Time- and pH- dependent proteolysis of Engineered Proteins by Collagenase

Hunter Berry
University of Mississippi, haberry1@go.olemiss.edu

Follow this and additional works at: https://egrove.olemiss.edu/hon_thesis

Part of the Biochemistry Commons

Recommended Citation
https://egrove.olemiss.edu/hon_thesis/1183

This Undergraduate Thesis is brought to you for free and open access by the Honors College (Sally McDonnell Barksdale Honors College) at eGrove. It has been accepted for inclusion in Honors Theses by an authorized administrator of eGrove. For more information, please contact egrove@olemiss.edu.
Time- and pH- dependent proteolysis of Engineered Proteins by Collagenase

By
Hunter Allen Berry

A Thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
April 2019

Approved By:

Advisor: Professor Susan Pedigo

Reader: Professor Gregory Tschumper

Reader: Professor Davita Watkins
(Under the direction of Dr. Susan Pedigo)

Pleiotropic effects from oral administration of anti-inflammatory drugs limit their effectiveness. The ultimate goal of this project is to develop a novel self-assembling protein-based hydrogel for in situ delivery of NSAIDs at the site of chronic inflammation. Since inflammation causes acidification and activation of collagenases in the inflamed tissue, we have designed the hydrogel to respond to both of these queues to effectively deliver drug at the site. Self-assembly of the protein hydrogel exploits the high affinity and specific interaction between the protein calmodulin (CaM) and its specific binding peptide, M13. The two components of the hydrogel, CCLP and PCLP contain repeats of CaM and M13 domains, respectively, which are separated by engineered collagen-like-sequences (CLS). Proteolysis of the protein hydrogel formed by PCLP and CCLP by collagenases will cause exposure of the protomers and release of drug at the site. This important aspect of this responsive material requires susceptibility to relevant proteases, and is the specific subject of the present study. Exposure of CCLP to collagenase, and MMP-1, indicates that each protomer is susceptible to cleavage in the CLS sequences. This conclusion was confirmed by SDS-PAGE of cleaved CCLP. Further proof, through use of MALDI-TOF, was required in order to more precisely characterize fragment identity; however, lack of access to current working negative mode MALDI-TOF prohibited precise molecular weight quantification of fragment bands. Initial studies have
prompted iterations of the component protomers. Further, we have demonstrated that the hydrogel is also susceptible to cleavage by collagenases. Results indicate the pH and Temperature dependent degradation of Engineered Proteins by collagenase require investigation into other degradable sequences as substitutes for current CLS to better improve lifetime in inflammatory conditions. Also, future research into kinetic profiles of the degradation of PCLP and CCLP by MMPs are necessary for optimizing the lifetime of the hydrogel for in-situ drug delivery.
# Table of Contents

Chapter 1: Introduction

Chapter 2: Materials and Experimental Procedure

Chapter 3: Results and Discussion

Chapter 4: Conclusion

Works Cited
CHAPTER 1

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most common nonprescription drugs in the US and internationally. Traditional oral administration of NSAIDs as anti-inflammatory therapeutics induces systemic harm in the gastrointestinal, renal, and cardiovascular systems.\(^1\) The adverse side effects of systemic administration of NSAIDs necessitate research into alternative methods for administering drug. In-situ drug delivery using a hydrogel scaffold system for spatial and time-sensitive drug release bypasses many systemic complications. Our lab is developing a novel protein-based hydrogel by taking advantage of the calcium-sensitive, high affinity interaction between calmodulin (CaM) and the peptide M13. A critical feature of our hydrogel is its ability to respond to the conditions in inflamed tissues, namely the lower pH and presence of particular proteases that are specific for remodeling the collagen network. Here, we will describe novel CaM- and M13-based protomers previously demonstrated to form a protein hydrogel, which include CCLP3, CCLP2, CCLP3 bis-Cys, and PCLP3. Each of these protomers is designed to be a substrate for Matrix Metalloproteinases (MMPs). MMPs are naturally present in the ECM to remodel the collagen network for tissue growth and repair of injuries.\(^4\) We have engineered collagen-like sequences into our hydrogel protomers to allow for steady degradation and drug release in inflamed tissues. Due to decreased pH in inflamed tissue, an understanding of pH- and time-dependent proteolysis of CCLP3, CCLP2 and CCLP3 bis-Cys is needed to assess the in vivo lifetime and responsiveness of the resultant hydrogels. PCLP3 was not included in initial
protomer proteolysis studies due to solubility issues. PCLP3 is only soluble in 6M urea, which denatures collagenase, rendering it inactive.

