T.C.

DOKUZ EYLÜL UNIVERSITY

IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

RECOMBINANT PRODUCTION AND CHARACTERIZATION OF MATRIX METALLOPROTEINASES - 1 AND - 13 FOR TREATMENT OF MACULAR CORNEAL DYSTROPHY

SANİYE GÜL KAYA

MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

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THESIS CODE: DEU.IBG.MSc-2019850019

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Supervisor: Assoc. Prof. Dr. Gökhan Karakülah

Co-Supervisor: Dr. Sibel Kalyoncu Uzunlar

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'RECOMBINANT PRODUCTION AND CHARACTERIZATION OF MATRIX METALLOPROTEINASES - *1* AND - 13 FOR TREATMENT OF MACULAR CORNEAL DYSTROPHY' on the date of 22/06/2021.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

CHST6: Carbohydrate sulfotransferase 6

ECM: Extracellular matrix

MCD: Macular corneal dystrophy

MMP: Matrix Metalloproteinase

Ni-NTA: Nickel - nitrilotriacetic acid

SDM: Site-directed mutagenesis

RT-PCR: Real time PCR

TIMP: Tissue inhibitor of metalloproteinases

RECOMBINANT PRODUCTION AND CHARACTERIZATION OF MATRIX METALLOPROTEINASES - 1 AND - 13 FOR TREATMENT OF MACULAR CORNEAL DYSTROPHY

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ABSTRACT

Macular corneal dystrophy (MCD) is a rare hereditary disease characterized by the accumulation of insoluble aberrant substances in the corneal stroma. MCD may result in a decrease of visual acuity that can lead to blindness. There is still no cure for the disease, and corneal transplantation is possible but it can lead to likelihood of dystrophy recurrence and graft-related risks. Matrix metalloproteinases -1 and -13, which are necessary for corneal reshaping, have been reported to be decreased in MCD. The goal of this study is to provide a new treatment method for MCD by topical application of MMP-1 and MMP-13 to the cornea. MMP-1 and MMP-13 enzymes were being produced using recombinant DNA technology as a preliminary step in this research. Human MMP-1 and MMP-13 genes were cloned into pET-17b plasmid for *E.coli* expression. Auto-induction media wass used for bacterial expression (*E. coli*). Using a novel auto-induction methodology, our data indicated that active MMP-1 and MMP-13 enzymes may be generated in *E. coli* periplasm and released into production media. Promising protein yields were found, and production parameters were further optimized. Purified MMP-1 and MMP-13 enzymes were characterized by biophysical and biochemical tests. Protein thermal shift assay and enzyme kinetics assay were used to examine the profiles of recombinant enzymes. For MMP-1 and MMP-13 enzymes, this work presents a unique autoinduction recombinant production and characterization approach. These enzymes are expected to be used as a treatment method for MCD after completion of proof-of-concept studies.

Keywords: Macular corneal dystrophy, Matrix metalloproteinase - 1, Matrix metalloproteinase - 13, recombinant enzyme production

MATRİX METALLOPROTEİNAZ - 1 VE - 13'ÜN MAKÜLER KORNEA DİSTROFİSİ TEDAVİSİ İÇİN REKOMBİNANT ÜRETİMİ VE KARAKTERİZASYONU

Saniye Gül Kaya, Dokuz Eylül Üniversitesi, İzmir Uluslararası Biyotıp ve Genom Enstitüsü, saniyegul.kaya@msfr.ibg.edu.tr

ÖZET

Maküler kornea distrofisi (MKD), stromada anormal, çözünmeyen maddelerin kademeli olarak birikmesi ile karakterize nadir görülen bir genetik hastalıktır. MKD ilerleyen durumlarda görme kaybına neden olabilir. Şu anda, bu hastalık için özel bir tedavi yoktur ve distrofinin tekrarlama riski ve nakille ilişkili riskler nedeniyle kornea nakli kesin bir çözüm değildir. Ekstrasellüler matrikste bulunan matriks metalloproteinaz -1 ve -13 (MMP) enzimleri korneanın yeniden şekillenmesi ve anormal birikimlerin giderilmesi için önemli olup MKD'de azaldığı gösterilmiştir. Bu araştırmanın amacı, korneaya MMP-1 ve MMP-13 enzimlerini uygulayarak MKD için yeni bir terapötik strateji geliştirmektir. MMP-1 ve MMP-13 enzimlerinin üretilmesi bu amaca yönelik ilk adımdır. Rekombinant DNA teknolojisi ile insan MMP-1 ve MMP-13 genleri, pET-17b plazmidine klonlandı. Bakteriyel (*E. coli*) ekspresyon, kendiliğinden indüklenebilir otoindüksiyon ortamında gerçekleştirilmiştir. MMP-1 ve MMP-13 enzimleri afinite kromatografisi ile saflaştırıldı. Veriler, aktif MMP-1 ve MMP-13 enzimlerinin yeni bir oto-indüksiyon tekniği kullanılarak üretim ortamına salındığını göstermektedir. Üretim parametreleri optimize edilerek umut verici protein verimleri elde edilmiştir. Rekombinant enzimin analizi, enzim aktivite testi ve protein termal stabilite testi ile gerçekleştirilmiştir. Bu çalışma, MMP-1 ve MMP-13 enzimleri için yeni bir üretim yöntemi (oto-indüksiyon) ve karakterizasyon teknikleri üzerine yeni bakış açıları kazandırır. Preklinik özellikleri daha fazla inceledikten sonra, bu enzimin MKD için terapötik bir strateji olarak kullanılması amaçlanmıştır.

Anahtar kelimeler: Maküler kornea distrofisi, Matrix metalloproteinaz - 1, Matrix metalloproteinaz - 13, rekombinant enzim üretimi

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1. INTRODUCTION AND AIM

1.1. Statement and Importance of the Problem

Macular corneal dystrophy (MCD) is a rare genetic disease that has no cure. Currently, only treatment strategy is corneal transplantation. The decreased amount of Matrix metalloproteinases-1 and -13 has been shown in MCD disease pathway. Our hypothesis is that when MMP-1 and MMP-13 are given topically to the MCD cornea, a novel treatment strategy would be developed. In this study, MMP-1 and MMP-13 were produced recombinantly via *E.coli* and purified. After purification steps, bio-chemical/physical characterizations were performed.

1.2. Aim of the Study

This study aims to develop a recombinant production and characterization process for human MMP-1 and MMP-13 enzymes to be used for a novel MCD treatment strategy.

1.3. Hypothesis of the Study

Recombinant human MMP-1 and MMP-13 enzymes can provide a new treatment by **s**topping / regressing abnormal deposits in MCD disease (Murab, Chameettachal, & Ghosh, 2016).

2. GENERAL INFORMATION

2.1. Matrix Metalloproteinases

Matrix Metalloproteinases are zinc and calcium dependent endopeptidases that degrade extracellular matrix components or/and non-ECM components. The zinc in the active site bounds to three histidine residues and it is highly conserved as a HEXXHXXGXXH sequence. Also they have a conserved cysteine switch motif PRCGXPD, in which the cysteine sulfhydryl Zn^{2+} group chelates the active site, preventing MMPs from being active (Cui et al., 2017) (Laronha & Caldeira, 2020). Although all MMPs have a conserved prodomain and a catalytic domain with a zinc-binding active site and three histidine residues, also they contain distinct domains that perform separate functions, as seen in Figure 1. There are 25 MMPs known. MMPs are divided into two categories depending on their cellular location: "secreted" and "membrane-bound". They are also classified into six categories based on their structure and substrate specificity: Collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), membrane-type MMPs (MMP-14, -15, -16, -17, -24, -25),

stromelysins (MMP-3, -10, -11), matrilizines (MMP-7, -26), others (MMP-12, -19, -21, -22, -23, -26 and -28) (Sekton, 2010).

MMPs have different physiological roles in different tissues. They have roles in angiogenesis, tissue repair, immune response and responsibilities in cell profiferation, migration and differentiation. They are expressed and produced in different tissues since play a essential role in ECM remodeling. Gene expression, zymogen activation (cysteine switch mechanism), and inactivation of enzymes with inhibitors such as endogenous tissue inhibitors (TIMPs) all play a role in MMP activity under physiological conditions. TIMPs, which are basic antagonists of MMPs, bind non-covalently to the active site of the enzyme to prevent it from being activated (Bode et al., 1999). TIMPs are divided into four sub-types (TIMP-1, -2, -3, and -4), and their expression is regulated by cytokines, growth factors, and cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 and -6 (IL-1 and IL-6) during tissue remodeling and development. Overexpression of MMPs can lead to diseases like atherosclerosis, fibrosis, and cancer, so the equilibrium between MMP and TIMP processes in tissues is important for ECM homeostasis and also diseases such as cancer, atherosclerosis and fibrosis (Gearing et al., 1994) (Li, Tay, & Yiu, 2020)(Vandenbroucke et al., 2013).

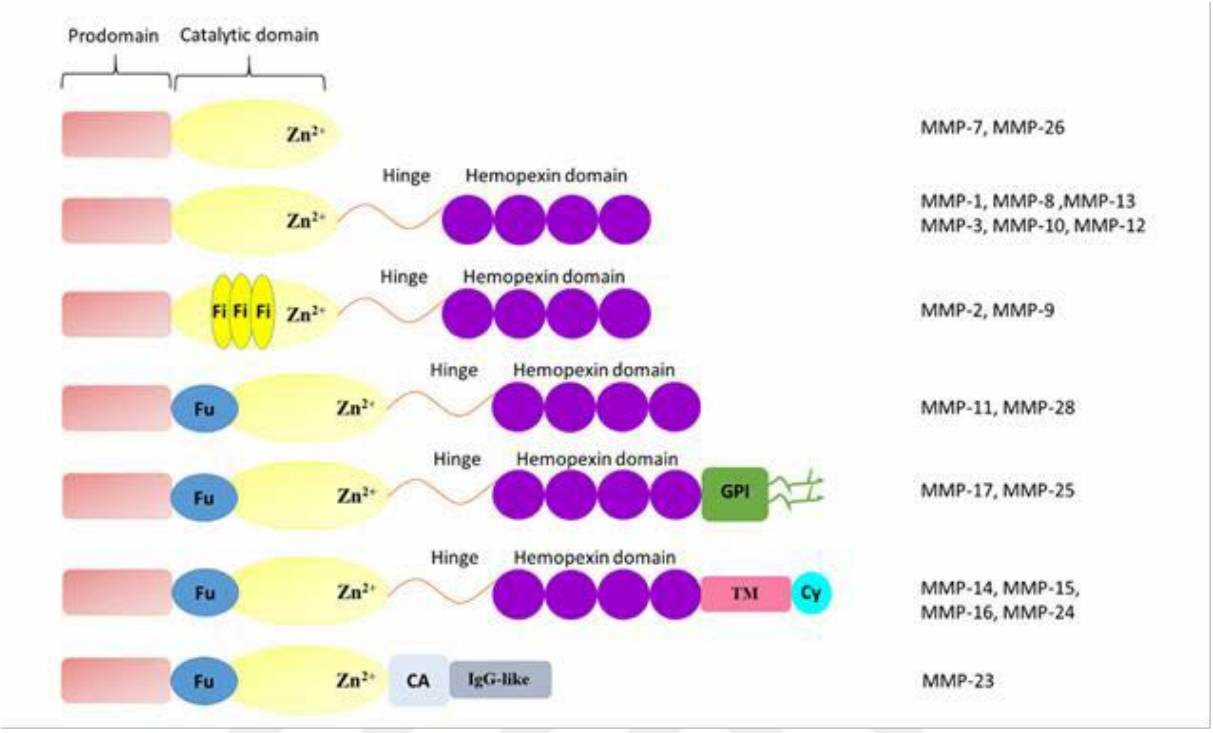

Figure 1. Domains of MMPs(Kaya et al., 2021).With the cysteine transfer, the prodomain acts as an auto-inhibitor, keeping MMPs inactive. The catalytic domain provides the necessary enzymatic action with a zinc-binding motif (HEXXH). The substrate positioning/binding responsible hemopexin (HPX)-like domain is connected to the catalytic domain through a flexible hinge region. Although some MMP groups lack HPX-like domains, others have anchoring domains for fibronectin (Fi), furin (Fu), transmembrane (TM), cytosolic (Cy), cysteine array (CA), immunoglobulin-like (IgG-like), and glycosylphospatidylinositol (GPI) (for membrane attachment)(Hadler-Olsen, Fadnes, Sylte, Uhlin-Hansen, & Winberg, 2011).

2.1.1. Collagenases

Collagenases consist of MMP-1 (interstitial collagenase), -8 (neutrophil collagenase), -13 and -18. MMP-1 shows enzymatic activity to collagen and gelatin. Also, it cleaves proMMP-9 to activate it. MMP-1 is upregulated in many autoimmune and inflammatory conditions. Inflammatory cytokines including tumor necrosis factor (TNF) and interleukin-1 (IL-1) increase MMP-1 production. MMP-13, also called collagenase-3, is responsible for degrading type II collagen. MMP-13 was first discovered in connective tissue, specifically cartilage and forming bone. MMP-13, on the other hand, has been found in epithelial and neuronal cells. MMP-13 is overexpressed in osteoarthritis patients' cartilage tissues, and increased MMP-13 expression in chondrocytes can lead to osteoarthritis growth. They have also various roles in several diseases and tissues (Cui et al., 2017).

