it had also β -sheet structures (Figure 3.13).

Since the DsbC-TNFR1 fusion protein is obtained from the host cell in soluble form, the protein has a folded structure. To show that the effect of urea used before the affinity purification step does not disrupt the structure of the protein, the secondary structures of fusion proteins were analysed with and without urea (Figure 3.13). The comparison of presence and absence of urea showed no structural differences between the samples, only a shift depending on the concentration differences was observed.

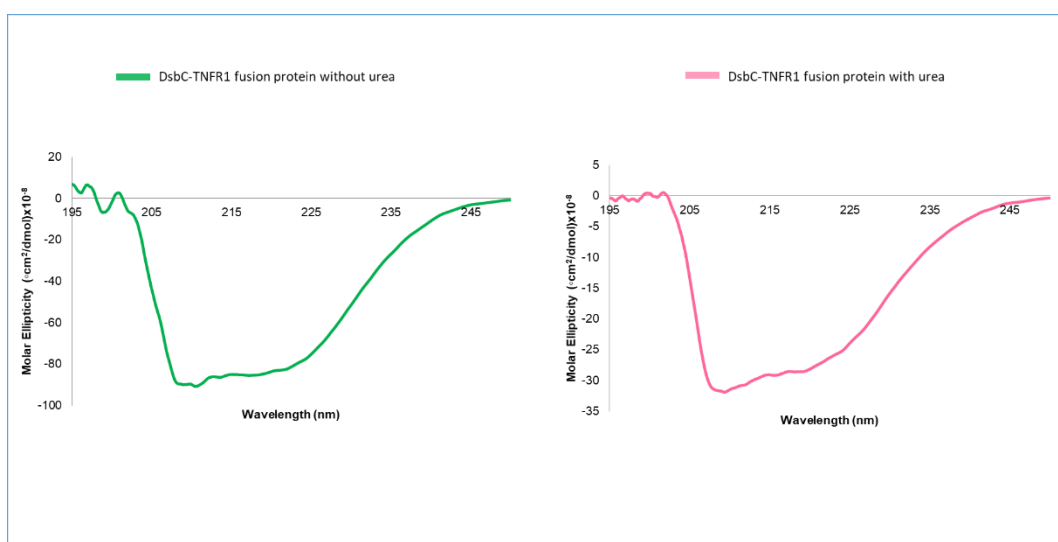


Figure 3.13: The circular dichroism spectra of DsbC-TNFR1 without urea (green), with urea (pink).

3.5 Purity Analysis by Capillary Electrophoresis

Purity analysis was performed by capillary electrophoresis. Figure 3.14 depicts the capillary electrophoresis results following affinity and anion exchange chromatography.

When one-step purification (affinity) was performed, the purity was 68.15%, which reaches 92.72% when AEX was added as a second step.

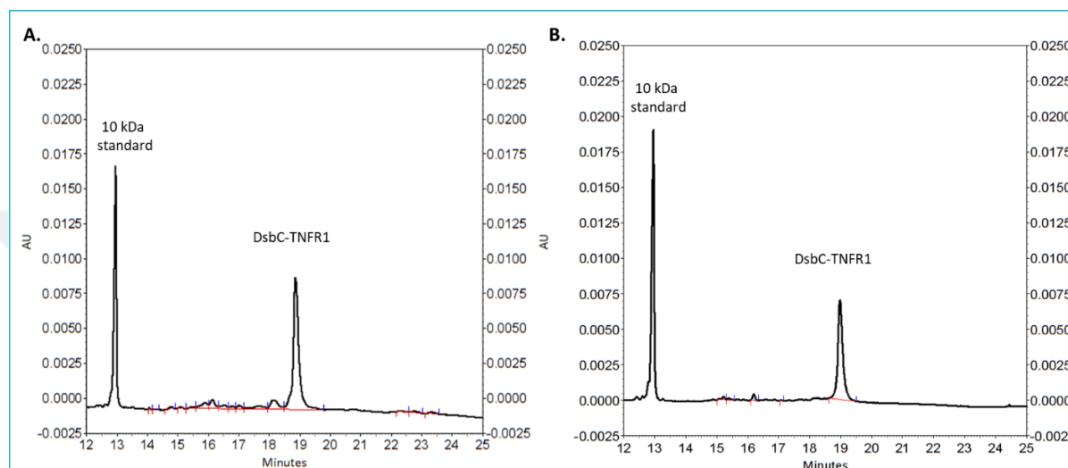


Figure 3.14: Electropherograms of the purity analysis of fusion protein. The results of the purity analysis electropherograms A. belongs to affinity purification and B. belongs to the AEX purification.

3.6 Isoelectric Point Determination by Capillary Electrophoresis

The capillary isoelectric focusing method was used to determine the isoelectric point (pI) of fusion protein. The theoretical pI of the fusion protein is 6.66. The pH 9.5, 7.0, and 5.5 pI markers, were selected according to the theoretical value, and used to calculate the pI of the protein, which was determined as 6.66 (Figure 3.15).

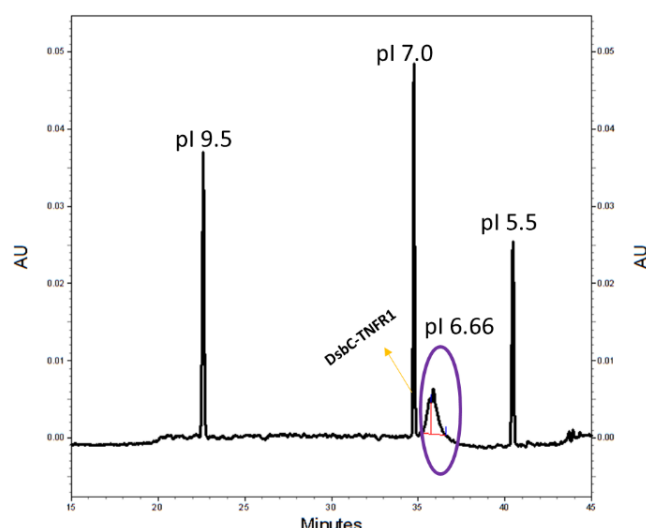


Figure 3.15: The results of the cIEF analysis of fusion protein.

3.7 Intact Mass Analysis

The intact mass analysis by Liquid Chromatography–Mass Spectrometry (LC-MS/MS) was used to measure the molecular weight of the DsbC-TNFR1 fusion protein. Figure 3.16 shows the mass spectrum and deconvolute mass data. The molecular weight of the fusion protein was confirmed using computed deconvolute mass as a consequence of intact mass analysis. Based on the analytical results, the molecular weight of DsbC-TNFR1 was determined as 43791.03436 Da, which is compatible with the 43791.3765 Da theoretical molecular weight of the protein.