**Inflammation**

Inflammation is a physiological response meant to address harmful stimuli such as infection, damaged tissue, or abnormal increases in stress. Due to the highly complex dynamic wound healing process that includes intricate interactions between ECM molecules, inflammatory mediators, resident cells and infiltrating leukocytes, the healing process is generally divided into three separate phases, which are time and spatially dependent on each other. Inflammation, Tissue formation, and Tissue remodeling are the three phases. Following tissue injury, acute inflammation begins with pro-inflammatory mediator and cytokine release from resident Mast Cells, Macrophages, monocytes, and non-immune cells such as fibroblasts and endothelial cells. Pro-inflammatory cytokines have been determined to be strong inducers of various Matrix-metalloproteinases. MMPs contribute to many of the inflammatory processes following induction. By modulating inflammatory mediators, such as chemokines and cytokines, regulating physical barriers, and establishing chemokine gradients for movement of leukocytes to site of injury and infection, MMPs assist in each step of the healing process. In order to aid the delivery of inflammatory mediators and inflammatory cells, Nitric oxide, and prostaglandin vasodilation are released. Histamine, Bradykinin, and leukotrienes act in conjunction with the vasodilators to force protein rich fluid into the inflamed tissue. These processes are accompanied by neutrophil delivery to the site of inflammation. Neutrophils and Macrophages then begin the process of phagocytizing infectious agents and debridement.
of dead tissue\textsuperscript{3}. MMPs also assist in the anti-inflammatory response, if the stimulation of
the inflammatory response has subsided, by acting to clear the inflammatory cells from
the healed wound\textsuperscript{5}. If the stimulation of the inflammatory response is terminated, the
acute inflammatory response is resolved, however, if the stimulation of the inflammatory
response continues, chronic inflammatory disease can arise, such as diabetes, cancer,
cardiovascular diseases and autoimmune diseases\textsuperscript{6}.

**Chemical and pH component of pain in inflammation**

There are many inflammatory mediators throughout the inflammatory response that are
related to the pathological aspect of inflammation, such as the pro-inflammatory
cytokines IL-1B, IL-6, and TNF-\textalpha\textsuperscript{7}. Studies have also shown that other inflammatory
mediators, such as Prostaglandin E\textsubscript{2}, Bradykinin, Histamine and serotonin also induce
nociception in inflamed tissue. Additionally, the inflammatory response increases the
acidity of the surrounding ECM, which also plays a role in the pathological response\textsuperscript{8}.

Studies have shown that lowered pH, which is a product of the inflammatory
response, also has an effect on pathological aspect of inflammation. The presence of high
proton concentration is apparent in inflammation, as well as in malignant tumors and
other acute injuries such as fracture-related hematomas, resulting in pH as low as 5.8\textsuperscript{9}.
This phenomenon is due to leukocytes present in the inflamed tissue that actively excrete
lactate into the ECM as they act to combat the harmful inflammatory stimuli,
Extracellular lactate decreases the pH of the inflamed tissue, which causes a painful
stimulation. A study of the combination of inflammatory mediators, Histamine, Bradykin,
5-HT, and Prostaglandin E\textsubscript{2} with low pH showed synergistic combination with regards to nociceptor stimulation\textsuperscript{9}.

This form of pain, that which is induced by the inflammatory response, is meant to be an adaptation that results in a protective mechanism. The increase in sensory sensitivity, during unavoidable tissue damage, such as with chronic inflammation of tissue, discourages physical contact to and movement of, the place of inflammation. The nociceptors, which are receptors for pain, have become sensitized during the inflammatory response in order to increase awareness and protection of the inflamed tissue\textsuperscript{10}.

Additionally, the onset of fever is also an adaptive mechanism, which works to aid in combatting invasive species and healing. Following the induction of the inflammatory response, other cytokines are produced that are called endogenous pyrogens. Through further signaling cascade, and production of prostaglandins, the endogenous pyrogens induce an increase in temperature. This increase in temperature is an adaptive mechanism that is meant to inhibit bacterial growth, increase bactericidal activities of neutrophils and macrophages. This adaptive mechanism is why inflamed and healing tissue feels hot to the touch\textsuperscript{10}.