Collagenases cleave some ECM proteins and other soluble proteins, but they are best known for cleaving fibrillar collagen types I, II, III, IV, and XI into two distinct segments, 1/4 C-terminal and 3/4 N-terminal. They first unwinds triple helical collagen before hydrolyzing the peptide bonds in a two-step procedure. The hemopexin domain is needed for the cleavage of native fibrillar collagen, while the catalytic domain is capable of cleaving non-collagen substrates (Laronha & Caldeira, 2020).

2.2. Macular Corneal Dystophy

Macular corneal dystrophy (MCD) is a rare disease, a corneal stromal dystrophy which is inherited by autosomal recessive. It is caused by mutations in the carbohydrate sulfotransferase (CHST6) gene that affects keratan sulfate (KS) metabolism and causes accumulation of non-sulfated KS in keratocytes and corneal stroma. It is characterized by cloudy cornea in the first decade of life and progressive loss of vision in the middle of the life, needing corneal transplantation. Nevertheless, recurrence after corneal transplantation and complications such as graft rejection and post-transplantation endothelial cell loss have been observed. Thus novel therapeutic strategies for MCD is needed (Zheng et al., 2020) (Feizi, Karjou, Abbasi, Javadi, & Azari, 2020).

CHST6 gene encodes for an enzyme called GlcNAc-6-sulphotransferase (C-GlcNAc6ST). Mutation on this gene results in development of inactive form of C-GlcNAc6ST enzyme responsible for the keratan sulfate's sulfation (Murab et al., 2016). In terms of the availability of sulfated KS epitopes in serum and cornea, MCD has three immunophenotypes (I, IA, and II). Sulfated corneal KS-specific mAb 5-D-4, which recognizes disulfated KS disaccharides, is used in clinical studies to diagnose MCD immunophenotypes, the use of this antibody to stain the samples reveals three potential MCD immunophenotypes. Immunophenotype I identified with undetectable result with the antibody both in serum and cornea (i.e. no antigen is detected), immunophenotype IA identified with undetectable or very low serum titers of KS and no KS antigen in stromal ECM while a few KS is detected withinside the keratocytes. In immunophenotype II, the serum stage of KS antigen is below regular or regular and the KS antigen withinside the stroma may be observed (Cursiefen et al., 2001)(Liu, Smith, Bowling, Jonasson, & Klintworth, 2006).

2.3. Recombinant Protein Production

Proteins are one of the most important macromolecules in biotechnology and they have crucial applications in biopharmaceutical market. As a drug, proteins are complex to synthesize when compared to the small molecules. Protein-based drugs are produced in host cells by recombinant DNA technology. They are called recombinant proteins because the DNA encoding for protein is given recombinantly into the host cells. One of the first recombinant protein produced by this system, was an human insulin hormone and was synthesized by *Escherichia coli (E.coli*) in 1977 (Overton, 2014).

Recombinant protein production is carried out mainly in prokaryotic or eukaryotic host cells. The most commonly used and preferred host in prokaryotic production is *E.coli* (Chen, 2012). Expression in eukaryotic cells are yeast, plant, and mammalian cells. Prokaryotic or eukaryotic expression systems have many different variables. Both expression systems have unique advantages and disadvantages. Prokaryotic expression systems have many advantages such as easy cultivation, relatively fast and inexpensive protein expression, rapid growth of cells and easy isolation and purification of produced proteins. However, obtaining functional protein can be challenging in prokaryotic systems depending on the characteristics of produced protein. It is common that proteins can be precipitated as inclusion bodies in bacteria. During purification, inclusion bodies can be harmful for protein (Fahnert, Lilie, & Neubauer, 2004). In addition, the post-translational modifications required for some proteins to be functional are absent in bacterial systems. Sometimes the protein produced can have a toxic effect on the bacterial cell (Feder & Hofmann, 1999).

Yeast and mammalian Chinese Hamster Ovary (CHO) cells are the leading eukaryotic expression systems. CHO cells have more complicated and expensive processes than *E.coli.* Yeasts, like bacterial cells, are easy-to-cultivate and easy-to-study organisms. In addition, it can also perform cell-specific post-translational modifications (Overton, 2014).

In recombinant protein production, bacterial expression systems are more preferred than eukaryotes because of their fast, efficient, cheap, and easy protein expression. There are more than one strain used for expression. Some of these are *Bacillus brevis, Bacillus megaterium, Bacillus*

subtilis, *Caulobacter crescentus*, other strains, and, most importantly, *Escherichia coli* BL21 (Terpe, 2006).

Escherichia coli (E.coli) is the most well-known and used microorganism because they are easy to culture, having simple and cheap medium ingredients, fast and easy to produce, morphologically, genetically and physiologically well-researched and an organism that offers possibilities such as strain selection in the widest scope. Apart from these, many proteins have the capacity to be expressed in high efficiency in *E.coli*. The ability of cloning and expression in different cells depends on the desired protein and the selected expression system (Sørensen & Mortensen, 2005).

E.coli BL21 (DE3) strain is used for this thesis. Generally, this strain is responsible for the expression of regions under the control of the T7 expression system. It expresses the desired protein under the *lac* promoter. The *lac* promoter is conventionally induced by isopropyl β-d-1-thiogalactopyranoside (IPTG) (Hausjell, Weissensteiner, Molitor, Halbwirth, & Spadiut, 2018). IPTG is now the most common inducer for recombinant protein expression in the *lac* operator/repressor mechanism. IPTG could be toxic for the cell. Autoinduction medium can be used instead of IPTG to induce a T7 or pTrc promoter. To produce autoinduction medium, glucose, glycerol, and lactose are added to a buffered broth. The glucose is used first by the *E. coli* as they grow. They are obligated to consume lactose when the glucose runs out, which stimulates expression of the T7 promoter. As glucose is almost depleted, the carbon source switches to lactose, which is followed by a regioselective chemical transition of lactose to allolactose, which serves as a catalyst for transcription activation by releasing the repressor. Autoinduction has many advantages (Studier, 2005),(Kataoka & Takasu, 2021). Auto-induction is more favorable than IPTG induction because the expression strain is only inoculated on auto-induction medium and expression is ensured without the need for culture growth monitoring and addition of inducers at the appropriate time. According to the unit culture volume, autoinduction with high density cultures produces a better outcome than IPTG induction (Studier, 2005).

2.4. Protein Purification Methods

Correct folding and solubility of the recombinantly expressed protein in the cell is an important criterion for protein quality. Recombinant proteins can easily be expressed in *E.coli.* In some situations, produced proteins can form aggregates in cell and become insoluble form of proteins, it is called inclusion body (IB). Chaperones are responsible for the correct folding of the protein. In the absence of sufficient chaperone expressing, the proteins are misfolded and form these inclusion bodies. Many methods are still being developed to prevent the formation of inclusion bodies. In addition, inclusion bodies may be formed in response to cellular stress. The formation of inclusion bodies is difficult to predict because it depends on more than one parameter such as; host microorganism, protein type, production and purification conditions (Fahnert et al., 2004).

Isolation of therapeutic active protein from inclusion bodies from *E. coli* cells consists of solubilization of protein aggregates, refolding and purification of the solubilized protein. High concentrations (6–8 M) of chaotropic reagents such as urea, guanidine hydrochloride, and detergents such as SDS are used to solubilize semi-pure protein aggregates and other contaminants. This keeps cysteine residues reduced, preventing the development of non-native intra- or inter-disulfide bonds in highly condensed protein solutions at alkaline pH. The important step is refolding step. After unfolding the protein, refolding is been processed slowly in appropriate buffer (S. M. Singh & Panda, 2005)

There are many chromatographic methods used in lab-scale and industrial scale of protein purification such as; gel filtration, ion exchange, affinity, etc. (Łącki & Riske, 2020). Affinity chromatography has been used to purify proteins for many years. It is based on specific adhesion processes (adsorption, desorption) between biomolecules and ligands.

Immobilized metal ion affinity chromatography (IMAC) is one of the most popular and well-known affinity chromatographic purification methods for recombinant proteins. This technique has been fabricated by using metal ions immobilized on a support or beads to purify proteins in aqueous solutions. It is based on affinity to cysteine and histidine of metal ions such as Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+}. Proteins can be specifically bound to the metal ions from specific sites such as cysteine, histidine and tryptophan on their surface, it is usually called tag. Well-known and used application

is the purification of polyhistidine labeled proteins through IMAC (Hundred, Garvey, Cremer, & Sussdorf, 2009).

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Cell Strains

E.coli BL21 (DE3) pLysS Competent Cells (Agilent, 200132) were used as expression strain.

3.1.2. Plasmids

MMP-1 & MMP-13's active and hemopexin sites were cloned into pet17b plasmids. The plasmids used in this study are listed in Table 1 and shown in Figure 2 for MMP-1 and Figure 3 for MMP-13.

Table 1. Plasmids used in this study.

Name	**Source**
pet17b_MMP-1	Genscript
pet17b_MMP-13	Genscript

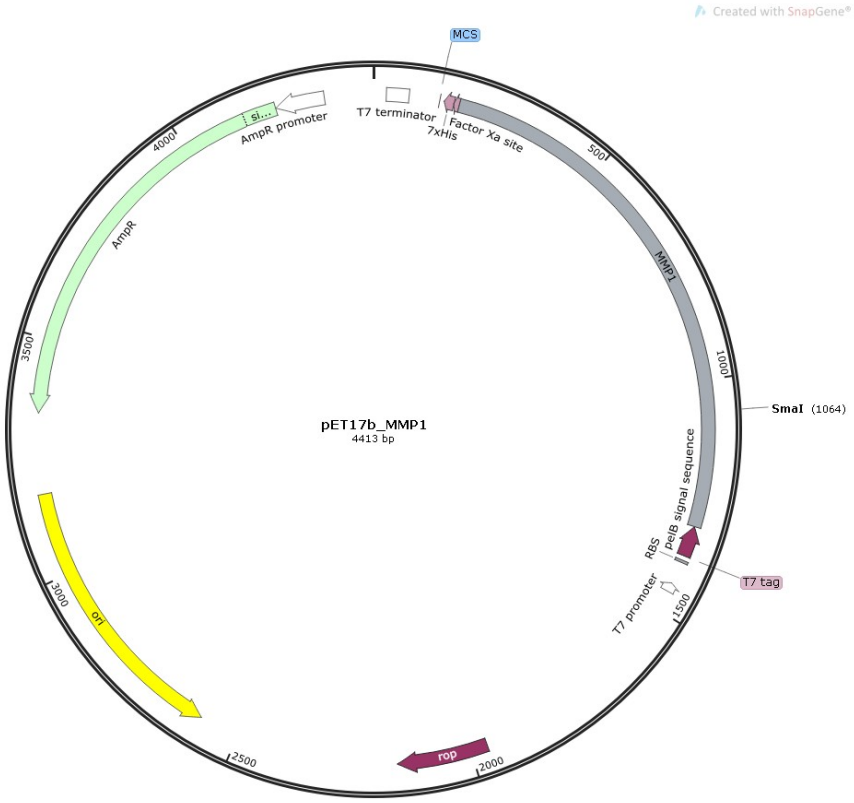

Figure 2. pET17b MMP-1 vector map.

Figure 3. pET17b MMP-13 vector map.

3.1.3. Reagents for Bacterial Experiments

Recipes of materials used in bacterial expression experiments are listed in Table 2.

Table 2. Recipes used in bacterial expression.

Reagents	**Recipe**
LB Broth Media	1,55 gr LB Broth dissolved in 100 mL ddH_2O. Sterilized in autoclave. Added 100 µM AMP, 25 µM CAM antibiotics before use.
LB Agar Plates	4 gr LB Agar dissolved in 100 mL ddH_2O. Sterilized in autoclave. Added 100 µM AMP, 25 µM CAM antibiotics before use.
AMP: Ampicillin Stock (100 mg/ml stock)	1 gr ampicillin was dissolved in 10 ml ddH_2O.

	Filtered and aliquots were stored at -20 ^{o}C.
CAM: Chloramphenicol Stock (25 mg/ml stock)	0,25 gr chloramphenicol was dissolved in 10 mL ethanol. Aliquots were stored at -20 ^{o}C.
20X NPS (pH 6.8)	6.6 gr $(NH_4)_2SO_4$ 13.6 gr KH_2PO_4 14.2 gr Na_2HPO_4 dissolved in 100 mL dH_2O.
50X 50/52	%25 Glycerol (25 mL) %2.5 Glucose (2.5 gr) %10 Alpha-Lactose monohydrate (10 gr) dissolved in 100 mL dH_2O. Used filter for sterilization.
Autoinduction Media (pH 6.8)	For 300 mL: 3 gr Tryptone, 1,5 gr Yeast Extract dissolved in 277,5 mL dH_2O. After autoclave sterilization, 15 mL of 20 X NPS (pH:6.8), 6 ml of 50 X 50/52, 300 µl of 1 M MgSO4, 300 µl of CAM, 300 µl of AMP are added.
SOC Media	For 100 ml: 0.5 gr NaCI, 20 gr Tryptone, 5 gr Yeast Extract dissolved in 950 mL dH_2O and sterilized via autoclave. Before use add 5 ml 2 M $MgCI_2$.