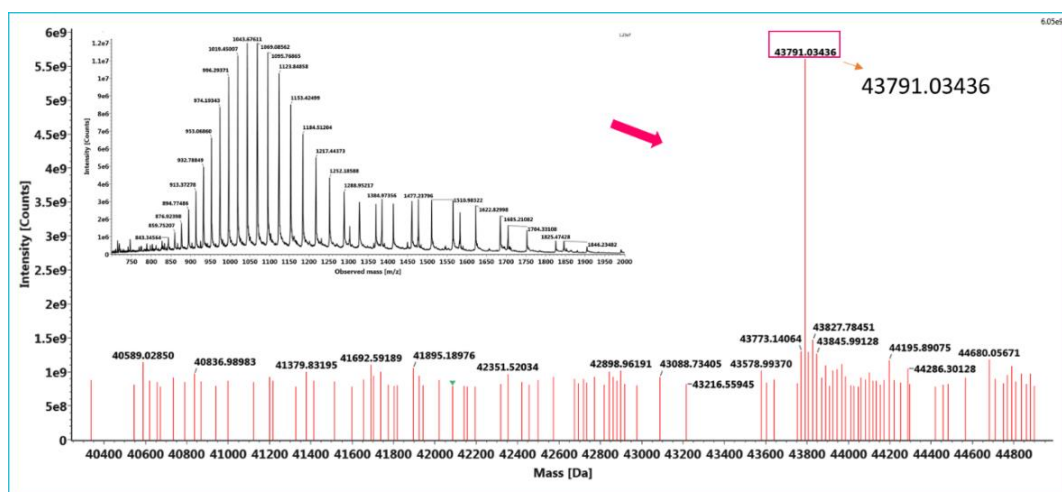


Figure 3.16: LC/MS spectrum of DsbC-TNFR1 (left) and the deconvoluted mass spectra of peaks corresponds to DsbC-TNFR1 peak (right).

3.8 Blue Native PAGE Analysis

After the purification, the DsbC-TNFR1 fusion protein was analysed by blue native PAGE to understand its natural state. When compared with the molecular size marker, the fusion protein was detected in dimer form and also different oligomeric structures (Figure 3.17).

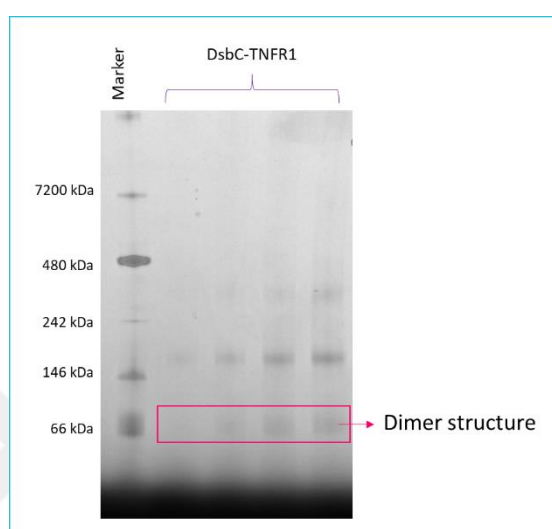


Figure 3.17: BN-PAGE result of the native DsbC TNFR1 fusion protein.

3.9 *In-vitro* Pull-Down Assay

The pull-down assay is used to detect or confirm interactions between proteins *in vitro*. The biological functionality of the fusion protein was analyzed using this method. The TNFR1 fusion protein was attached to the Ni-NTA beads via their His-tag, then, a commercial ligand was incubated with Ni-NTA-bound TNFR1. First of all, the interaction of TNFR1 fusion protein and commercial ligand was checked (Figure C.5), then the experiment was repeated by creating control groups. As a negative control, ligand was incubated with Ni-NTA beads in the absence of TNFR1. Empty beads were also used for another negative control (Figure 3.18).

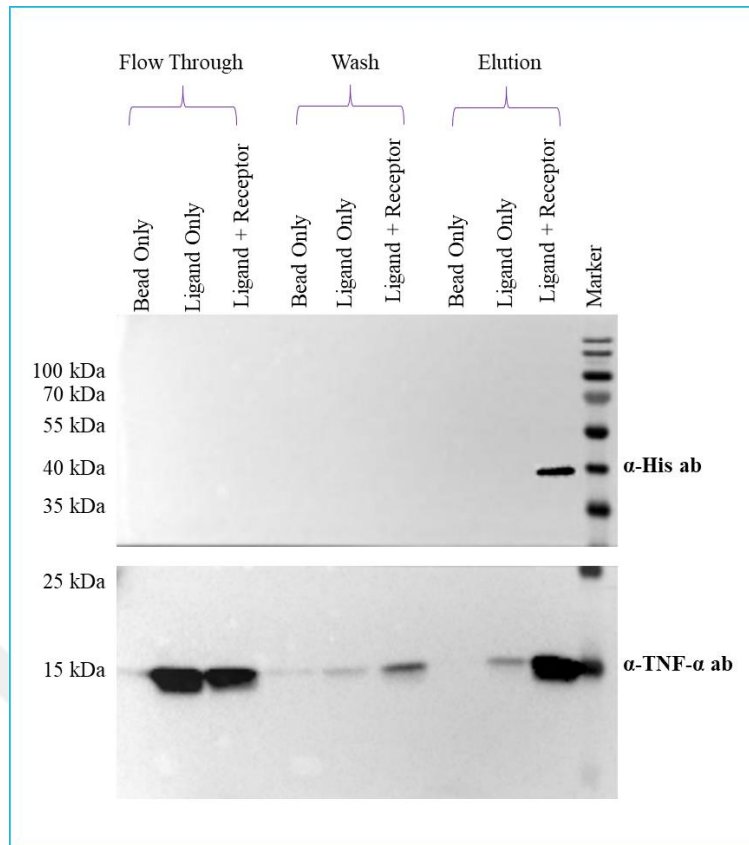


Figure 3.18: The immunoblotting results of a pull-down assay using purified DsbC-TNFR1 (bait) and commercial TNF- α (prey) protein. Nickel bound agarose beads were incubated with pure DsbC-TNFR1 (load lane). Following bead washing, pure TNF- α was loaded onto the bead and incubated. After incubation, unbound ligand was discarded via centrifugation (flow-through). After washing, bead-bound proteins were eluted with elution buffer containing imidazole (elution lane). In addition, ligand protein was applied to the unbound blank bead (ligand only), and it was checked whether the ligand was bound non-specifically.

The results revealed that the fusion protein interacted with the commercial ligand. In addition, it is seen that the commercial ligand binds to Ni-NTA beads, albeit slightly and non-specifically (Figure 3.18).

4. DISCUSSION

Small proteins including disulfide-bonds are versatile bioactive molecules with major pharmacological, medicinal, and/or biotechnological properties. Small disulfide-rich proteins (DRPs) are gaining popularity in the treatments of autoimmune diseases due to their benefits over small molecule drugs such as high selectivity and affinity. Molecular imaging agents, antibacterial and host-defense peptides, and ion channel blockers are some examples of these DRPs (Nozach et al., 2013).

Disulfide linkages are required for the tertiary and quaternary structure formation of extracytoplasmic proteins, which have more than one cysteine residue (Raina & Missiakas, 1997). A recent research revealed that the therapeutic protein market consists most of proteins with disulfide bonds (Lobstein et al., 2016). For this reason, it is important to obtain proteins with disulfide bonds with low cost, high yield, and in an easy way.

As the extracellular domain of TNFR1 consists of 4 cysteine-rich domains (CRDs), it is one of the disulfide rich therapeutic proteins (Naismith et al., 1995). TNFR mediated signaling is important for immunological response, cell death, and cell proliferation (G. Chen & Goeddel, 2002). Because of all of these crucial activities, TNFRs and their ligands are potential candidates for the therapy of a various disorders (Monaco et al., 2015). In the recent years, the anti-TNF therapy method gained popularity in the clinical field of autoimmune diseases (Ramos-Casals et al., 2008).

Many approaches for recombinant protein overexpression have been improved since the late 1970s, and they are still being developed with *E. coli*, which is the most popular among the various hosts employed for protein overexpression in prokaryotic systems (Banaś et al., 2019). Such bacterial system is a common and well-defined prokaryotic host mechanism for heterologous protein expression due to its ease of handling, large-scale manufacturing, and low culture costs (Kong & Guo, 2014). Another reason why *E. coli* is such a preferred tool in biopharmaceutical production of heterologous

recombinant proteins is attributed to its approval of use by Food and Drug Administration (FDA) (Fathi-Roudsari et al., 2016).