**Acute and Chronic inflammation**

There are two stages of the inflammation response depending on the length of time that pathological effects persist. Acute inflammation, as previously mentioned, is an adaptive response meant to resolve any disease, harmful stimulus or foreign stimulus and persists for approximately 24 hours. Chronic inflammation, which persists for a
prolonged period of time, is harmful due to the inability to terminate the initiating inflammatory stimulus. An overly activated immune or repair system is an expensive energy consuming mechanism that is marked by sickly prognoses, as well as loss of appetite. On a cellular level, the continual inflammatory response leads to chronic inflammatory diseases, such as cancer, diabetes, cardiovascular disease, and autoimmune diseases.  

**Adverse side effects of systemic administration of NSAIDs**

NSAIDs are prescribed for their painkilling, anti-inflammatory, therapeutic effects, but have adverse side effects such as gastrointestinal, cardiovascular, and renal complications (Figure 1.1). The therapeutic benefit comes primarily from the ability of NSAIDs to block specific prostaglandin synthesis, through inhibition of the cyclooxygenase enzymes COX-1 and COX-2. COX-1 is expressed in normal cells, and primarily deals with physiological housekeeping mechanisms, whereas COX-2 is not normally expressed, unless inflammation occurs, when it is then induced and is significantly unregulated. COX-1 inhibition is an undesired effect of the use of NSAIDs; this is because COX-1 produces prostaglandins and thromboxane A2, which control physiological functions as well as the mucosal barrier in the GI tract, renal homeostasis and platelet aggregation. Inhibition of COX-1 is what leads to gastrointestinal and renal toxicities, as well as cardiovascular complications. COX-2 inhibition is the intended effect of NSAIDs, due to its production of prostaglandins that are related to the pathological aspect of inflammatory responses such as pain and fever.
Figure 1.1. The adverse side effects of inhibition of COX-1 and COX-2 inhibition by NSAIDS. Inhibition of COX-1 and COX-2 has inflammatory pathologically non-specific effects, such as prostaglandin synthesis inhibition, which has adverse side effects on the kidney, GI and cardiovascular systems. (Figure taken from http://tmedweb.tulane.edu/pharmwiki/doku.php/nsaid_side_effects)

Utilizing ECM conditions for drug release

In order to effectively bypass the systemic side effects that occur when NSAIDs are administered orally, specific conditions of the site of inflammation can be exploited to delivery drugs locally. In inflamed tissue, Matrix Metalloproteinases expression is significantly upregulated by many inflammatory cytokines, such as tumor necrosis factor and by growth factors such as, epithelial growth factor. Normally secreted as an inactive form, proteinases, reactive oxygen and hypochlorous acid generated by leukocytes, all due to the inflammatory response, activate Matrix-Metalloproteinases to aid in healing. By utilizing the conditions and enzymatic composition of the ECM in inflamed tissue,
novel protein hydrogels formed from CCLP and PCLP, which contain MMP substrate cleavage sites, can be used for inflammation responsive drug release\(^4\).

**Matrix Metalloproteinases (MMPs)**

MMPs family contains 23 related human proteinases that are not only involved in tissue remodeling, but also in cleavage of specific substrates capable of secondary biological activity, and in indirect and direct modification of signaling molecules and in creating the means for cellular migration the 3D-space of tissues. There are three domains in the structure of all MMP’s, the pro-peptide, hemopexin and catalytic domains. The pro-domain contains a cysteine residue that inhibits catalysis by coordinating with the active site zone. The pro-domain is removed, exposing the active site, which controls MMP’s ability to cleave substrates. The hemopexin domain mediates protein-protein interactions, and plays a large role in proper substrate recognition, activation of the enzyme, protease localization, internalization and degradation. By regulating the proteins composing the extracellular matrix, MMPs can affect a wide range of extracellular and intracellular functions of cells. There are countless substrates of MMPs, such as peptide growth factors, cell-adhesion molecules, cytokines and chemokines, producing varying secondary mechanisms, leading to a complex, yet diverse characterization of MMPs. Further research into the MMP family began upon finding that MMPs are unregulated in many forms of human diseases, such as arthritis and cancer\(^5\).

Inflammation is crucial for the protection of the human body, however, if unregulated or excessive in nature, inflammation can lead to further tissue injury, chronic disease or organ dysfunction, as previously mentioned. Matrix Metalloproteinases are
crucial to the inflammatory process, by either stopping or furthering the pathological effects of the inflammation. MMPs regulation of inflammation is dependent on the location, specific MMP, and availability of substrate. In nearly all forms of human disease that utilize inflammation, increased expression of MMPs has been observed. Although specific substrates of MMPs have been, for the most part, undefined, catalytic effectiveness of specific cleavable sequences has been determined for target substrates. In particular, the collagen type-1 derived sequence, GPQG / IWGQ, which is present in CCLP3, CCLP2 and CCLP bis-Cys, has been shown to be catalytically degraded by both MMP-1 and MMP-8.