3.1.4. Chemicals & Commercial Kits

Chemicals and Commercial kits used in thesis are listed in Table 3.

Table 3. Chemicals and Commercial Kits used in experiments.

Name	**Catalog Number**	**Vendor**
TGX FastCast Acrylamide Kit, 12%	1610175	Biorad
Gelcode blue safe protein stain	LSG-89898	Thermo
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	LSG-A32955	Thermo
Triton X-100	T8787	SIGMA
Trizma® base	T1503	SIGMA
NaCl	9690335000	ISOLAB
NaOH	SHY999.500	Bioshop Canada
Potassium Phosphate Monobasic	062834243	SIAL AFGSCI
Potassium Chloride	9600361000	ISOLAB
Di-Sodium Hydrogen Phosphate Anhydrous	71636	SIGMA
Yeast Extract	YEX555.205	Bioshop Canada
A-Lactose Monohydrate	L2643	SIGMA
Tryptone	95039	SIGMA
LB Broth	L1900	SIGMA
LB Agar	LBA408	Bioshop Canada
Skim milk	SKI400.500	Bioshop Canada
HisPur™ Ni-NTA Resin	88222	Thermo

Imidazole	I202	SIGMA
Guanidine Hydrochloride	BP178-1	Fisher
Ampcillin, Sodium Salt	AMP201.25	Bioshop Canada
Chloramphenicol	CLR201.10	Bioshop Canada
Sypro Orange	S5692	SIGMA
Precision Plus Protein™ Dual Color Standard	1610374	Bio-Rad
SDS	L3771	SIGMA
Glycine	GLN001.500	Bioshop Canada

3.1.4. Western Blot Antibodies

Western blot antibodies used in this thesis are listed in Table 4.

Table 4. Western blot antibodies.

Name	**Catalog Number**	**Vendor**
Anti His Taq Antibody	GTX30500	GENETEX
Anti MMP-1 Primary Antibody	AB6002	Merck Millipore
Anti MMP-13 Primary Antibody	SC-515284	Santa Cruz Biotechnology
Anti-rabbit IgG (H+L) (DyLight™ 680 Conjugate)	5366	Cell Signaling
Anti-mouse IgG (H+L) (DyLight™ 800 4X PEG Conjugate)	5257	Cell Signaling
HRP- Conjugated Anti-Mouse Secondary	SAB3701066	Sigma

3.1.5. Enzyme Activity Assay Substrates

Enzyme activity assay substrates are listed in Table 5.

Table 5. Enzyme Activity Assay Subtrates.

Name	Catalog Number	Vendor
MMP-13 Substrate, Fluorogenic	SCP0189	SIGMA
MMP Substrate III, Fluorogenic	44256	Calbiochem

3.1.6. Buffers & Solutions

Buffers and solutions used in this thesis are listed in Table 6.

Table 6. Buffer & solutions that used in this study.

Buffer	Recipe
20X PBS (pH 7.4)	160 gr NaCI, 4 gr KCI, 4.8 gr KH_2PO_4, 28.8 gr Na_2HPO_4 dissolved in 1 L dH_2O. Use filter for sterilization.
10X TBS	24.23 gr Trizma HCl 80.06 g NaCl. Mix in 800 mL of dH_2O. pH to 7.6 with pure HCl. Complete to 1 L.
TBST	Dilute 10X TBS to 1X with dH_2O. Add 1:1000 Tween-20.
10X Running Buffer	250 mM Tris base 1,9 M Glycine 1% SDS in 1 L dH_2O

	pH~ 8,3
10X Transfer Buffer	250 mM Tris base 1,9 M Glycine 1 L dH_2O pH~ 8,3
Western Blot Blocking Solution	5% Skimmed milk powder in 1X TBST.
Antibody Dilution Solution	1% Skimmed milk powder (w/v) in 1X TBS-T.
2 M Imidazole	34.04 gr Imidazole dissolved in 250 mL H_2O.
1 M $MgSO_4$	6.02 gr MgSO4 dissolved in 50 mL ddH_2O. Sterilize via filter.
10X Purification Buffer (200 mL)	5.67 gr Sodium Phosphate, 58,44 gr NaCl dissolved in 200 mL dH_2O.
Buffer A	25 mL 10X PB 2.5 mL 2 M Imidazole 222.5 mL dH_2O
Buffer B	5 ml 10X PB 6.25 ml 2 M Imidazole 38.75 ml dH_2O
Enzyme Activity Assay Buffer	50 mM TrisHCl, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 0.1% PEG600, 0.05% Brij-35
Lysis Buffer	50 mM TrisHCl, %1 Triton X-100,

	10 mM $MgCl_2$, 1 Tablet protease inhibitor for each 10 mL (2 mL lysis buffer for 200 mg wet cell)
8 M GdnCl	382.12 g GdnCl is dissolved in approximately 200 mL dH_2O. (Total volume should be 500 mL)
Dialysis Buffer	50 mM Tris-HCl buffer (pH 7.6) with 10 mM $CaCl_2$, 0.1 mM ZnCl, 300 mM NaCl

3.1.7. Site Directed Mutagenesis Reagents

The reagents used in SDM are given in Table 7.

Table 7. Site directed mutagenesis reagents.

Name	Catalog Number	Vendor
DpnI Enzyme	R0176S	NEB
KOD Hot Start DNA Polymerase	71086	Sigma
NucleoSpin Plasmid MiniPrep Kit	740588	MN
PCR-Clean-Up Kit	28104	Qiagen

3.1.8. Equipments

Equipments used in this study are listed in Table 8.

Table 8. Equipments.

Equipments	Vendors
Basic Power Supply	Biorad
MySpinTM 6 Mini Centrifuge	Thermo Scientific

Incubator MaxQ 4000	Thermo Scientific
Microwave Oven	Beko
pH Meter	Hanna
SimpliAmp Thermal Cycler	Applied Biosystems
Vortex Mixer	Thermo Scientific
Electrophoresis Gel System	Biorad
Centrifuge 5810R	Eppendorf
Centrifuge MicroCL 17R	Thermo Scientific
Microplate Reader Synergy H1	Biotek
Nanodrop 2000	Thermo Scientific
Li-Cor Odyssey Imaging System	Li-Cor
ABI 7500 Fast thermal cycler	Thermo Scientific
GelDoc XR+ with Image Lab Software	Biorad
Water Bath and Lid	Nüve
TissueLyser II	QIAGEN
Orbital Shaker	Thermo
Avanti J-25i Centrifuge	Beckman Coulter

3.2. METHODS

3.2.1. Visual Abstract of the Methods

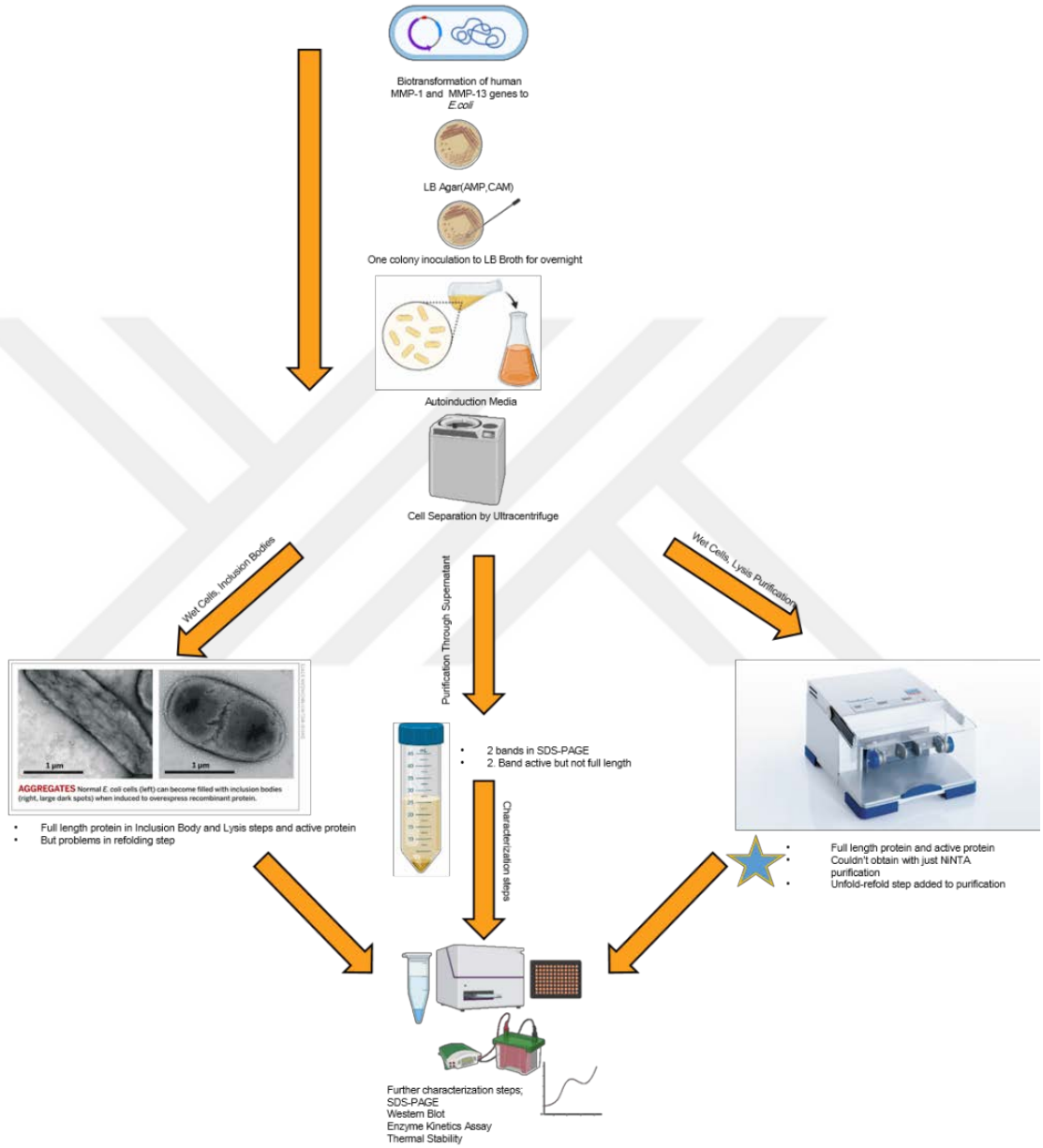

Figure 4. Overall scheme of methods.

3.2.2. Time and Place of the Study

All experiments were performed between October 2019 – June 2021 at Izmir Biomedicine and Genome Center.

3.2.3. Plasmid design

Plasmids containing genes for human MMP-1 and MMP-13 proteins have been designed at SnapGene. Those genes were cloned into the pET17b expression plasmid (gene synthesis was done with GenScript) by adding the necessary restriction sites and tags containing both catalytic and hemopexin regions of MMP-1 and MMP-13. The designed human MMP-1 and MMP-13 protein sequences are given in Figure 5.

Figure 5. Aminoacid sequences of MMP-1 and MMP-13.

3.2.4. Cell cultivation and recombinant protein production

For the production of MMP-1 and MMP-13 in bacterial cells, firstly, a *E.coli* BL21 (DE3) pLysS 100 µL cells in tube was taken and thawed on ice. After taking 50 µL of the thawed cells, 1.2 µL 0.4 M β-Mercaptoethanol was added and kept on ice for 10 more minutes. Then 3-5 µL of plasmid from 110 ng/µL stock was added to this mixture and kept on ice for 20 minutes. This mixture was subjected to thermal shock at 42 °C for 42 seconds in a water bath heated to 42 °C, and then immediately left on ice for 2 minutes. 500 µL of SOC medium was added to the mixture and shaken at 250 rpm for 1 hour at 37 °C. This mixture was spread over the surfaces of 20 mL LB-Agar plates containing 100 µg /mL ampicillin and 35 µg/mL chloramphenicol. Agar plates were incubated at 37 °C for 18 hours. One of the colonies formed on agar surfaces was selected and grown for 18 hours in 20 mL LB-Broth containing ampicillin and chloramphenicol at 37 °C

with shaking at 225 rpm. From the ready inoculum, the 300 mL auto-induction production medium in a 500 mL flask containing 100 µg /mL ampicillin and 35 µg/mL chloramphenicol was inoculated with an initial cell density of 0.05 OD_{600} (optical density). Unless otherwise noted, autoinduction production was incubated at 18 °C with shaking at 250 rpm for 72 hours. Bacterial concentration was determined by measuring the cell at 600 nm wavelength with a 96-plate reader (Microplate Reader Synergy H1, Biotek).

3.2.5. Optimization Conditions

Temperature optimization: Production flasks at 4, 18, 30 °C were incubated at 250 rpm for different times. OD_{600} and pH values were checked during the production. The flasks at 4° C were done at cold room and followed for 1 week.The flasks at 18, 30 °C were done for 48 hours. Ni-NTA column purification from directly from supernatant was done for all flasks.

pH optimization: Production flasks at pH values of 6.8, 7.8 were incubated at 250 rpm for 48 hours. pH alteration was achieved by NPS in different pH. Ni-NTA column purification from directly from supernatant was done for all flasks.