However, that does not neglect the fact that mammalian proteins are not easily expressed in bacterial systems due to their disulfide bonds, which are critical for stability, proper folding and activity (Kong & Guo, 2014). Some disadvantages of employing the *E. coli* system include the lack of a post translational protein mechanism, and formation of inclusion bodies due to high amount of protein expression. Therefore, expression of heterologous proteins, especially those containing disulfide bonds, has always been challenging (Kong & Guo, 2014).

Thus, many techniques are being explored to create native and soluble proteins using *E. coli* as a bacterial host. Several high-throughput techniques have been employed in recent years to determine the optimum conditions for soluble protein production by using fusion partners, various strains, or different temperature conditions (Nozach et al., 2013).

To overcome the disulfide bond formation problem in *E. coli*, several strains overexpressing DsbC or DsbA proteins that promote disulfide bond formation have been employed. Following implementation of this strategy, many eukaryotic proteins were successfully produced in their correct folded structure (Joly et al., 1998; Kurokawa et al., 2001; Zhuo et al., 2014).

Herein, due to the importance of DsbC on the solubilization of the disulfide-rich proteins, TNFR1 protein that is normally forms inclusion bodies, was produced in the SHuffle cell line that developed for DRP. However, TNFR1 was not obtained in soluble form, which could be due to the Shuffle cell line's ability to produce more disulfide bonded protein than wild-type strains, but at a lower yield and quality.

Previously, it is reported that DsbC is the best fusion partner among the 12 studied fusion partners (Nozach et al., 2013). For this reason, it is decided to construct a fusion vector containing both DsbC and TNFR1 to be able to produce TNFR1 in a soluble form. Different host strains, temperature and induction (IPTG or auto induction) conditions were performed to optimize production of soluble TNFR1, which showed that the higher yield was obtained when the *E. coli* Rosetta (DE3) strain was grown in auto-induction medium (ZYP-5052) at 17°C for 24 hours.

Disulfide bond oxidation in non-reducing cytoplasm is said to be inefficient and sluggish (Saaranen & Ruddock, 2013). The optimization trials using different strains showed that the strains with oxidizing cytoplasm, such as Rosetta-gami and SHuffle T7, were found to be unefficient in producing TNFR1 in a soluble form, but Rosetta (DE3) cells with reducing cytoplasm provided a higher yield. The reason why the strains with oxidizing cytoplasm were found inefficient might be related to the fact that they are less robust than other *E. coli* strains and frequently have different growth parameters (Nozach et al., 2013). Furthermore, while oxidation can occur in the non-reducing cytoplasm by transferring disulfide bonds from oxidized thioredoxin to folding proteins, this is inefficient and slow (Saaranen & Ruddock, 2013). In a *trxB*-/*gor* background, correct folding has been thought to be deleterious to yield, and as a result, these strains have been shown to have exceedingly poor yields (Hatahet et al., 2010).

As an initial stage in purification, affinity chromatography (Ni-NTA affinity) method was used (Figure 3.9, Figure 3.10). The elution fractions chosen based on the SDS-PAGE results and purification chromatogram were pooled and dialyzed with Tris buffer. As a second step of purification, the purity of fusion protein was increased using anion exchange chromatography (Figure 3.11, Figure 3.12).

Purification of the protein was maintained by using a binding buffer containing 8M urea, which favored to reduce hydrophobic interactions and increase the binding of the target to the affinity column. Since no reducing agents was used during the purification, the disulfide bonds were preserved. After the purification, the characterization studies were performed.

The structure of TNFR1 is known to be consist of β -strands and omega loops (Naismith et al., 1996), and a crystal structure of DsbC showing the that the protein has both α -helix and β -sheet structure is available in the Protein Data Bank (PDB ID: 1EEJ) (McCarthy et al., 2000). However, no structural analyses for the DsbC-TNFR1 fusion protein has been previously reported. Herein, the secondary structure analyses performed between 195 nm and 250 nm wavelengths revealed that α -helix structure was dominant in the DsbC-TNFR1 fusion protein, but β -sheet configurations were also present (Figure 3.13).

The purity of fusion protein was assessed using capillary electrophoresis showed that after first step of the purification 65% purity was obtained. As DsbC-TNFR1 protein was obtained in soluble form from *E. coli*, it is challenging to get rid of host cell proteins. Thus, an additional purification step was utilized, which increased the purity up to 92% (Figure 3.14).

The pI of the fusion protein was determined as 6.67 by using isoelectric focusing (Figure 3.15), which was consistent with its theoretical value.

The intact mass analysis predicted that DsbC-TNFR1 fusion protein has a monomeric structure (Figure 3.16). The reason of obtaining the monomeric structure was thought to be caused from disruption of the hydrophobic interaction due to the mobile phase supplemented with acetonitrile and formic acid. Furthermore, it is also verified that oligomerization of TNFR1 is mediated by the hydrophobic interactions rather than the disulfide bonds. The intact mass analyses also showed the lack of disulfide bond between the DsbC monomers and TNFR1 monomers.

It is previously reported that DsbC is found in the homodimer structure (McCarthy et al., 2000) and TNFR1 tends to dimerize and form aggregates (Albogami et al., 2021). Blue-Native PAGE analysis revealed that the DsbC-TNFR1 fusion protein has both dimer and different oligomeric structures (Figure 3.17).

A pull-down assay was performed for the functional analysis of the DsbC-TNFR1 fusion protein, and it was determined that the DsbC-TNFR1 protein interacted with TNF- α . It was also observed that the fusion structure did not interfere with the function of TNFR1, so, it was not needed to cleave DsbC and 6xHis tag from the target protein.

In conclusion, this study revealed a novel fusion system for TNFR1 production. By using DsbC-TNFR1 fusion system, TNFR1 was produced in soluble form, purified using tandem chromatography and characterized successfully.

REFERENCES

- Albogami, S., Todd, I., Negm, O., Fairclough, L. C., & Tighe, P. J.** (2021). Mutations in the binding site of TNFR1 PLAD reduce homologous interactions but can enhance antagonism of wild-type TNFR1 activity. *Immunology*, 164(3), 637–654. <https://doi.org/10.1111/imm.13400>
- Alksne, L. E., Keeney, D., & Rasmussen, B. A.** (1995). A mutation in either dsbA or dsbB, a gene encoding a component of a periplasmic disulfide bond-catalyzing system, is required for high-level expression of the *Bacteroides fragilis* metallo-beta-lactamase, CcrA, in *Escherichia coli*. *Journal of Bacteriology*, 177(2), 462–464. <https://doi.org/10.1128/jb.177.2.462-464.1995>
- Bader, M. W., Hiniker, A., Regeimbal, J., Goldstone, D., Haebel, P. W., Riemer, J., Metcalf, P., & Bardwell, J. C. A.** (2001). Turning a disulfide isomerase into an oxidase: DsbC mutants that imitate DsbA. *EMBO Journal*, 20(7), 1555–1562. <https://doi.org/10.1093/emboj/20.7.1555>
- Banaś, A. M., Bocian-Ostrzycka, K. M., & Jagusztyn-Krynicka, E. K.** (2019). Engineering of the Dsb (disulfide bond) proteins—contribution towards understanding their mechanism of action and their applications in biotechnology and medicine. *Critical Reviews in Microbiology*, 45(4), 433–450. <https://doi.org/10.1080/1040841X.2019.1622509>
- Bardwell, J. C., McGovern, K., & Beckwith, J.** (1991). Identification of a protein required for disulfide bond formation in vivo. *Cell*, 67(3), 581–589. [https://doi.org/10.1016/0092-8674\(91\)90532-4](https://doi.org/10.1016/0092-8674(91)90532-4)
- Berkmen, M.** (2012). Production of disulfide-bonded proteins in *Escherichia coli*. *Protein Expression and Purification*, 82(1), 240–251. <https://doi.org/10.1016/j.pep.2011.10.009>
- Berkmen, M., Boyd, D., & Beckwith, J.** (2005). The nonconsecutive disulfide bond of *Escherichia coli* phytase (AppA) renders it dependent on the protein-disulfide isomerase, DsbC. *Journal of Biological Chemistry*, 280(12), 11387–11394. <https://doi.org/10.1074/jbc.M411774200>
- Bessette, P. H., Cotto, J. J., Gilbert, H. F., & Georgieou, G.** (1999). In vivo and in vitro function of the *Escherichia coli* periplasmic cysteine oxidoreductase DsbG. *Journal of Biological Chemistry*, 274(12), 7784–7792. <https://doi.org/10.1074/jbc.274.12.7784>
- Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., & Cerretti, D. P.** (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. In *Nature* (Vol. 385, Issue 6618, pp. 729–733). <https://doi.org/10.1038/385729a0>