**MMP-1 and MMP-8**

Matrix Metalloproteinase 1 was first discovered to degrade fibrillar collagen in tadpole tales in 1962, and was then characterized as interstitial collagenase. Following the discovery, a large field of study began to determine the role of MMP-1 in a pathological and physiological setting. MMP-1 is a part of a subgroup of soluble proteases known as collagenases. With regards to substrate specificity, MMP-1 is able to degrade a wide variety of substrates, allowing it to be the most versatile of all the MMPs. MMP-1 is catalytically active in the process of overturning the extracellular matrix; therefore, it has been hypothesized that many diseases that result from excessive or insufficient extracellular matrix turnover, such as arthritis are linked to deregulation of MMP-1. MMP-1 expression is correlated to oxidative stress, which is significantly higher in inflamed tissue.
Matrix Metalloproteinase 8 is a soluble proteinase, known as a collagenase, similar to MMP-1 in both structure and function. The differences lie in either MMP having subtlety greater affinities for specific substrates. MMP-8 is unregulated during oxidative stress, just as MMP-1, which corresponds to the increased turnover of ECM during inflamed tissue.

**Alternative method of drug delivery**

In order to combat the adverse effects associated with systemic administration, targeted drug delivery systems (DDSs) have been under extensive research. Targeted DDSs have many benefits, such as improvement in absorption, distribution, metabolism, and excretion of drugs. By increasing the solubility of drugs that have hydrophobic molecular structures or altering the pathway of drug administration, targeted DDSs help to increase the overall absorption of specific drugs. Traditionally, the systemic distribution of drugs allows no control for the spatial aim or utilization of a time-based release to optimize the analgesic and anti-inflammatory effects of NSAIDs. Targeted DDSs allow for specific targeting of inflamed tissue, as well as a response mediated by inflammation, providing an efficient drug effect. Alteration of drugs so that excretion is possible is primarily the job of the liver and kidneys. Drugs that are administered orally are absorbed through the gastrointestinal tract and must first pass through the liver before being released into systemic circulation. In the liver, many drugs undergo chemical reactions that alter or increase the activity of enzymes before release into the bloodstream for intended effects on target tissues. Following the intended effects of the NSAIDs, the kidneys must also work to clear them from the blood. The metabolic pathways through
which drugs enter and exit the systemic network of the body leads to potential for liver
and kidney toxicity. Administration through targeted DDSs could allow for alternate
administration and clearance mechanisms that could negate the systemic issues associated
with NSAIDs\textsuperscript{15}.

**Calmodulin and M13**

Calmodulin is a 148 residue long, dumbbell shaped molecule, with two EF-hand
Ca\textsuperscript{2+}-binding sites in both the N- and C-terminal domains and a central helix connecting
the two. In solution, the central helix adopts an unstructured conformation\textsuperscript{16}, which
allows the Calmodulin-Ca\textsuperscript{2+} complexes freedom to conform completely to the structure
of the target peptide (Figure 1.2). M13 is a 26 residue long peptide that binds the
Calmodulin-binding domain of skeletal muscle myosin light chain kinase. Upon binding
of M13 to the Calmodulin-Ca\textsuperscript{2+} domain, the conformational change in the central helix of
Calmodulin-Ca\textsuperscript{2+} causes the Calmodulin- Ca\textsuperscript{2+} binding domain to fold around the M13,
forming a tight hold\textsuperscript{17}. M13 also undergoes a conformational change upon complex with
Calmodulin-Ca\textsuperscript{2+}, from an undefined random coil in its apo state to a helical
conformation upon binding to Calmodulin-Ca\textsuperscript{2+}, further assisting in the strength of
binding\textsuperscript{18}. The Calmodulin-M13 complex is primarily stabilized by hydrophobic
interactions\textsuperscript{19}. Utilization of the Calcium dependent Calmodulin-peptide interaction
causes self-assembly of Engineered protein components, CCLP and PCLP, resulting in
non-covalent cross-linking formation of a hydrogel.
Figure 1.2. Complex of Calmodulin and M13. A. The ribbon drawing of calcium bound Calmodulin. B. The schematic depiction of apo Calcium-Ca\textsuperscript{2+} binding domains. C. The ribbon drawing of Calmodulin-Ca\textsuperscript{2+} complex with the M13 peptide. D. The schematic depiction of the Calmodulin-Ca\textsuperscript{2+} complex with the M13 peptide depicts the conforming of the Calmodulin-Ca\textsuperscript{2+} domain tightly to the M13 domain.