Induction optimization: One flask in Autoinduction media, other flask in LB Broth medium were incubated at 18 °C with shaking at 250 rpm. IPTG (Isopropyl β-D-1-thiogalactopyranoside) induced production was done in 300 ml LB medium, when the cell concentration is in the range of 0.4-0.6 OD, by adding 300 µl of IPTG solution to the production environment. It has been brought to a concentration of 1 mM IPTG. Cells were exposed to IPTG medium for 4 hours. After finishing the production for both flasks, Ni-NTA column purification from directly from supernatant was done.

Time optimization: Production flasks at 18 °C were incubated for 48 and 72 hours at 250 rpm. Protein was purified both from 48th and 72nd hours via Ni-NTA column purification from directly from supernatant.

3.2.6. Sugar Analysis of the culture

SHIMADZU LC-2010 HPLC was used as a device, RID-10A refractive index detector was used as a detector, and Transgenomic ICSep ICE-ION-300 column was used as a column. For the method, the oven temperature was 72 °C, the flow was determined as isocratic 0.4 mL / min, the

mobile phase was 0.0085 N sulfuric acid. For sugar analysis, lactose, glucose, glycerol, acetic acid, methanol, ethanol standards were prepared and mixed as 10000 ppm. The mixed standard was then diluted 5 times in half to calculate the standard. Supernatant samples taken from MMP-1 and MMP-13 production media every 12 hours were filtered and loaded on HPLC. The assay for glucose, lactose and glycerol used in the medium was calculated for the supernatant samples.

3.2.7. Protein Purification

Supernatant purification

The culture terminated in the extraction medium was separated from the cells by centrifugation (11,000 x g, 30 minutes). Supernatant was passed through Ni-NTA resins (1 mL of resin per 100 mL of supernatant) by vacuum method. The resin was first washed with 100 ml of 1X PB buffer (pH 7.4), then with 100 mL of Buffer A (this is done to get rid of specific non-binding proteins). To separate the proteins from the resin, the resin was washed with 5 mL Buffer B and the filtrate collected. The collected proteins are concentrated in 1X PBS with Amicon Ultra 5 mL filters (Millipore®). Concentrations of purified proteins are measured by nanodrop device (absorbance at 280 nm).

Inclusion Body Purification

Centrifugation was used to collect the cells. Wet cells (200 mg) were suspended in 2 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X-100, 10 mM $MgCl_2$, and protease inhibitor (1 tablet in 10 mL solution, rest stored at -80 °C).Glass bead protocol was performed for 15 sec at 30 Hz for three times (Tissue Lyser II®), chilled for 1 min on ice after glass bead. Insoluble material was washed by sequentially suspending it in 1 mL each of the following solutions and centrifuging the washed mixtures: 5 min for each 11,000 x g:

1) 50 mM glycine buffer (pH 10.0), 10 mM EDTA, 1% Triton X-100 (3 times),

2) 50 mM glycine buffer (pH 10.0), 10 mM EDTA,

3) 50 mM Tris-HCl buffer (pH 8.0), 10 mM dithiothreitol,

4) deionized water (2 times).

The protein pellet was dissolved by suspending it in 1 mL of 50 mM Tris-HCl buffer (pH 7.6) plus 6 M guanidine, first homogenized by pipetting then kept it for 1h in orbital shaker at RT. After centrifugation, the supernatant was kept (~1 mL), 1 ml Buffer A with 20 mM Guanidinium Chloride was added. NiNTA resin (100 μL) was added and, incubated at 4 °C for 2 hours with orbital shaking. It was centrifuged at 5000 x g for 5 min, supernatant was discarded. Resin was washed by 2 times with 2 mL Buffer A with 20 mM Guanidium Chloride. It was centrifuged at 5000 x g for 5min, supernatant was discarded. Elution was applied with 2 mL Buffer B with 20 mM Guanidium Chloride. It was centrifuged at 5000 x g for 5 min, supernatant was kept. Elution was diluted by adding 8 mL Buffer B with 20 mM Guanidium Chloride (10 mL total). This 10 mL protein was put this in dialysis bag. It was dialysed in 4 L 50 mM Tris-HCl buffer (pH:7.6) with 10 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 300 mM NaCl in MEMBRA-CELL(SERVA) dialysis tube. Overnight dialysis was applied at 4 °C (in cold room) with slow stirring. Protein was concentrated with Amicon (Millipore) to ~250 μL, concentration was flash-frozen and aliquots were kept at –80 °C.

Lysis Purification

Cells were collected by centrifugation. Wet cells (200 mg) were suspended in 2 mL 50 mM Tris-HCl buffer (pH 8.0) plus 1% Triton X-100, 10 mM $MgCl_2$, + protease inhibitor (2 tablets in 40 mL buffer). 1.5 mL x 16 Eppendorf tubes were subjected to glass bead protocol in TissueLyser II® device. Chilled three times at 30 Hz for 15 seconds, 1 minute on ice after the glass bead. It was incubated at 4 °C for 2 hours with Nickel beads (1 mL beads). It was centrifuged at 5000 x g for 5 minutes, the supernatant was discarded. The resin was washed 2 times with 2 mL of 1X PB. It was centrifuged at 5000 x g for 5 minutes, the supernatant was discarded. The resin was washed 2 times with 2 mL of Buffer A. Centrifuged at 5000 x g for 5 minutes, the supernatant was discarded. Elution was applied with 2 mL of Buffer B. It was centrifuged at 5000xg for 5 minutes, supernatant was stored and buffer exchanged in Amicon with PBS.

Lysis Purification via unfold-refold

In the last step of lysis purification, it was treated with 4 M Guanidinium hydrochloride and then the folding of the protein was achieved by dialysis method explained in the previous section.

Dialysis buffer solution was prepared as 1 L. Dialysis was applied in 2 different steps. The first step buffer additionally contained 2 M Guanidinium hydrochloride and was applied for 18 hours. The second step buffer contains 50 mM TrisHCl, 10 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 300 mM NaCl and the second step was applied for approximately 1 hour. Performing dialysis in 2 steps protects the protein against precipitation.

3.2.8. Size Exclusion Chromatography

Separation was performed using size exclusion chromatography with a Superdex 75 pg (GE Healthcare) column via ÄKTA™ Avant. MMP-1 and MMP-13 proteins are seperated in 1X PBS, pH 7.4.

3.2.9. Western Blotting

Sample preparation

6x laemmli buffer was added to each sample once the concentrations were equalized. 30% β-Mercaptoethanol was added to laemmli buffer prior to use or 350 mM DTT added to the sample. The samples were boiled for 10 min at 95 ^{O}C and kept at -20 ^{O}C for further use.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE (%12) was applied for the samples. BIO-RAD TGX FastCast Acrilamide Kit (%12) was used (Table 9). About 500 mL 1X Running Buffer was added the tank.

Table 9. SDS-PAGE (%12) FastCast Acrilamide Kit Recipe.

	Resolver A	Resolver B	Stacker A	Stacker B	%10 APS	TEMED
Resolver	3 mL	3 mL			30 µL	3 µL
Stacker			1 mL	1 mL	10 µL	2 µL

Running

Samples were run at 90 V until they exited the stacking gel, after which they were run at 110 V. It took approximately 2 hours to complete the run.

Transfer of proteins to PVDF membrane

Tank was filled with 1X transfer buffer containing 20% methanol . The sandwich transfer mechanism (gel, PVDF membrane, filter paper, sponges) was set up. The sandwich was firmly held together by the plastic holder. In 1X transfer buffer, the transfer was conducted at 350 mA for 90 minutes.

Blocking

Membrane was cut properly and were placed in a western blot incubation tray. The membranes were blocked for 1 hour at room temperature with 5% skim milk powder dissolved in 1X TBS.

Blotting

Antibodies were diluted in 1% BSA in a proper dilution ranges. Dilutions for antibodies;

Primary antibodies: 1:2,000-1:5,000

Secondary antibodies: 1:10,000-1:15,000

Membranes were incubated with primary antibodies at 4 ^{O}C overnight. After primary antibody incubation, membranes were washed with 1X TBS-T for 10 min 3 times. They were incubated with secondary antibody, at room temperature for 1 hour then membranes were washed with 1X TBS-T for 10 min 3 times.

Imaging

The membranes were imaged at 700 and 800 channels using the Li-Cor Odyssey Clx imaging equipment. Image Studio software was used to examine the band signals.

3.2.10. Thermal shift assay

Thermal stability (T_m, melting temperature) of MMP-1 and MMP-13 was characterized by using Real Time PCR Device (ABI 7500 Fast Thermal cycler). SYPRO™ Orange (Invitrogen) detection dye was used, it binds to the hydrophobic parts of the proteins. Dye was diluted 1:1000 to 50X prior to use. 5 µM protein was added to the 5X dye and mixed (for optimizations 1-10X dye and 1-5 µM protein trials were made and the condition with the best signaling was selected).

The fluorescence signal of the protein mixture heated from 0 °C to 96 °C at certain intervals was recorded and its derivative was calculated, and its thermal stability (T_m) was determined. Graphics were plotted in OriginPro software.

3.2.11. Enzyme activity assay

Fluorogenic MMP-1 substrate (Calbiochem 444256) suitable for MMP-1, MMP-13 substrate (SIGMA SCP0189) suitable for MMP-13 were dissolved in DMSO. First, the excitation and emission wavelengths suitable for the substrate were determined (excitation 340 nm for MMP-1 substrate, emission 485, excitation 330 nm, emission 393 nm for MMP-13 substrate). After that, MMP-1/13 enzymes (50-100 nM) and enzyme activity assay buffer were mixed in 96-well black plates with fluorescent substrates of different concentrations (1-50 μM). Fluorescence readings were taken on the microplate reading device (BIOTEK-SYNERGY-H1) together with the necessary controls for MMP-1 and MMP-13 enzymes (after Ni-NTA purification) produced according to these wavelengths. GraphPad Prism software was used to analyse the data.

3.2.12. Primers and Site-Directed Mutagenesis

After trying to produce hemopexin and catalytic sites of the enzymes together, we have decided to produce only catalytic site. For that aim, we designed primers on SnapGene to add HindIII region to the catalytic and hemopexin site of the enzymes. We purchased forward and reverse primers from Eurofins. Table 11 shows primer sequences.

SDM is processed with commercial KOD enzyme kit. Ingredients for reaction is given in Table 11. The thermal cycle was applied in Table 12 at SimpliAmp Thermal Cycler. After reaction 0.6 μL DpnI enzyme and 3 μL 10X NEB Buffer were added and processed at 37 °C for 30 min PCR. Lastly PCR Clean-up procedure is performed via using Qiagen Clean-up Kit. 100 μl Buffer NTI was added to PCR-product. The sample was loaded into a column in a collection tube. For 30 seconds, centrifuged at 11,000 x g. The column was reinstalled after the storage tube was emptied. To wash the sample, 700 μl Buffer NT3 was applied and centrifuged at 11,000 x g for 30 seconds. To dry the column, the flow through was discarded and the tube was centrifuged for 1 minute at 11,000 x g. The DNA was eluted by placing the column into a new clean 1.5 mL tube, adding 30 μl Buffer NE, incubating for 1 minute, and centrifuging for 1 minute at 11,000 x g.

Table 10. KOD mix recipe.

Reagent	Volume
KOD 10X Master Mix	2.5 µL
Forward Primer	0.75 µL
Reverse Primer	0.75 µL
Ds-DNA template	1 µL
25 mM MgSO4	1.5 µL
KOD DNA Polymerase	0.5 µL
dNTP	2.5 µL
Sterile dH2O	Make 25 µL total

Table 11. Primers used in SDM.

Primers	Sequence (5'→3')
MMP-1_HPX_F	GTGGCGTCCCTCGATAAGCTTGTTCTTCCGACAG
MMP-1_HPX_R	CTGTCGGAAGAACAAGCTTATCGAGGGACGCCAC
MMP-13_HPX_F	GTGGCGTCCCTCGATAAGCTTGCACCATAATATGCTG
MMP-13_HPX_R	CAGCATATTATGGTGCAAGCTTATCGAGGGACGCCAC
MMP-1_CAT_F	CACGGGGTTTTGCGAAAGCTTGTATATAGCCTGGATG
MMP-1_CAT_R	CATCCAGGCTATATACAAGCTTTCGCAAAACCCCGTG
MMP-13_CAT_F	CGGGTCCTCGTCACCAAGCTTGTACAGACTCTGGATAC
MMP-13_CAT_R	GTATCCAGAGTCTGTACAAGCTTGGTGACGAGGACCCG

Table 12. SDM cyle procedure.