- Boissier, M.-C., Semerano, L., Challal, S., Saidenberg-Kermanac'h, N., & Falgarone, G.** (2012). Rheumatoid arthritis: From autoimmunity to synovitis and joint destruction. *Journal of Autoimmunity*, 39(3), 222–228. <https://doi.org/https://doi.org/10.1016/j.jaut.2012.05.021>
- Bremer, E.** (2013). Targeting of the tumor necrosis factor receptor superfamily for cancer immunotherapy. *ISRN Oncology*, 2013, 371854. <https://doi.org/10.1155/2013/371854>
- Brenner, D., Blaser, H., & Mak, T. W.** (2015). Regulation of tumour necrosis factor signalling: live or let die. *Nature Reviews Immunology*, 15(6), 362–374. <https://doi.org/10.1038/nri3834>
- Chan, F. K. M., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L., & Lenardo, M. J.** (2000). A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science*, 288(5475), 2351–2354. <https://doi.org/10.1126/science.288.5475.2351>
- Chen, G., & Goeddel, D. V.** (2002). TNF-R1 signaling: a beautiful pathway. *Science (New York, N.Y.)*, 296(5573), 1634–1635. <https://doi.org/10.1126/science.1071924>
- Chen, J., Song, J. L., Zhang, S., Wang, Y., Cui, D. F., & Wangt, C. C.** (1999). Chaperone activity of DsbC. *Journal of Biological Chemistry*, 274(28), 19601–19605. <https://doi.org/10.1074/jbc.274.28.19601>
- Chen, X., Bäuml, M., Männel, D. N., Howard, O. M. Z., & Oppenheim, J. J.** (2007). Interaction of TNF with TNF Receptor Type 2 Promotes Expansion and Function of Mouse CD4⁺CD25⁺ T Regulatory Cells. *The Journal of Immunology*, 179(1), 154–161. <https://doi.org/10.4049/jimmunol.179.1.154>
- Cheung, T. C., & Ware, C. F.** (2013). Tumor Necrosis Factor Receptors. In *Encyclopedia of Biological Chemistry: Second Edition* (2nd ed., Vol. 4). Elsevier Inc. <https://doi.org/10.1016/B978-0-12-378630-2.00358-3>
- David J. MacEwan.** (2002). A matter of life and death. *Journal of the American Dental Association* (1939), 133(2), 142–143. <https://doi.org/10.14219/jada.archive.2002.0127>
- Fathi-Roudsari, M., Akhavian-Tehrani, A., & Maghsoudi, N.** (2016). Comparison of three Escherichia coli strains in recombinant production of reteplase. *Avicenna Journal of Medical Biotechnology*, 8(1), 16–22.
- Fischer, R., Kontermann, R. E., & Maier, O.** (2015). Targeting sTNF/TNFR1 signaling as a new therapeutic strategy. *Antibodies*, 4(1), 48–70. <https://doi.org/10.3390/antib4010048>
- Gleason, F. K., & Holmgren, A.** (1988). Thioredoxin and related proteins in procaryotes. *FEMS Microbiology Reviews*, 4(4), 271–297. <https://doi.org/10.1111/j.1574-6968.1988.tb02747.x>
- Grivennikov, S. I., Tumanov, A. V., Liepinsh, D. J., Kruglov, A. A., Marakusha, B. I., Shakhov, A. N., Murakami, T., Drutskaya, L. N., Förster, I., Clausen, B. E., Tessarollo, L., Ryffel, B., Kuprash, D. V., & Nedospasov, S. A.** (2005). Distinct and Nonredundant In Vivo Functions of TNF Produced by T Cells and Macrophages/Neutrophils:

- Protective and Deleterious Effects. *Immunity*, 22(1), 93–104. <https://doi.org/https://doi.org/10.1016/j.immuni.2004.11.016>
- Guo, Z.-Y., Jia, X.-Y., & Feng, Y.-M.** (2004). Replacement of the interchain disulfide bridge-forming amino acids A7 and B7 by glutamate impairs the structure and activity of insulin. *Biological Chemistry*, 385(12), 1171–1175. <https://doi.org/10.1515/BC.2004.151>
- Haebel, P. W., Goldstone, D., Katzen, F., Beckwith, J., & Metcalf, P.** (2002). The disulfide bond isomerase DsbC is activated by an immunoglobulin-fold thiol oxidoreductase: Crystal structure of the DsbC-DsbDa complex. *EMBO Journal*, 21(18), 4774–4784. <https://doi.org/10.1093/emboj/cdf489>
- Hatahet, F., Nguyen, V. D., Salo, K. E. H., & Ruddock, L. W.** (2010). Disruption of reducing pathways is not essential for efficient disulfide bond formation in the cytoplasm of E. coli. *Microbial Cell Factories*, 9, 67. <https://doi.org/10.1186/1475-2859-9-67>
- Hawari, F. I., Rouhani, F. N., Cui, X., Yu, Z. X., Buckley, C., Kaler, M., & Levine, S. J.** (2004). Release of full-length 55-kDa TNF receptor 1 in exosome-like vesicles: A mechanism for generation of soluble cytokine receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 101(5), 1297–1302. <https://doi.org/10.1073/pnas.0307981100>
- Hehlgans, T., & Pfeffer, K.** (2005). The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: Players, rules and the games. *Immunology*, 115(1), 1–20. <https://doi.org/10.1111/j.1365-2567.2005.02143.x>
- Idriss, H. T., & Naismith, J. H.** (2000). TNF α and the TNF receptor superfamily: Structure-function relationship(s). *Microscopy Research and Technique*, 50(3), 184–195. [https://doi.org/10.1002/1097-0029\(20000801\)50:3<184::AID-JEMT2>3.0.CO;2-H](https://doi.org/10.1002/1097-0029(20000801)50:3<184::AID-JEMT2>3.0.CO;2-H)
- Jang, D. I., Lee, A. H., Shin, H. Y., Song, H. R., Park, J. H., Kang, T. B., Lee, S. R., & Yang, S. H.** (2021). The role of tumor necrosis factor alpha (Tnf- α) in autoimmune disease and current tnf- α inhibitors in therapeutics. *International Journal of Molecular Sciences*, 22(5), 1–16. <https://doi.org/10.3390/ijms22052719>
- Joly, J. C., Leung, W. S., & Swartz, J. R.** (1998). Overexpression of Escherichia coli oxidoreductases increases recombinant insulin-like growth factor-I accumulation. *Proceedings of the National Academy of Sciences of the United States of America*, 95(6), 2773–2777. <https://doi.org/10.1073/pnas.95.6.2773>
- Joly, J. C., & Swartz, J. R.** (1997). In vitro and in vivo redox states of the Escherichia coli periplasmic oxidoreductases DsbA and DsbC. *Biochemistry*, 36(33), 10067–10072. <https://doi.org/10.1021/bi9707739>
- Kadokura, H., & Beckwith, J.** (2010). Mechanisms of oxidative protein folding in the bacterial cell envelope. *Antioxidants and Redox Signaling*, 13(8), 1231–1246. <https://doi.org/10.1089/ars.2010.3187>