PCLP and CCLP and hydrogel formation

Hydrogels are hydrophilic, polymer-based substances with physiologically compatible characteristics, capable of countless therapeutic benefits. Constituents of hydrogels can be synthetic in origin, such as poly(ethylene glycol) (PEG), polyacrylamide (PAM), poly(vinyl alcohol) (PVA), and poly(methyl methacrylate) (PMMA), or naturally occurring polymers, such as collagen, gelatin, chitosan, and alginate. There are limitations to these single polymer, synthetic, and naturally occurring hydrogels, which prompts the engineering of more complicated, but promising, hydrogels composed of co-polymers. Co-polymer hydrogels are composed of crosslinking polymers that are a mesh-like network formed by binding oligomer chains within a polymeric
scaffold that was previously assembled. Hydrogel materials of this nature are susceptible to specific modification to mimic conditions in the extracellular matrix to better control the therapeutic outcome\textsuperscript{22}. CCLP and PCLP are engineered proteins capable of forming a co-polymer cross-linking that exhibit physical hydrogel properties capable of degradation by ECM enzymes. A schematic of the formation of our protein-based hydrogels is shown in Figure 1.3. Actual photographs of our hydrogels are shown in Figure 1.4.

**Figure 1.3. Schematic of hydrogel formation.** The multiple peptide complex of CCLP and PCLP (n=2 or n=3) form a mesh-like hydrogel with multiple sites of interaction. The CCLP and CCLP proteins and the resultant hydrogel are susceptible to enzyme specific degradation (Figure taken from Pedigo lab).

**Figure 1.4.** The formation of a 10% hydrogel from PCLP and CCLP (Courtesy of Christopher Fox). Three images of the same hydrogel, with and without overlaying aqueous buffer.
Goal of Experiments

The specific goal of the experiments reported here is to test the susceptibility of the engineered protomers to degradation by collagenase, and various MMPs. The studies address susceptibility to cleavage of specific target sequences by collagenase, MMP-1 and MMP-8, and the effects of degradation rate as a function of pH and time. All assays of enzyme activity are performed by gel assays of degradation products. Finally, initial studies of the proteolytic degradation of the gels themselves as a function of pH and time. Results from these studies create a foundation for re-engineering the component protomers to change the in vivo lifetime of the gel matrix.
CHAPTER 2

MATERIALS AND EXPERIMENTAL PROCEDURES

Materials

All materials were standard chemicals available from suppliers unless otherwise noted.

Materials for Overexpression

Bacto Agar
Tryptone
Yeast Extract
NaCl
250 mL Erlenmeyer Flasks
100 x 20 mm polystyrene culture dishes
Kanamycin (1000x stock- 30 mg/ml in water)
Inoculation loops
1M potassium Phosphate, pH 7.4, sterile
Isopropylthiogalactoside (IPTG) 0.4M, sterile
Spectrophotometer
Disposable Cuvettes, narrow width, 1cm path length
0.8 L centrifuge bottles
Centrifuge

Materials for Purification

Stationary Phases
His-Tag: Immobilized Nitrilotriacetic Acid with Ni(II) (Thermofisher)

Hydrophobic Interaction: Phenyl Sepharose (Amershan Biosciences)

HisTag Buffers:

Binding Buffer: 20mM Tris, 0.5 M NaCl, 5 mM Imidazole, 6 M Urea, pH 7.9
Wash Buffer: 20mM Tris, 0.5 M NaCl, 40 mM Imidazole, 6 M Urea, pH 7.9
Elution Buffer: 10mM Tris, 250 mM NaCl, 0.5 M Imidazole, 6 M Urea, pH 7.9

*Buffers were filtered through 0.45 µm filter prior to use.

Hydrophobic Interaction Buffers:

A: 20 mM Tris, 50 mM CaCl₂, 50 mM NaCl, pH 7.4.
B: 20 mM Tris, 50 mM CaCl₂, 500 mM NaCl, pH 7.4.
C: 20 mM Tris, 10 mM EDTA, 50 mM NaCl, pH 7.4.