Cycle	Temperature (°C)	Time
1	95	2 min
18	95	10 sec
	55	30 sec
	72	30 sec per 1 kb

1	72	5 min

3.2.13. Mini Culture & Plasmid isolation

Confirmed colonies were grown overnight at 37 ºC, 250 rpm shaker in the 20 mL LB Broth media. At the end of the incubation, the cells were centrifuged in 50 mL tubes at 11100 g, +4 ºC for 10 minutes. After centrifugation, the supernatants were decanted and the pellets were saved for plasmid isolation. Plasmids were isolated by using MN NucleoSpin® Plasmid kit. For the elution of the plasmids, 50 µl of Elution buffer heated to 70 ºC was added to the center of the column. It was incubated for 1 min at room temperature and centrifuged for 1 min at 11,000 x g. After this step, 100 ng/µl 15 µl DNA samples is sent to Eurofins for sequencing.

3.3. Study Plan and Calender

All wet-lab experiments was conducted at İzmir Biomedicine and Genome Center, between on November 2019 and June 2021.

3.4. Ethics Committee Approval

We have taken a permission to conduct the experiments and studies from the Izmir Biomedicine and Genome Center non-interventional Ethical Committee. Our proposed study titled "Recombinant production and characterization of matrix metalloproteinases – 1 and 13 for treatment of macular corneal dystrophy".

Previous ethical committee approval date = 20.04.2020

Decision number= 2020-011

4. RESULTS

4.1. Optimization of the conditions for Protein Production

Produced proteins were purified via Ni-NTA chromatography method from 300 mL Autoinduction Media supernatant unless otherwise specified.

4.1.1. Temperature

For temperature optimization, 300 mL Autoinduction Media was incubated at 4°C (cold room) for one week, 18°C for 72 h, and 30 °C for 72 h at 250 rpm. After the protein purification from different conditions, protein yields are shown in Figure 6.

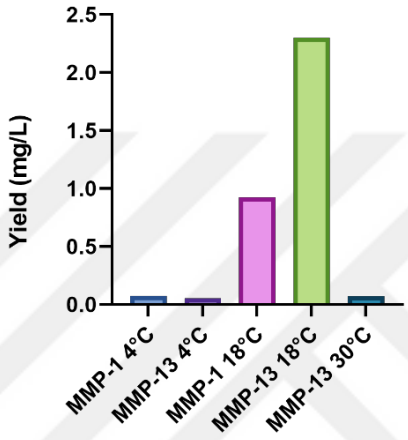

Figure 6. Yields under the temperature optimization.

After trying different temperature conditions, protein yield shows that the best temperature condition is 18 °C for the production process.

4.1.2. pH

In the Autoinduction Media, pH was adjusted by 20X NPS buffer. pH 6.8 and 7.8 were tried for MMP-13 production. Protein yield shows that optimum pH for this production process is 6.8 because total protein amount is higher according to SDS-PAGE analysis (Figure 7).

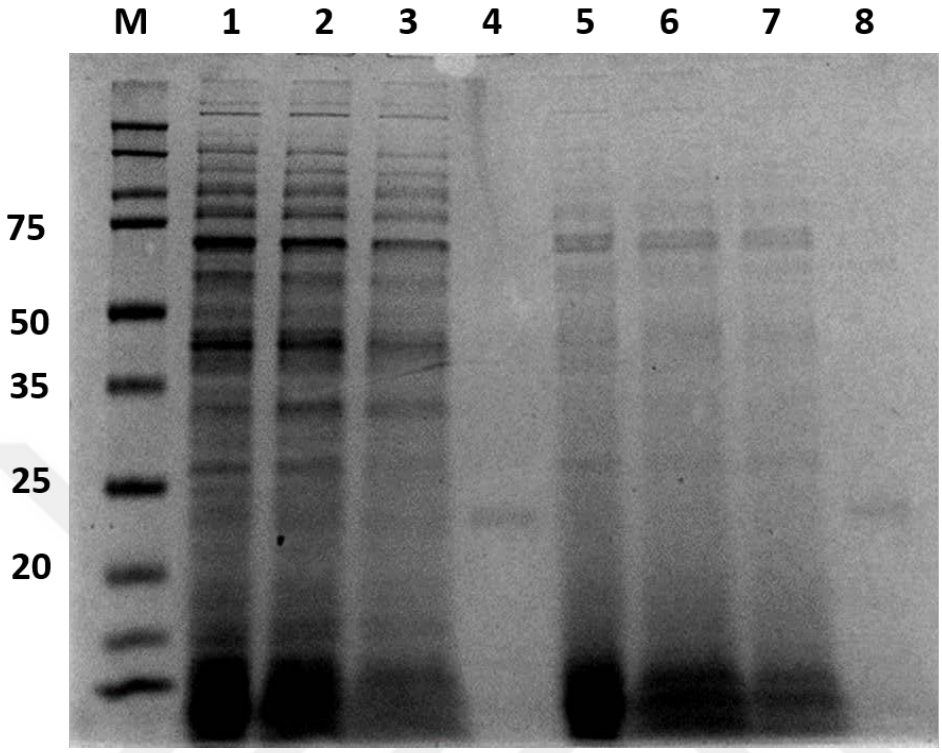

Figure 7. SDS-PAGE for pH optimization of MMP-13 production. M: Molecular weight ladder, 1: Total protein at pH 6.8, 2: Flow-through at pH 6.8, 3: Wash at pH 6.8, 4: Purified protein at pH 6.8, 5: Total protein at pH 7.8, 6: Flow-through at pH 7.8, 7: Wash at pH 7.8, 8: Purified protein at pH 7.8.

As seen from SDS-PAGE result, there is no significant difference between pH 6.8 and 7.8 purified proteins. We have decided to continue with pH 6.8 because total protein amount was higher and it was recommended to use pH 6.8 for autoinduction medium (Studier, 2005).

4.1.3. Induction method

IPTG was used for an alternative induction method. IPTG was added when OD_{600} reached 0.4-0.6. Final concentration of IPTG was 1 mM and production continued for 4 hours after IPTG addition. When they were compared, protein yields show that the best induction method is Autoinduction for both MMP-1 and MMP-13 production processes (Figure 8). Autoinduction was used for further experimental setup.

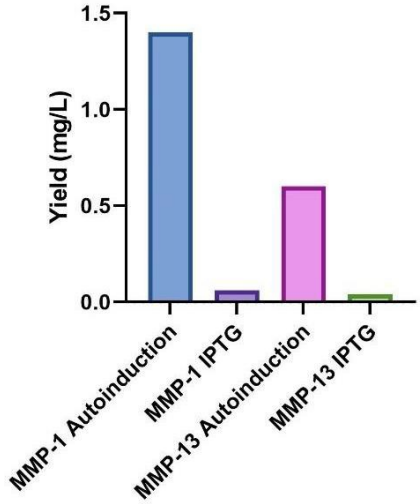

Figure 8. Protein yields under different induction methods.

4.1.4. Time

Autoinduction Media was incubated at 18 °C for 72 hours. During the production, OD_{600} and pH were checked (Table 13). Purification was performed from supernatants of both 48th hour and 72th hour. After the purification, protein yield shows that 72th hour had higher protein yields for both MMP-1 and MMP-13 (Figure 9).

Eventually, after 72 h production, more protein was obtained than 48th hour purification. Unless otherwise specified, purification was done at 72th hour of production for both MMP-1 and MMP-13.

Table 13. pH and OD_{600} during production.

	0h OD_{600}	**0 h pH**	**24 h OD_{600}**	**24 h pH**	**48h OD_{600}**	**48 h pH**	**72 h OD_{600}**	**72 h pH**
MMP-1 18°C	0,06	6,69	3,60	6,32	4,00	5,91	4,64	6,53
MMP-13 18°C	0,06	6,60	3,30	6,43	6,24	5,98	7,04	6,72

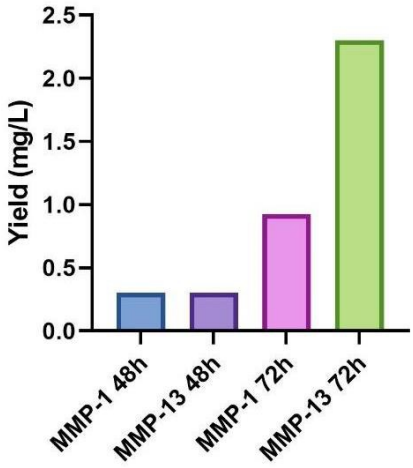

Figure 9. Protein yields under the time optimization.

4.2. Sugar analysis of the production

During the 72 h production, every 12 h, samples were taken from the supernatant and filtered for the HPLC analysis. After the analysis, chromatographs of standards and sugar profiles of MMP-1 and MMP-13 productions were given in Figure 10.

As seen from the sugar analysis, glucose was consumed in the first 24 hours for both MMP-1 and MMP-13 as expected. Glucose is the best carbon source for *E.coli* and it was consumed first. Lactose which induces protein expression, was consumed at 24th hour for MMP-1 and at 35th hour for MMP-13 productions. It was interestingly shown that *E.coli* can metabolize lactose as glucose consumption continues. This might mean that protein induction happens right after inoculation into autoinduction media. Since glycerol, which is a secondary carbon source, was consumed after approximately 50 hours for both MMP-1 and MMP-13 productions, culture growth should be stopped after 50 hours for better protein yield.

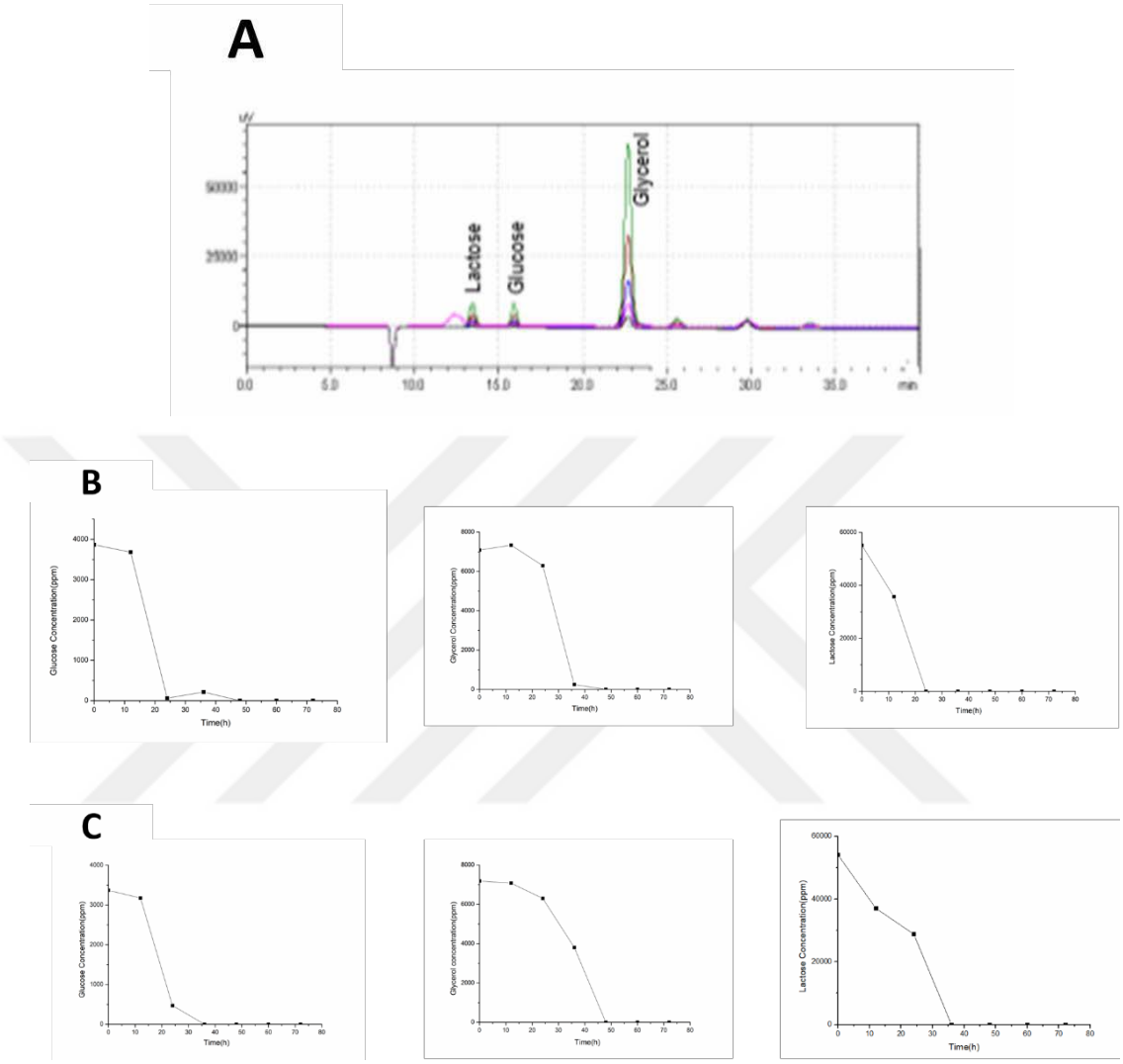

Figure 10. Sugar analysis of production medium.Chromatographs of standards (Lactose, Glucose, Glycerol) for HPLC sugar analysis (A), MMP-1 sugar consuming graphs (B), MMP-13 sugar consuming graphs (C).