- Kadokura, H., Katzen, F., & Beckwith, J.** (2003). Protein disulfide bond formation in prokaryotes. *Annual Review of Biochemistry*, 72, 111–135. <https://doi.org/10.1146/annurev.biochem.72.121801.161459>
- Kong, B., & Guo, G. L.** (2014). Soluble expression of disulfide bond containing proteins FGF15 and FGF19 in the cytoplasm of *Escherichia coli*. *PLoS ONE*, 9(1), 1–8. <https://doi.org/10.1371/journal.pone.0085890>
- Kriegler, M., Perez, C., DeFay, K., Albert, I., & Lu, S. D.** (1988). A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell*, 53(1), 45–53. [https://doi.org/https://doi.org/10.1016/0092-8674\(88\)90486-2](https://doi.org/https://doi.org/10.1016/0092-8674(88)90486-2)
- Kurokawa, Y., Yanagi, H., & Yura, T.** (2000). Overexpression of protein disulfide isomerase DsbC stabilizes multiple-disulfide-bonded recombinant protein produced and transported to the periplasm in *Escherichia coli*. *Applied and Environmental Microbiology*, 66(9), 3960–3965. <https://doi.org/10.1128/AEM.66.9.3960-3965.2000>
- Kurokawa, Y., Yanagi, H., & Yura, T.** (2001). Overproduction of Bacterial Protein Disulfide Isomerase (DsbC) and Its Modulator (DsbD) Markedly Enhances Periplasmic Production of Human Nerve Growth Factor in *Escherichia coli*. *Journal of Biological Chemistry*, 276(17), 14393–14399. <https://doi.org/10.1074/jbc.M100132200>
- Lobstein, J., Emrich, C. A., Jeans, C., Faulkner, M., Riggs, P., & Berkmen, M.** (2016). Erratum to: SHuffle, a novel *Escherichia coli* protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm. *Microbial Cell Factories*, 15(1), 1–16. <https://doi.org/10.1186/s12934-016-0512-9>
- Locksley, R. M., Killeen, N., & Lenardo, M. J.** (2001). The TNF and TNF Receptor Superfamilies: Integrating Mammalian Biology. *Cell*, 104(4), 487–501. [https://doi.org/https://doi.org/10.1016/S0092-8674\(01\)00237-9](https://doi.org/https://doi.org/10.1016/S0092-8674(01)00237-9)
- Loetscher, H., Pan, Y. C., Lahm, H. W., Gentz, R., Brockhaus, M., Tabuchi, H., & Lesslauer, W.** (1990). Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell*, 61(2), 351–359. [https://doi.org/10.1016/0092-8674\(90\)90815-v](https://doi.org/10.1016/0092-8674(90)90815-v)
- Luettig, B., Decker, T., & Lohmann-Matthes, M. L.** (1989). Evidence for the existence of two forms of membrane tumor necrosis factor: an integral protein and a molecule attached to its receptor. *Journal of Immunology (Baltimore, Md. : 1950)*, 143(12), 4034–4038.
- Martin, J. L., Bardwell, J. C. A., & Kuriyan, J.** (1993). Crystal structure of the DsbA protein required for disulphide bond formation in vivo. *Nature*, 365(6445), 464–468. <https://doi.org/10.1038/365464a0>
- McCarthy, A. A., Haebel, P. W., Törrönen, A., Rybin, V., Baker, E. N., & Metcalf, P.** (2000). Crystal structure of the protein disulfide bond isomerase, DsbC, from *Escherichia coli*. *Nature Structural Biology*, 7(3), 196–199. <https://doi.org/10.1038/73295>
- Merdanovic, M., Clausen, T., Kaiser, M., Huber, R., & Ehrmann, M.** (2011). Protein quality control in the bacterial periplasm. *Annual Review of*

Microbiology, 65, 149–168. <https://doi.org/10.1146/annurev-micro-090110-102925>

- Missiakas, D., Georgopoulos, C., & Raina, S.** (1994). The *Escherichia coli* dsbC (xprA) gene encodes a periplasmic protein involved in disulfide bond formation. *EMBO Journal*, 13(8), 2013–2020. <https://doi.org/10.1002/j.1460-2075.1994.tb06471.x>
- Monaco, C., Nanchahal, J., Taylor, P., & Feldmann, M.** (2015). Anti-TNF therapy: past, present and future. *International Immunology*, 27(1), 55–62. <https://doi.org/10.1093/intimm/dxu102>
- Naismith, J. H., Devine, T. Q., Brandhuber, B. J., & Sprang, S. R.** (1995). Crystallographic evidence for dimerization of unliganded tumor necrosis factor receptor. *Journal of Biological Chemistry*, 270(22), 13303–13307. <https://doi.org/10.1074/jbc.270.22.13303>
- Naismith, J. H., Devine, T. Q., Kohno, T., & Sprang, S. R.** (1996). Structures of the extracellular domain of the type I tumor necrosis factor receptor. *Structure*, 4(11), 1251–1262. [https://doi.org/10.1016/S0969-2126\(96\)00134-7](https://doi.org/10.1016/S0969-2126(96)00134-7)
- Nozach, H., Fruchart-Gaillard, C., Fenaille, F., Beau, F., Ramos, O. H. P., Douzi, B., Saez, N. J., Moutiez, M., Servent, D., Gondry, M., Thaï, R., Cuniasse, P., Vincentelli, R., & Dive, V.** (2013). High throughput screening identifies disulfide isomerase DsbC as a very efficient partner for recombinant expression of small disulfide-rich proteins in *E. coli*. *Microbial Cell Factories*, 12(1), 1–16. <https://doi.org/10.1186/1475-2859-12-37>
- Puimège, L., Libert, C., & Van Hauwermeiren, F.** (2014). Regulation and dysregulation of tumor necrosis factor receptor-1. *Cytokine and Growth Factor Reviews*, 25(3), 285–300. <https://doi.org/10.1016/j.cytogfr.2014.03.004>
- Raina, S., & Missiakas, D.** (1997). Making and breaking disulfide bonds. *Annual Review of Microbiology*, 51, 179–202. <https://doi.org/10.1146/annurev.micro.51.1.179>
- Ramos-Casals, M., Brito-Zerón, P., Soto, M.-J., Cuadrado, M.-J., & Khamashta, M. A.** (2008). Autoimmune diseases induced by TNF-targeted therapies. *Best Practice & Research. Clinical Rheumatology*, 22(5), 847–861. <https://doi.org/10.1016/j.berh.2008.09.008>
- Rietsch, A., & Beckwith, J.** (1998). The genetics of disulfide bond metabolism. *Annual Review of Genetics*, 32, 163–184. <https://doi.org/10.1146/annurev.genet.32.1.163>
- Rietsch, A., Belin, D., Martin, N., & Beckwith, J.** (1996). An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. *Proceedings of the National Academy of Sciences*, 93(23), 13048–13053. <https://doi.org/10.1073/pnas.93.23.13048>
- Rietsch, A., Bessette, P., Georgiou, G., & Beckwith, J.** (1997). Reduction of the periplasmic disulfide bond isomerase, DsbC, occurs by passage of electrons from cytoplasmic thioredoxin. *Journal of Bacteriology*,