Materials for Proteolysis

MMP and Collagenase Digest Buffers:

Tris digest Buffer: 100 mM Tris, 100 mM NaCl, 10 mM CaCl₂, pH 7.4
MES digest Buffer: 100 mM MES, 100 mM NaCl, 10 mM CaCl₂, pH 5.7 & pH 6.5

200 nM Collagenase Stock (Sigma Aldrich)

20 nM MMP-1 & MMP-8 Stocks (Anaspec)

2X SDS Reducing Loading Buffer

SEC Buffer: 10 mM HEPES, 140 mM NaCl, 6 M urea, pH 7.4
Experimental Procedures

The purpose behind this experiment is to produce the PCLP and CCLP proteins, which are the two required components for the formation of the hydrogel, and characterize their degradation under certain physiological conditions. We synthesized the proteins using a bacterial expression system of genes in engineered plasmids. We prepared overnight cultures, which were inoculated with freshly plated, pre-transformed cells, which were then used to inoculate large LB-Kanamycin cultures. Large cultures were grown to mid log phase, and then induced with IPTG allowing bacteria to express protein. Following expression period, cells were separated from supernatant, sonicated and centrifuged in order to isolate protein. Protein was purified using either His-Tag chromatography or hydrophobic interaction chromatography. Following purification, protein was lyophilized and stored in freezer at -20°C.

Lyophilized protein was weighed and re-suspended in three different digest buffers: MES Buffer pH 5.7, MES Buffer pH 6.5 and Tris Buffer pH 7.4, resulting in a final concentrations of 200 µM. We made a collagenase stock in each of the respective buffers, resulting in a 2 µM solution. We performed digests of CCLP3, CCLP2 and CCLP3 bis-Cys by collagenase in 100 µL to test whether the proteins were susceptible to proteolysis. Sample points for general collagenase proteolysis were 0 hr and 48 hr. We performed SDS-PAGE on pre- and post-digest protein samples in order to visually observe digest occurring. In order to accurate visualize band density diminishing, digest samples were diluted to a final concentration of 10 µM, corresponding to a 20:1 dilution of all other components of the digest before running on SDS-PAGE. Subsequently, we tested pH dependent proteolysis by collagenase of engineered proteins. We made 230 µM
CCLP3, and CCLP2 stocks and 200 µM collagenase stocks at pH 5.7, 6.5, and 7.4. Engineered proteins and collagenase were combined to form 200 µL of digests of 200 µM CCLP3 and CCLP2 and 200 nM collagenase at pH 5.7, 6.5 and 7.4. Time points were taken on an exponential scale as follows- 0 min, 10 min, 20 min, 40 min, 80 min, 160 min, 320 min, 640 min, 1280 min. Due to concern of Ca²⁺ having an impact on band migration distances in SDS-PAGE, additional 1280 min samples were treated with 600 µM EDTA in order to chelate the Ca²⁺ ions. (Calcium concentration following dilutions was 500 µM.) Samples were then run on SDS-PAGE, and trend lines for all digests were created. Following general collagenase digests, we made 230 µM stocks of CCLP3, and CCLP2 and 100nm stocks of MMP-1 and MMP-8. Following apparent activation of MMP-1, and MMP-8, Engineered proteins were combined with MMP-1 and MMP-8 in separate 25µL digests of 20nm MMP-1, and MMP-8, and 50 µM engineered protein. We took sample time points, 0 min, 30 min, and 1, 2, 4, 8, 16, 32, and 64 hrs in order to test the susceptibility of engineered proteins to proteolysis of specific MMPs. SDS-PAGE was performed on each protein digest in order to visualize proteolytic fragments.

**Methods**

The following section provides details of the procedures that were followed.

**Preparation of Overnight**

We inoculated LB Agar plates with the pre-transformed cells, preparing isolated colonies by using a streaking method throughout the plate. Cultures were grown overnight at approximately 37° C. The following day, we acquired a well-defined colony
from the agar plates, and then inoculated into the 50 mL LB containing Kanamycin. The 50mL culture grew overnight at 37°C in the incubator, rotating at 250 rpm.

**Protein Overexpression**

Experimenters inoculated 1L LB Kanamycin with 5 mL from the 50 mL overnight culture, then placed them in 37°C incubator at 250 rpm. Absorbance of the expression cultures was monitored at 600 nm. When the AU reached 0.6-1.0, expression was induced by addition of 1 mL of 0.4 M IPTG into each large culture, resulting in a final concentration of 0.4 mM IPTG. Large cultures then incubated at 37°C for 2 additional hours at 250 rpm.