4.3. Purification through Supernatant

Ni-NTA purification was done from the supernatant. After purification in the SDS-PAGE, two main bands were observed (Figure 11). Theoretical molecular weights of MMP-1 and MMP-13 are around 44 kDa. While the first band was around 70 kDa and the second band was around 24 kDa. This two-band profile was observed for purified proteins from different batches (Figure 11). Western blot shows that Anti-His antibody binds to those two bands in supernatant (Figure 12).

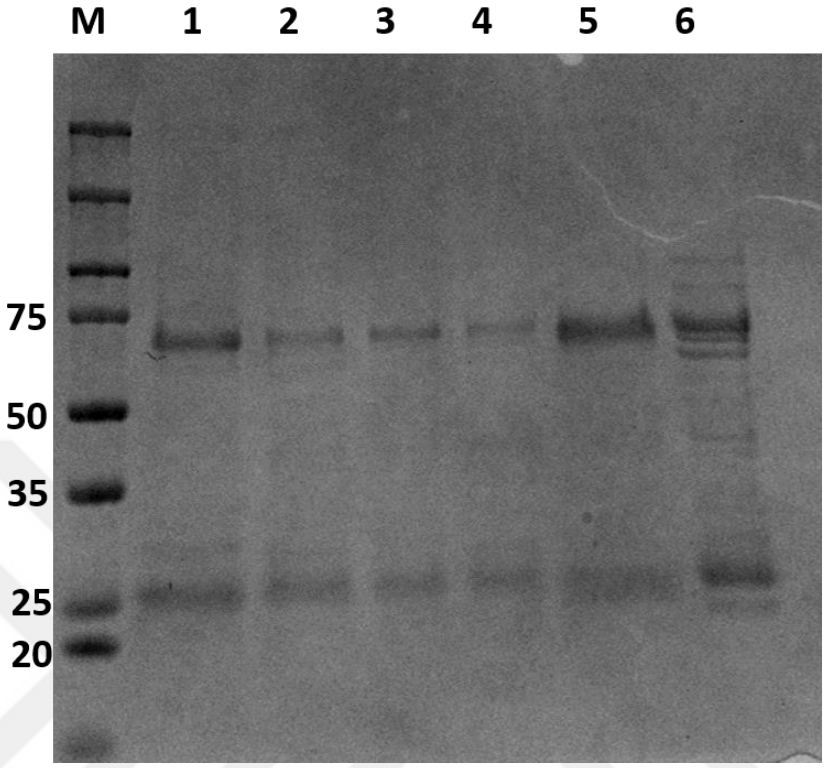

Figure 11. SDS-PAGE showing proteins after supernatant purification M: Molecular weight ladder, 1: MMP-1 from batch 1, 2: MMP-1 from batch 2, 3: MMP-1 from batch 3, 4: MMP-13 from batch 1, 5: MMP-13 from batch 2, 6: MMP-13 from batch 3.

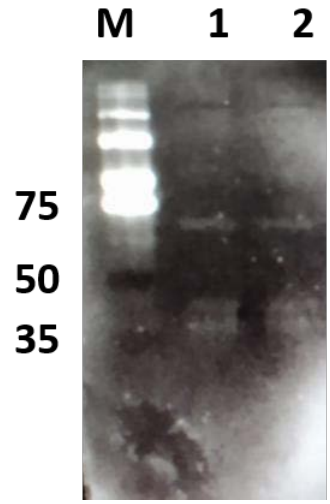

Figure 12. Western blot showing, M: Molecular weight marker, 1: MMP-1 supernatant, 2: MMP-13 supernatant. As primary antibody, Anti-His (Mouse) antibody was used. As a secondary Anti-mouse HRP-conjugated antibody was used.

According to the results, we could not get the full-length protein from the supernatant, instead we got two separate bands. In order to see which band is active, we did second-step purification

through size exclusion chromatography (SEC) and analyzed those bands accordingly as described in the following sections.

4.3.1. Thermal shift assay

Thermal shif assay was performed for MMP-1 and MMP-13 proteins purified from supernatant in ABI 7500 Fast Thermal cycler. Firstly different dye and protein concentrations were tried to achieve good profile. 2X, 5X, 10X dye, 2 μM, 5 μM, 10 μM protein concentrations were tried. 10X dye and 2 μM protein concentrations were the optimal condition to observe T_m curve for MMP-13. 10X dye and 5 μM protein concentrations were the optimal condition to observe T_m curve for MMP-1. Data were fit to Hill1 equation in OriginPro 8.5.

Table 14. T_m values for MMP-1 and MMP-13 purified from supernatant.

Proteins	T_m (°C)
MMP-1	**41.73°C ± 0.02**
MMP-13	**44.72°C ± 0.12**

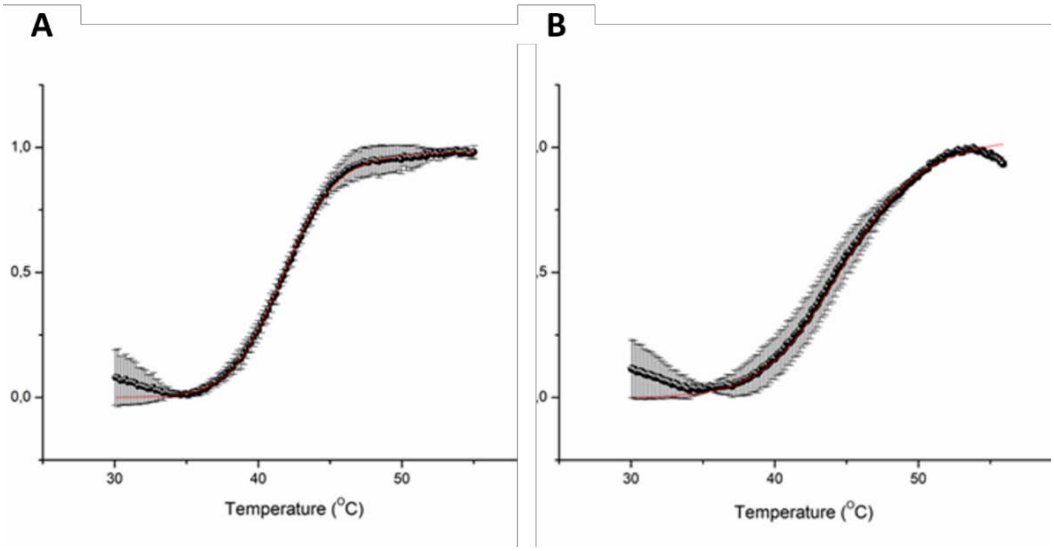

Figure 13. Thermal shift assay graphs for proteins purified from supernatant. MMP-1(A), MMP-13(B).

4.3.2. Size exclusion chromatography

The second step purification method was used to separate different two bands mentioned in the previous section. Separation was performed using size exclusion chromatography (SEC) with a Superdex 75 pg (GE Healthcare) column. As it can be seen from Figure 14, the first and second bands were well separated for both MMP-1 and MMP-13. First peak was obtained around 55 mL and the second peak was obtained around 75 mL elution volume. SDS-PAGE analysis confirmed the separation of those two bands (Figure 15). To determine if these proteins are active, an enzyme activity assay was performed as described in the next section.

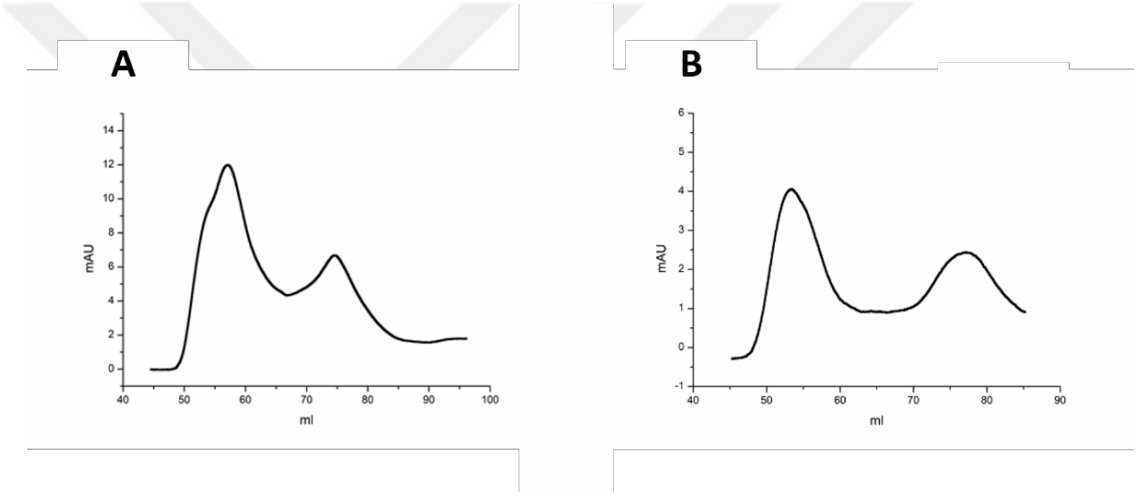

Figure 14. SEC chromatographs for MMP-1(A), MMP-13(B).

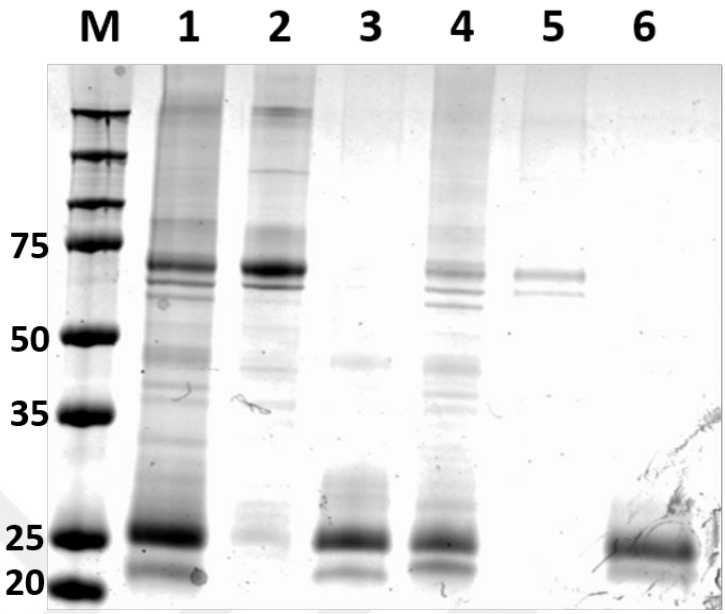

Figure 15. SDS-PAGE showing proteins after SEC M: Molecular weight ladder, 1: MMP-1 before SEC, 2: MMP-1 first peak, 3: MMP-1 second peak, 4: MMP-13 before SEC, 5: MMP-13 first peak, 6: MMP-13 second peak.

4.3.3. Enzyme activity assay for proteins purified from supernatant

After separation of these two bands, enzyme activity assay was processed to see which band has the enzymatic activity. For both enzymes, 50 nM enzyme was choosen as a optimal enzyme concentration and 10 μM substrate concentration was used. In the result of assay, it was seen that second band (~24 kDa) was active for both MMP-1 and MMP-13 (Figure 16). While mixed (before SEC) samples has activity over time, it was seen that this activity only came from second peak.

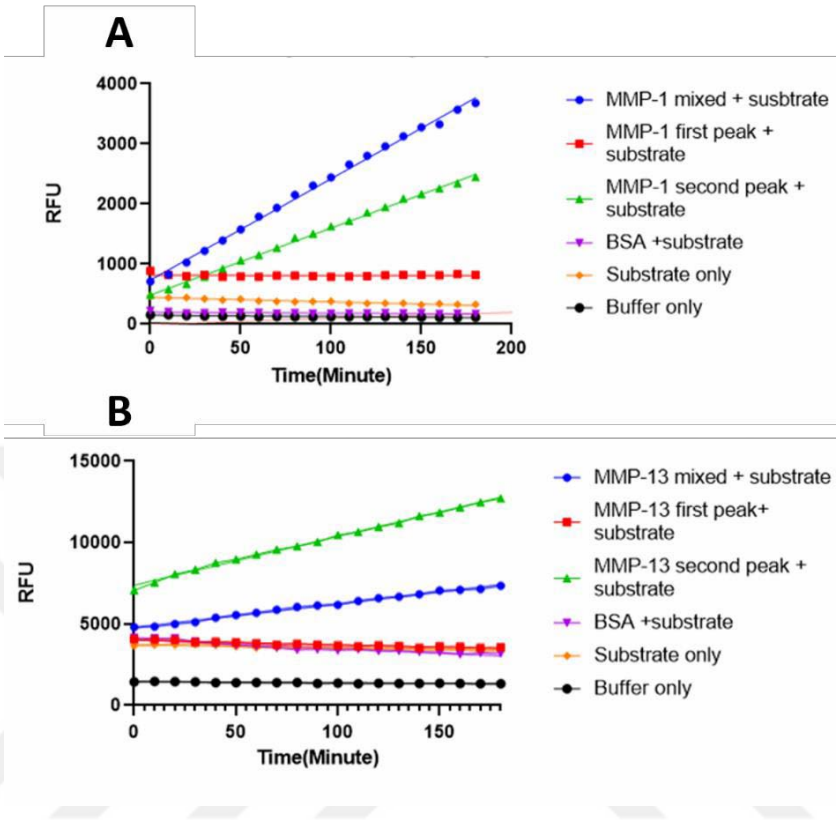

Figure 16. Enzyme activity assay for MMP-1(A), MMP-13(B) purified from supernatant.