179(21), 6602–6608. <https://doi.org/10.1128/jb.179.21.6602-6608.1997>

- Saaranen, M. J., & Ruddock, L. W.** (2013). Disulfide bond formation in the cytoplasm. *Antioxidants and Redox Signaling*, 19(1), 36–43. <https://doi.org/10.1089/ars.2012.4868>
- Sevier, C. S., & Kaiser, C. A.** (2002). Formation and transfer of disulphide bonds in living cells. *Nature Reviews Molecular Cell Biology*, 3(11), 836–847. <https://doi.org/10.1038/nrm954>
- Vandenabeele, P., Declercq, W., Beyaert, R., & Fiers, W.** (1995). Two tumour necrosis factor receptors: structure and function. *Trends in Cell Biology*, 5(10), 392–399. [https://doi.org/10.1016/S0962-8924\(00\)89088-1](https://doi.org/10.1016/S0962-8924(00)89088-1)
- Wajant, H., Pfizenmaier, K., & Scheurich, P.** (2003). Tumor necrosis factor signaling. *Cell Death & Differentiation*, 10(1), 45–65. <https://doi.org/10.1038/sj.cdd.4401189>
- Wajant, H., & Siegmund, D.** (2019). TNFR1 and TNFR2 in the Control of the Life and Death Balance of Macrophages. *Frontiers in Cell and Developmental Biology*, 7. <https://doi.org/10.3389/fcell.2019.00091>
- Walczak, H.** (2011). TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. *Immunological Reviews*, 244(1), 9–28. <https://doi.org/https://doi.org/10.1111/j.1600-065X.2011.01066.x>
- Xu, J., Chakrabarti, A. K., Tan, J. L., Ge, L., Gambotto, A., & Vujanovic, N. L.** (2007). Essential role of the TNF-TNFR2 cognate interaction in mouse dendritic cell-natural killer cell crosstalk. *Blood*, 109(8), 3333–3341. <https://doi.org/10.1182/blood-2006-06-026385>
- Zhang, L., Chou, C. P., & Moo-Young, M.** (2011). Disulfide bond formation and its impact on the biological activity and stability of recombinant therapeutic proteins produced by Escherichia coli expression system. *Biotechnology Advances*, 29(6), 923–929. <https://doi.org/10.1016/j.biotechadv.2011.07.013>
- Zhuo, X. F., Zhang, Y. Y., Guan, Y. X., & Yao, S. J.** (2014). Co-expression of disulfide oxidoreductases DsbA/DsbC markedly enhanced soluble and functional expression of reteplase in Escherichia coli. *Journal of Biotechnology*, 192(Part A), 197–203. <https://doi.org/10.1016/j.jbiotec.2014.10.028>

APPENDICES

APPENDIX A : PDB ID of DsbC

APPENDIX B : PDB ID of TNFR1 Extracellular Domain

APPENDIX C : Replicates of the Results



APPENDIX A

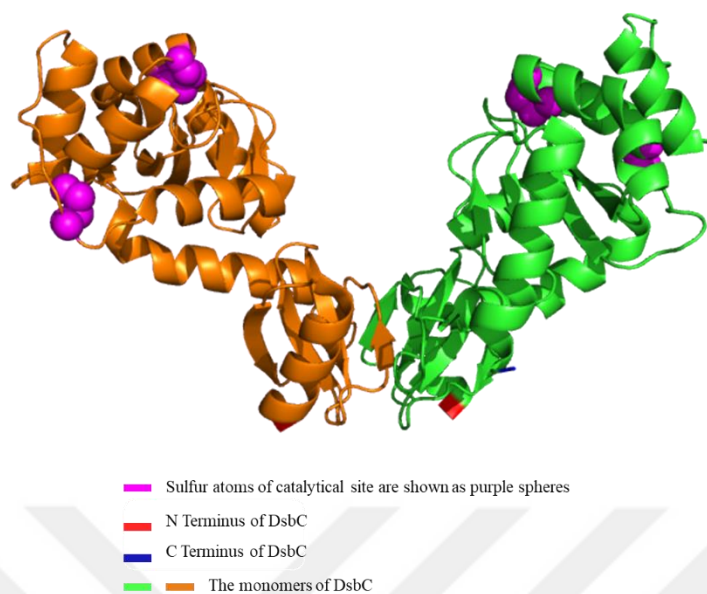


Figure A.1: The DsbC was adopted from the crystal structure with PDB id 1EXT.

APPENDIX B

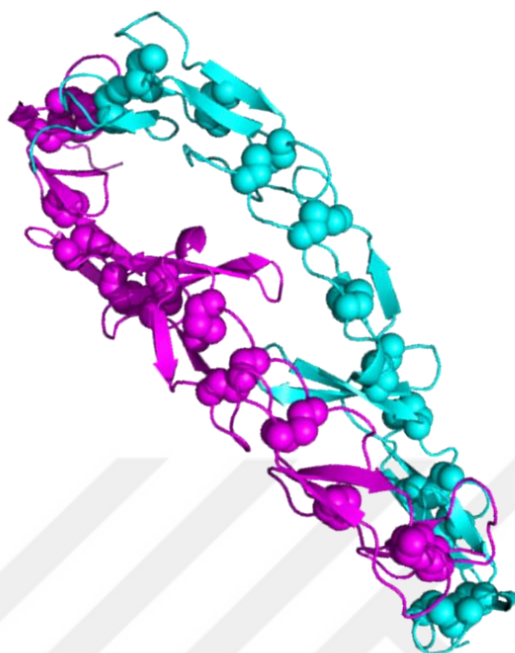


Figure B.1: The extracellular conformation of TNFR1 was adopted from the crystal structure with PDB id 1EXT