**Centrifugation**

We harvested the cells by transferring the cultures into 1L centrifuge bottles, which can hold approximately 800 mL. A 1mL post induction sample was taken, and cultures were centrifuged for 15 minutes at 3000 rpm. We decanted and discarded the supernatant. The cells were re-suspended in approximately 10 mL of 20 mM HEPES, 100 mM KCl, pH 7.4 buffer. We then transferred the cells to a metal beaker, covered them with parafilm and froze them at -20°C.

**Fractionation and Purification**

We thawed the cells, and then sonicated them for 5 minutes at 50% amplitude, 5 seconds on and 5 seconds off. We took a post sonication sample for SDS-PAGE analysis, and then centrifuged the sonicated cells at 13,000 rpm for 45 minutes. Soluble protein
was decanted from the pellet and ready for chromatography. Insoluble protein was in the pellet, and required Triton-X washes. Following decanting of the supernatant, we added 15 mL of 10% Triton-X to the pellet, stirred the pellet up, and incubated it at room temp for 10 minutes. We centrifuged the 10% Triton-X suspension at 13,000 rpm for 20 minutes. Following centrifugation, we took a supernatant sample, and then decanted the rest. We added 15 mL of 1% Triton-X to the pellet, resuspended the pellet, and then incubated at room temp for 10 minutes. We centrifuged the 1% Triton-X suspension at 13,000 rpm for 20 minutes. The pellet was resuspended in 1% Triton-X, and then incubated it at room temp for 10 minutes. The 1% Triton-X suspension was then centrifuged at 13,000 rpm for 20 minutes. Following centrifugation, we took a sample and decanted off the supernatant, leaving a washed pellet with high protein content. The pellet was then dissolved in His Tag Binding Buffer containing 6M urea.

His Tag chromatography was utilized in order to purify the insoluble protein following centrifugation and fractionation. The histidine residues cause the protein to adhere to Nickel (II) in the column’s stationary phase. Column was equilibrated in 25 mL of Binding Buffer containing 6 M urea. Once columns are equilibrated, we loaded 5 mL of crude protein in 6 M urea onto the column and collected the eluent in a conical labeled Flow Through. We loaded a total of 25 mL Binding Buffer with 6 M Urea onto the column, collecting 5 mL fractions labeled BB-1 through BB-5. A total of 25 mL of Wash Buffer with 6 M urea was loaded onto the column and collected in 5 fractions labeled WB-1 through WB-5. We loaded 40 mL of Elution Buffer onto the column, collecting 5 mL in EB-1, then 10 mL in EB-2 and EB-3, and 5 mL in EB-4, EB-5 and EB-6. We determined which elution fractions contained protein by checking absorbance against
corrected for a Binding Buffer sample. After determining which EB fractions contained protein, we dialyzed the protein into SEC Buffer (10 mM HEPES, 140 mM NaCl, 6 M urea, pH 7.4). Protein stock was stored in the freezer.

Soluble protein is first heated in the presence of calcium to 80° C for 10 minutes to precipitate contaminating proteins. The supernatant contains soluble protein. The hydrophobic residues present in the protein allow the protein to adhere to the hydrophobic stationary phase of the column. This technique was used to purify the CCLP constructs. In the presence of calcium and low salt (Buffer A), calmodulin adsorbs to the column. Then the column is washed with buffer B (high calcium and low salt) to remove nonspecifically adsorbed contaminants. The column is then washed with Buffer A again. Finally protein is eluted with Buffer C (low calcium, low salt). Elution fractions contain purified protein, which is then dialyzed into water and lyophilized.

**Proteolysis Experiments**

We used the lyophilized protein stocks to make 200 µM solutions of CCLP3, CCLP2 and CCLP bis-Cys (in SEC Buffer), and also made a 100 µM collagenase stock and a 13 mM sodium azide solution. Stock solutions were diluted and combined into a 100 µL solution of 200µM protein, 2µM collagenase, and 0.05% sodium azide. We then took 10 µL samples at 0hr digest time point and a 48hr digest time point, using 10µL of 2X SDS reducing buffer to quench the reaction. Once time points were gathered for each protein, we ran each sample on a gel in order to observe the degree of cleavage.

Following observation of cleavage of engineered proteins by collagenase, we then tested the pH dependence of collagenase. We made 230 µM stocks of CCLP3, and
CCLP2, and made a 1.48 µM stock of collagenase at three different pH 5.7, 6.5, and 7.4. We calculated the volume of each required component stock, 230 µM CCLP3, and CCLP2, and 1.48 µM collagenase stock, in order to result in a 200 µL digest consisting of 200 µM CCLP3, and CCLP2, and 200 nM collagenase. We then combined the CCLP3, CCLP2, and Collagenase to result in three different 200 µL of a 200 µM CCLP3/CCLP2, 200 nm collagenase digests, at pH 5.7, 6.5 and 7.4.