4.4. Purification through Inclusion Bodies

The recombinant protein may not fold correctly and aggregate in *E. coli* during recombinant expression. If the three-dimensional structure of the protein is not formed properly, then insoluble aggregates called inclusion bodies are formed in the system. For this reason, problems are encountered in the biological activity of the protein. Recovery of the protein from inclusion bodies can be performed, but folding the protein correctly in the experimental setup is a difficult process. After inclusion body pruification, aggregates were observed by eye in the dialysis membrane. In order to obtain full-length MMP-1 and MMP-13, we tried to isolate our proteins from inclusion bodies. As seen from SDS-PAGE analysis, we couldn't obtained any protein in elution step. (Figure 17) but in Western Blot we saw full length protein in lysis and flowthrough samples (Figure 17).

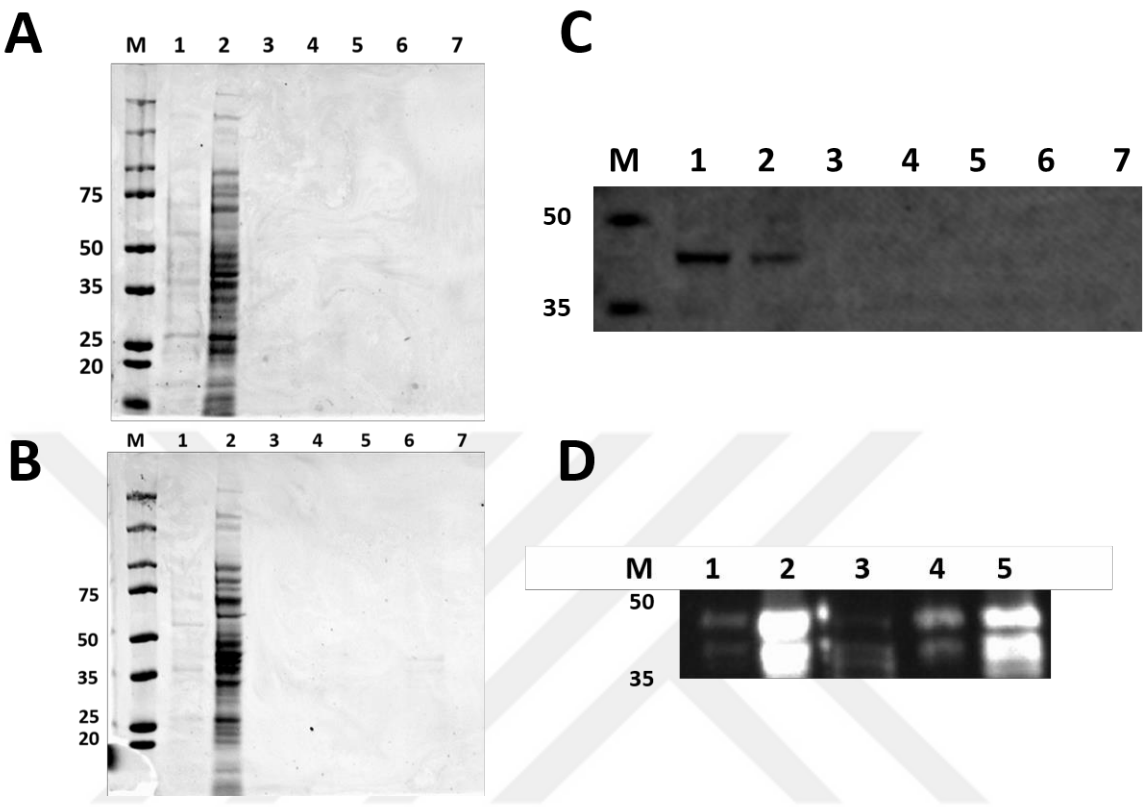

Figure 17. SDS-PAGE showing inclusion body purification samples. MMP-1 M: Molecular weight ladder, 1: Culture sample, 2: Lysis sample, 3: Flowthrough sample, 4: Wash 1 sample, 5: Wash 2 sample, 6: Elution sample, 7: Refolded protein (A), MMP-13 M: Molecular weight ladder, 1: Culture sample, 2: Lysis sample, 3: Flowthrough sample, 4: Wash1 sample, 5: Wash2 sample, 6: Elution sample, 7: Refolded protein (B). Western Blot showing, MMP-1 M: Molecular weight ladder, 1: Culture sample, 2:Lysis sample, 3: Flowthrough sample, 4: Wash 1 sample, 5: Wash 2 sample, 6: Elution sample, 7: Refolded protein (C), MMP-13, M: Molecular weight ladder, 1:Culture sample, 2:Lysis sample, 3: Flowthrough sample, 4: Wash sample 5: Elution sample (D). As primary antibody, anti-human MMP-1 (rabbit) and MMP-13 (mouse) antibodies were used. As a secondary Anti-mouse (800), anti-rabbit (680) antibodies were used.

4.5. Purification through Lysis

After we couldn't observe full lenght enzyme at the elution step via Inclusion body purification, we continued to purify directly from the lysis step. After lysed supernatant obtained, Ni-NTA purification was performed. In the SDS-PAGE, unpure and low protein amount was obtained for both MMP-1 and MMP-13 (Figure 18).

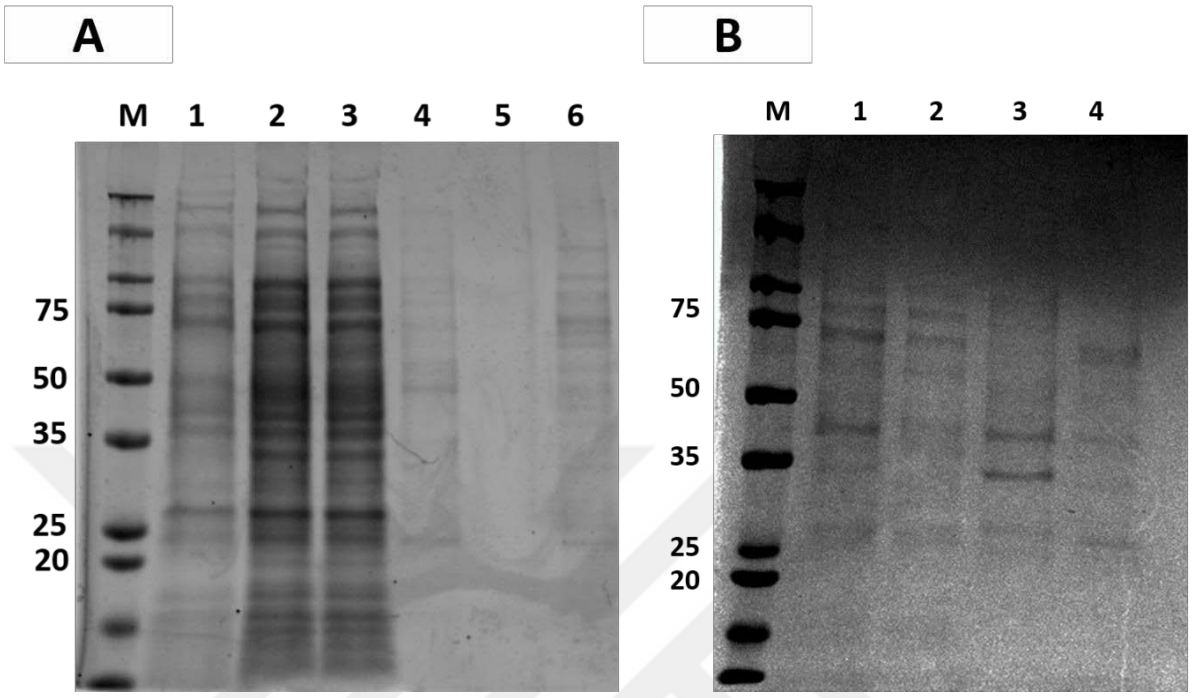

Figure 18. SDS-PAGE showing, MMP-1 M: Molecular weight ladder, 1:Culture sample, 2:Lysis sample, 3: Flowthrough sample, 4: Wash1 sample, 5: Wash2 sample, 6: Elution sample(A), MMP-13 M: Molecular weight ladder, 1:Culture sample, 2: Flowthrough sampple, 3: Wash sample 4: Elution sample(B).

4.5.1. Enzyme activity assay for lysis samples

To see if there is any enzyme in the samples obtained from lysis purification, enzyme activity assay was performed previously described. We clearly found that lysis samples included active enzymes (Figure 19). Because their purities and yields were not good, we decided to continue with MMP-1 and MMP-13 purified from supernatant.

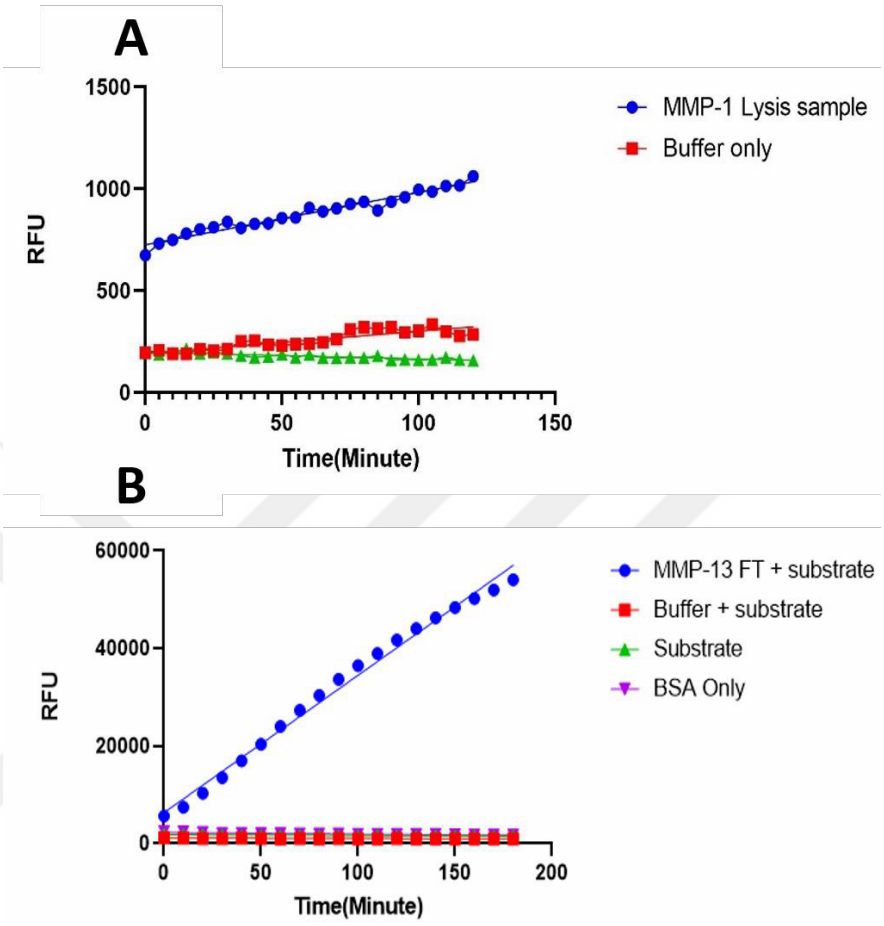

Figure 19. Enzyme activity assay for MMP-1 (A), MMP-13 (B) obtained from lysis samples.

4.6. Purification via lysis unfold-refold

Because purification from lysis samples were unpure, we tried to perform unfold-refold during purification to get proteins with higher purity. Unlike directly purification from lysis samples, at the bead incubation step, 4 M GdnCl added to the lysis supernatant and incubated for 2h at room temperature. Since refolded protein could be stored at room temperature, bead incubation was processed at room temperature. After bead incubation step, traditional Buffer A and Buffer B purification (with 4 M GdnCl) was applied. Final step was 2 step dialysis; first overnight dialysis in 2 M GdnCl added dialysis buffer, second is approximately 2h dialysis in dialysis buffer. After final concentration via Amicon, you can see the protein profile in SDS-PAGE (%12) in Figure 20. Both MMP-1 and MMP-13 proteins were obtained as degraded.

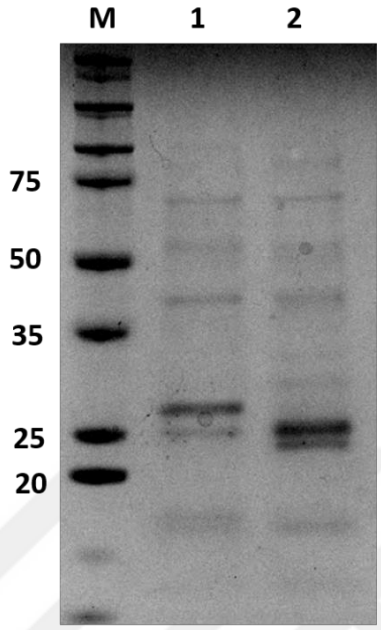

Figure 20. SDS-PAGE showing, M: Molecular weight marker, 1: MMP-1 pure after purification, 2: MMP-13 pure after purification.