APPENDIX C

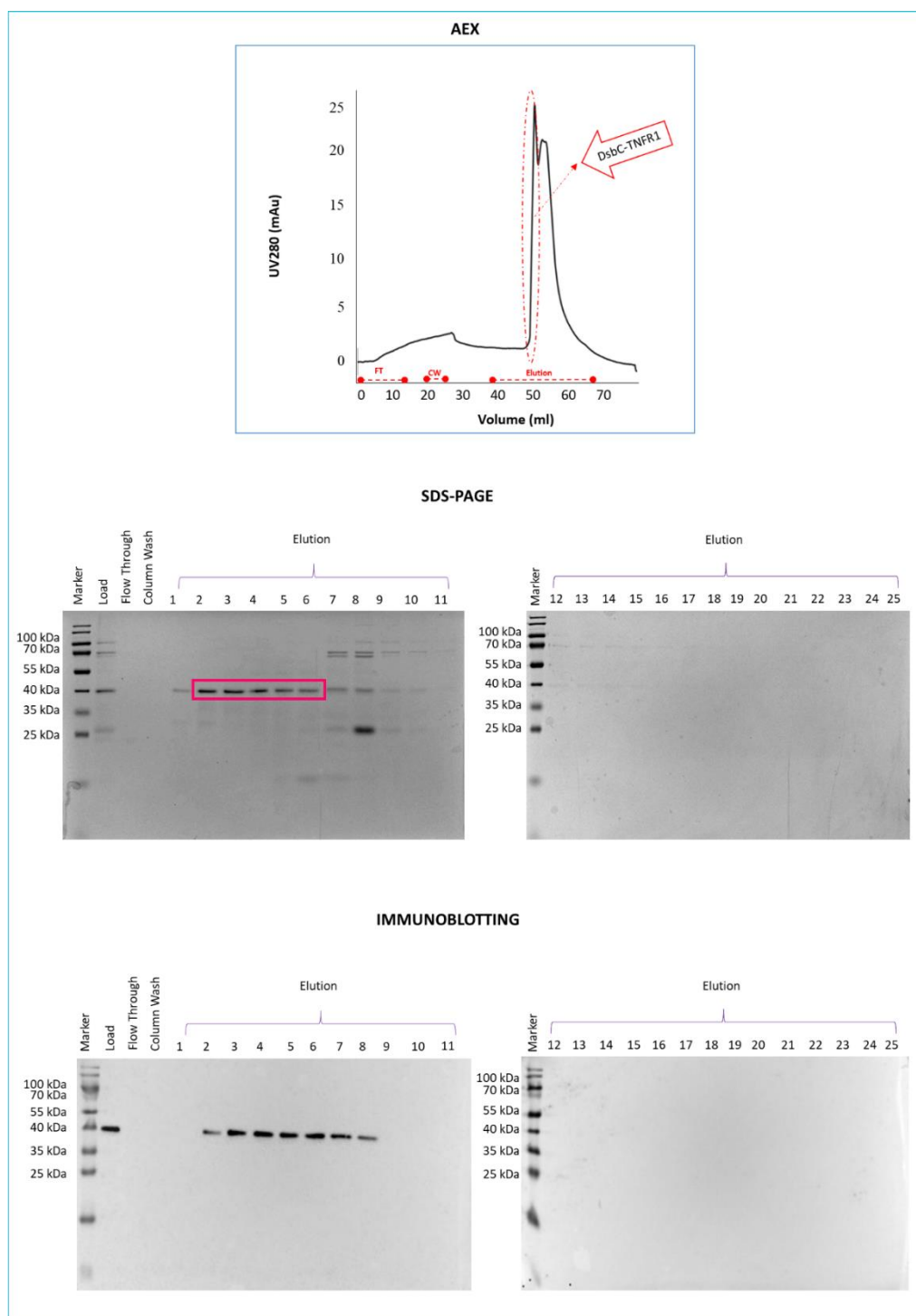


Figure C.1: Replicate of purification of DsbC-TNFR1. SDS-PAGE (12%) and immunoblotting results of the AEX purification of DsbC-TNFR1. The elution fractions are indicated numerically. The collected fractions were shown. FT is Flow-through, CW is Column Wash.

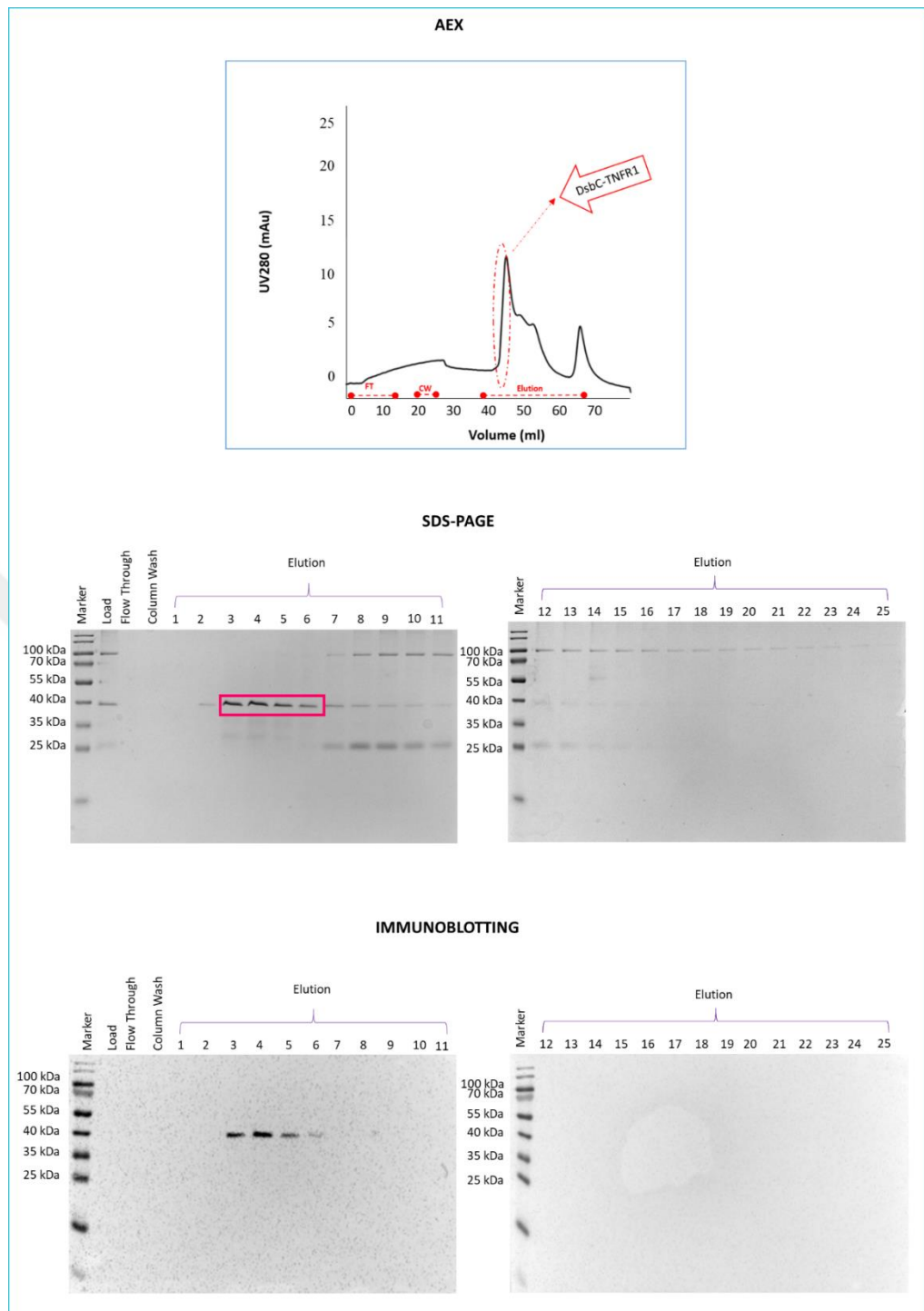


Figure C.2: Replicate of purification of DsbC-TNFR1. SDS-PAGE (12%) and immunoblotting results of the AEX purification of DsbC-TNFR1. The elution fractions are indicated numerically. The collected fractions were shown. FT is Flow-through, CW is Column Wash.

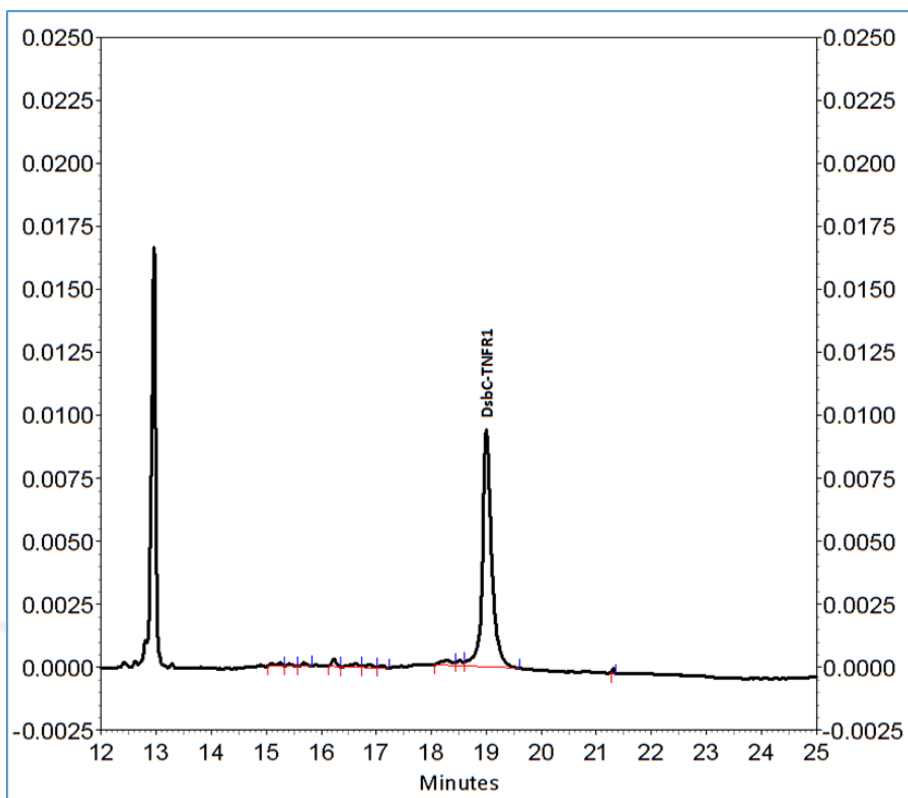


Figure C.3: Replicate of capillary electrophoresis purity analysis of DsbC-TNFR1. Electropherograms belongs to two step purification.

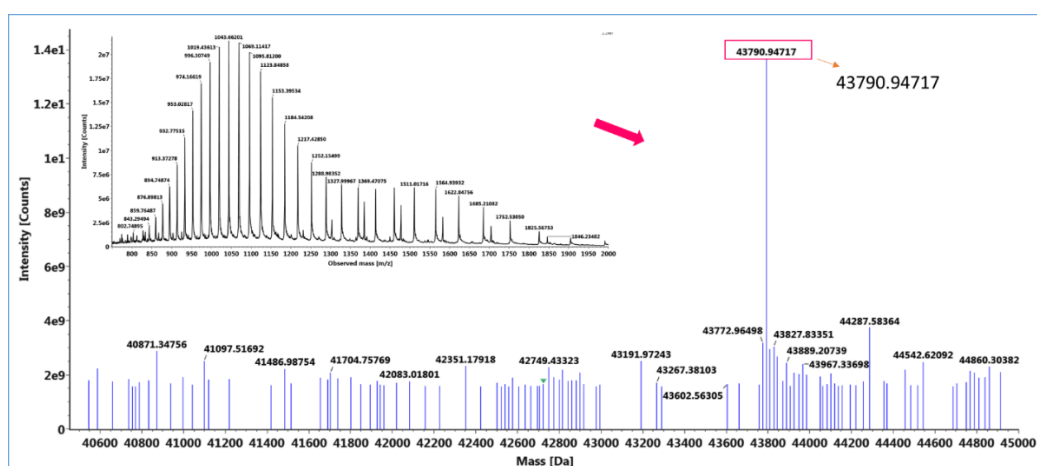


Figure C.4: Replicate of intact mass analysis of DsbC-TNFR1 protein. LC/MS spectrum of DsbC-TNFR1 (left) and the deconvoluted mass spectra of peaks corresponds to DsbC-TNFR1 peak (right).

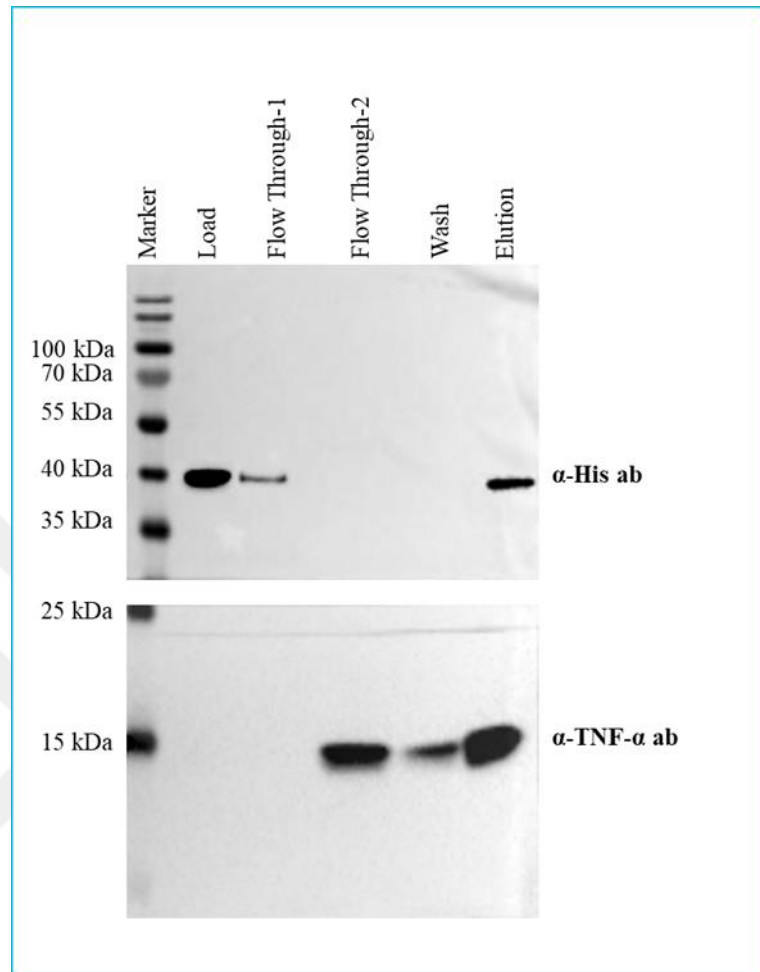


Figure C.5: Replicate of *in-vitro* pull down assay. The immunoblotting outcome of a pull-down assay using purified DsbC-TNFR1 (bait) and commercial TNF- α (prey) protein. Nickel bound agarose beads were incubated with pure DsbC-TNFR1 (load lane). Then unbound proteins were removed (flow-through 1 lane) and the agarose beads were washed with binding buffer. Pure TNF- α were loaded onto the bead and incubated. After incubation, unbound ligand was discarded via centrifugation (flow-through 2 lane) and the bead washed with binding buffer (wash lane). After washing, bead-bound proteins were eluted with elution buffer containing imidazole (elution lane)



CURRICULUM VITAE

Name Surname : Derya HATİPOĞLU



EDUCATION :

- **B.Sc.** : 2019, Istanbul University,
Faculty of Science and Literature,
Department of Molecular Biology and Genetics

PROFESSIONAL EXPERIENCE AND REWARDS:

- 2019 - 2021 Istanbul Technical University, Istanbul - TUBITAK 1007 KAMAG Project: Biosimilar Drug Development, Scholar
- 2021 Istanbul Technical University, Istanbul - TUBITAK 1001, Scholar

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- Yasar, S., **Hatipoğlu, D.**, Çakır, Ö., (2019). Determination of the effect of Curcumin on superoxide dismutase and catalase activity in tomato, MolBiyKon'19, İstanbul Technical University, Turkey
- Yalçinkaya, Y. D., **Hatipoğlu, D.**, Doğanay, D. G., (2020). Development of a Practical Capillary Zone Electrophoresis Method to Determine Charge Variant Profiles Under Forced Degradation Conditions for Monoclonal Antibodies9., İlaç Kimyası Kongresi, Antalya
- **Hatipoğlu, D.**, Dınler Doganay, G., (2021). Expression of Soluble Tumor Necrosis Factor Receptor 1 (TNFR1) in *E. coli*, BIO Turkey'21 International Biotechnology Congress.