4.6.1. Michaelis-Menten profile of the proteins

The Michaelis-Menten equation is $V = V_{max}[S]/ (K_m + [S])$ where V is the initial velocity, V_{max} is the maximum velocity at infinite substrate concentration, [S] is substrate concentration, and K_m is the substrate concentration at half maximal velocity. This equation is the basic definiton of the enzyme kinetics and it is widely used. Other equation is $V_{max}=k_{cat}E_{T.}$ E_T means enzyme concentration in the reaction and k_{cat} means how much substrate an enzyme can catalyze in per unit time. k_{cat} is also called turnover number and does not vary in different enzyme amounts (Choi, Rempala, & Kim, 2017). The affinity of the substrate for the enzyme's active site is defined by the K_m value. k_{cat}/K_m ratio shows how powerful the enzyme is for the specific substrate.

In this experimental set up, different enzyme concentrations were tried from the proteins in Figure 20 (50 nM, 100 nM, 200 nM) and 50 nM was chosen as the best ([E]<<[S]). Among of different subtrate concentrations, firstly 10 μM and 100 μM substrate concentrations were used. Then, other substrate concentrations were tried for both MMP-1 & MMP-13; 5 μM, 25 μM, 35 μM, 50 μM, 75 μM. Data were fit using the Michaelis-Menten analysis and k_{cat} analysis options with GraphPad Prism 9.1.0. In the Figure 21 and Table 15, Michaelis-Menten profiles of the enzymes were shown.

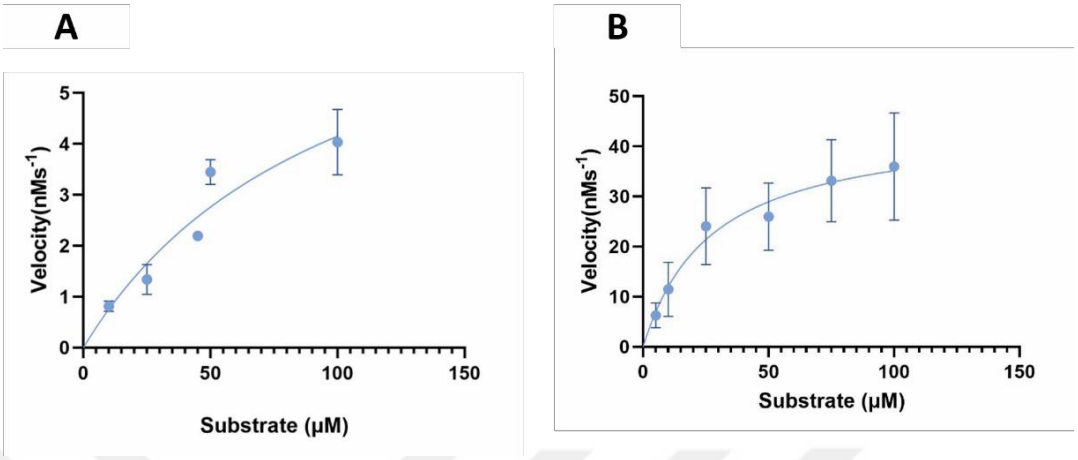

Figure 21. Michaelis Menten graphs of MMP-1(A), MMP-13(B).

Table 15. Kinetic values of proteins.

	$V_{max}(nMs^{-1})$	$k_{cat}(s^{-1})$	$K_m(\mu M)$
MMP-1	**8.37 ± 2.84**	**0.1675 ± 0.06**	**101.7 ± 56.08**
MMP-13	**44.72 ± 7.75**	**0.8940.15**	**27.25 ± 13.38**

4.6.2. Thermal shift assay

Based on our previously T_m experiments 10X dye concentration was used. Experiment was done from the proteins obtained from lysis purification (Figure 20). MMP-1 worked on 2 µM protein concentration, MMP-13 worked on 5 µM protein concentration. Data were fit Hill1 equation in OriginPro 8.5.

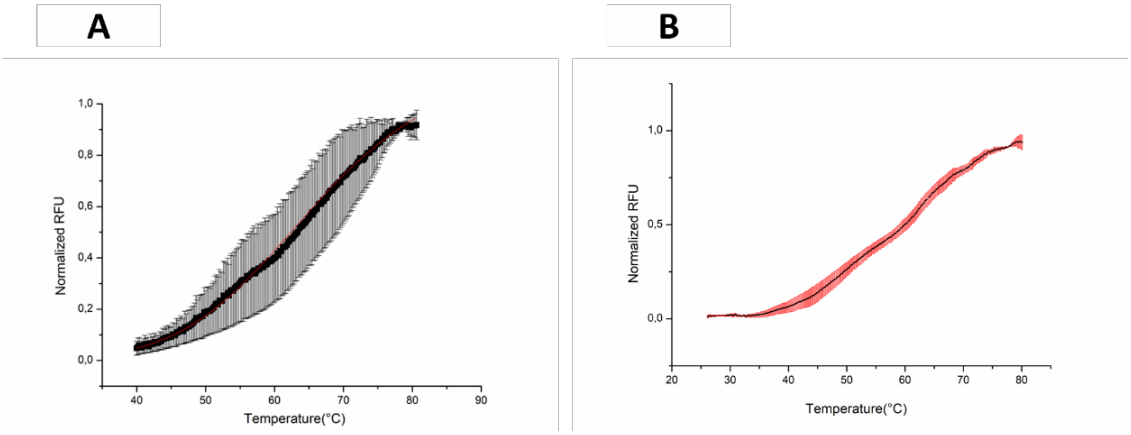

Figure 22. Thermal shift assay graphs for MMP-1 (A), MMP-13 (B).

Table 16. Tm values for full length enzymes.

Proteins	Tm (°C)
MMP-1	**65.53 ± 0.34**
MMP-13	**60.11 ± 0.12**

5. DISCUSSION

MMPs are important for ECM and tissue remodeling, and they involve many diseases. In some diseases, MMPs are suitable targets for the treatment method. Also MMPs have significant roles in cell transformation, signal transmission, apoptosis, cell proliferation, and immunological control, cell invasion, angiogenesis, and metastasis. Thus, MMP inhibitors are getting more popular. The first peptidomimetic MMP inhibitor is Batimastat which has promising results in several different cancer cell lines. Another example is Marimastat which inhibits MMP-1, -2, -3, -7 and -9 in breast and lung metastasis models. (Mondal, Adhikari, Banerjee, Amin, & Jha, 2020). We can say that MMP inhibitors are promising agent against cancer. It could be achieved by MMP neutralizing antibodies or directly giving MMPs to the relevant tissue instead of MMP inhibitors. (Dong, Song, Liu, & Guo, 2009). In our case, MMP-1 and MMP-13 are known to be downregulated in MCD (Murab et al., 2016). In the light of *Murab, et al.*'s paper, our aim is to produce and charactarize MMP-1 & MMP-13 enzymes. Afterwards as a continuation of this thesis, MCD disease organ on-a-chip model has been developed by other research group in IBG. The enzymes that we produced will be tried on this MCD on a-chip model. Effects of MMPs will be observed by histolocigal characterizations for a proof-of-concept treatment strategy.

Our aim was to produce and characterize MMP-1 and MMP-13. In the literature there are many cases about producibility of MMP-1 and MMP-13 in *E.coli*. The cases includes findings about autoproteolysis, occurrence of truncated form and fragmentation of these enzymes in purification steps. (Parkar et al., 2000), (Kumar, Colomb, Czerski, Cox, & Sarkar, 2018). In our conventional supernatant purification setup, we obtained different 2 bands in SDS-PAGE (Figure

11). We found that the smaller band is active for both enzymes and it might be catalytic site of the enzyme as its degraded form (Figure 16). Our work is the first that can produce active form of MMP-1 and MMP-13 enzymes from supernatant. Although they were not full-length, they were shown to be active. In this case, we planned to clone only catalytic domain of MMP-1 and MMP-13 for supernatant production which would be an ideal process for recombinant production of such enzymes. Another alternative approach would be trying different expression organism such as *Pichia Pastoris* which is known for its high yield recombinant protein production.

After seeing degradation and truncated forms, we have decided to purify from inclusion bodies. In the literature, MMPs are generally purified from inclusion bodies (Fu et al., 2001). When we unfold the protein with 6 M GdnCl and refold via dialysis method, no full-length protein was observed. Unfold-refold step was not efficient. Later on, we tried to obtain full length enzyme from directly from lysis step since unfold-refold is harmful to protein, problems were observed after purification obtaining low amount unpure proteins. When we did the enzyme activity assay, we saw that there was some activity for both enzymes. After that, purification was processed from directly lysis step added unfold-refold to obtain higher purity. However, this time we have changed the unfold-refold strategy. In one article (Zhou & He, 2020) they decreased the denaturant concentration in dialysis step gradually. We applied this to our protocol, and we did 2 step dialysis. However, proteins were still degraded and truncated.

Determining the T_m is crucial for biopharmaceutical protein characterization. In the literature, unfortunately there is no reported T_m values of MMP-1 & MMP-13. We applied SYPRO-Orange protocol which binds hydrophobic parts of the proteins (Krintel, Mörgelin, Logan, & Holm, 2009). We did thermal melt for both supernatant and lysis samples. Because enzymes purified from supernatant is supposed to be only catalytic site, its T_m was lower than full-length as expected.

For Michaelis-Menten kinetics, K_m value of MMP-1 is determined as 101.7 μM that differed from other studies in the literature and 27.25 μM for MMP-13. Obtained parameters of Michaelis-Menten is similar to the ones in the literature (Falconer et al., 2019) (Figure 21). In our experimental design n=2 and two independent experiments were conducted. The difference between kinetic values could be due to degradation of proteins. In one paper, proteins include pro site and activated

by APMA before the assay (Falconer et al., 2019). However, in our case, protein includes just hemopexin and active site without pro site which means already active when it is produced.

Apart from these, we have decided to produce and isolate just catalytic site of enzymes from supernatant of *E.coli.* For that purpose, primers are designed and purchased for SDM to clone only catalytic sites of MMP-1 and MMP-13. After first SDM, colonies were observed and chosen colony was processed for plasmid-DNA isolation. Second SDM was processed and colonies are observed. DNA was sent to the sequencing. After confirming correct DNA sequences of MMP-1 and MMP-13, we will produce only catalytic domains. In the literature, they have produced catalytic site of the MMP-13 as a inclusion body and used it for further studies (Pathak, Hu, & Koehn, 1998).

Macular corneal dystrophy is a disease that inherited by autosomal recessive. It is caused by mutation in CHST6 gene which encodes for carbohydrate sulfotransferase enzyme. This enzyme is located in Golgi (Fukuda, Hiraoka, Akama, & Fukuda, 2001). Enzyme replacement therapy approach is complicated since Golgi is not reachable easily. The other approaches to treat MCD could be gene therapy (S. Singh, Das, Kannabiran, Jakati, & Chaurasia, 2020) and enzyme replacement theraphy. MMP-1 and MMP-13 are located in the stroma of the cornea which is easy to reach topically. In this thesis project, a treatment approach has been tried to be developed against MCD. In the continuation of this project, if the relevant enzymes are tested in the disease model and positive results are obtained, there will be a light of hope for the treatment of the disease. At this point, the enzymes need to be produced and characterized on a large scale in accordance with the biopharmaceutical aspect. All in all, we will broaden our process of producing of these enzymes and make it ready for the treatment.

6. CONCLUSION AND FUTURE ASPECTS

We showed that MMP-1 and MMP-13 can be recombinantly expressed and purified from supernatant in *E.coli*. There were degradation after supernatant purification but those degraded enzymes were proven to be active. We tried to obtain full-lenght enzyme through different purification techniques but it was challenging. MMP autoproteolysis and degradation is common in the literature. We showed that we were able to produce enzyme which was active according to Michaelis-Menten analysis. Further cloning was performed to obtain recombinant DNA sequences of only catalytic sites of MMP-1 and MMP-13. Next steps are applying these enyzmes on a Macular corneal dystrophy disease model on a chip and characterization of its efficacy through biochemical assays. If it is successful, it gives a hope for therapeutic approach to MCD and opens a new way of treatment for other rare diseases.

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APPENDIX I

İZMİR BİYOTIP VE GENOM MERKEZİ
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU (İBG-GOEK)
KARARI

Toplantı Tarihi	: 24/04/2020	**Toplantı Günü**	: Cuma
Toplantı Sayısı	: 5	**Toplantı Saati**	: 10:30

Sayın Gökhan KARAKÜLAH,

2020-011 Protokol No'lu; sorumlusu olduğunuz "Maküler kornea distrofisinin tedavisinde kullanılmak üzere Matriks Metalloproteinaz -1, -13'ün rekombinant üretimi ve karakterizasyonu" başlıklı araştırmanın uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Prof. Dr. H. Alper BAĞRIYANIK
Başkan

Pandemi süresince online ortamda gerçekleştirilen toplantımızda alınan kararlar tek imzalı olarak düzenlenmektedir. Pandemi sona erdikten sonra ıslak imzalı karar belgesi teslim edilecektir.