After observing pH dependent cleavage by collagenase, we began proteolysis experiments with MMP-1 and MMP-8. We activated MMP-1, and MMP-8 by incubating them at 37°C with 1mM APMA for 3 hours and 1 hour respectively. Following activation, we began the digest of CCLP3, and CCLP2 by MMP-1, and MMP-8. CCLP3 and CCLP2 concentrations were 50 µM, and MMP-1 and MMP-8 concentrations were 20 nM. Leftover activated MMP time points were taken for 0hr, 1 hr, 2 hr and 4 hr post activation, and digest time points were taken at 0 hr, 16 hr, 24 hr, 48 hr, and 72 hr. Samples were run on SDS-PAGE in order to visualize cleavage.
CHAPTER 3

RESULTS AND DISCUSSION

The objective was to prove that the theoretical cleavage of novel proteins was possible by collagenase. In order to analyze the proteolytic susceptibility of our engineered proteins by collagenase, we used SDS-PAGE to visualize the effects of a digest. We ran a standard molecular weight protein ladder, with known molecular weight bands, to use as a comparative tool for determining if cleavage of the main protein bands was occurring, and if so, what fragment pieces were being produced from the cleavage. As shown in Figure 2.1, each engineered protein and its subsequent digests are grouped on the gel. For each digest, a protein stock and a collagenase stock were run in order to eliminate the possibility of incorrectly identifying protein or collagenase bands as digested protein fragments on the gel. The engineered protein stocks before and after digestion with collagenase were the same concentration to accurately assess whether a band’s density was diminished. As depicted in Figure 2.1, the CCLP3, and CCLP3 bis-Cys protein stocks, which correspond to lanes 2 and 8 respectively, migrated according to their molecular weight, right above the 45,000 D band in the molecular weight standard. CCLP2 stock, which corresponds to lane 5, migrated just below the 45,000 D, indicating it is also migrating according to its molecular weight. As shown by the 48-hour digest point in lanes 4, 7, and 10, the main band formed migrated to approximately 16,000 D, indicating that collagenase cleaved at the correct points, producing Calmodulin. We initially planned to use MALDI-TOF in order to identify the precise molecular weights of each respective band, but the negative mode of the MALDI-TOF machine was not working, prohibiting us from precision quantification of band molecular weight.
Figure 2.1. Proteolysis of 200 μM CCLP3, CCLP2 and CCLP3 bis-Cys by 2 μM Collagenase in Tris Buffer pH 7.4. Lane 1 contains MW standard. Lanes 2, 3, and 4 contain CCLP3 stock, CCLP3 0 hr, and CCLP3 72 hr, respectively. Lanes 5, 6, and 7, contain CCLP2, CCLP2 0 hr, and CCLP2 72 hr respectively. Lanes 8, 9, and 10 contain CCLP3 bis-Cys, CCLP3 bis-Cys 0 hr, and CCLP3 bis-Cys 72 hr. Lane 11 contains collagenase.

After observing cleavage, Collagenase concentration was decreased in order to more accurately observe cleavage of engineered proteins by physiologically relevant concentrations. The objective was now to determine the lifetime of CCLP2 and CCLP3 when subjected to cleavage in extracellular matrix conditions that may occur during inflammation. Each gel corresponds to a specific construct at a pH of 5.7, 6.5, or 7.4 at 37°C. We ran a standard protein ladder in the first lane of each gel so that we could accurately identify the main construct bands, as well as fragment bands, that are produced from the cleavage by collagenase. As seen in Graphs 2.1 and 2.2, we created a trend line from the standard protein ladder by plotting the logarithm base 10 of the molecular weight of each protein band vs. the relative mobility of each respective protein band. Using the equation of the trend line, we were able to accurately identify the molecular
weights of the band fragments that are formed, in order to determine if the large, Calmodulin-containing products were produced.

Figures 2.2, 2.3 and 2.4 show the results of the pH dependence of the proteolysis of CCLP3 by Collagenase. At pH 5.7, there was very little proteolysis of CCLP3 indicating that the protease has low activity at this pH. At pH 6.5 (Figure 2.3) significant proteolysis is observed, including two intermediates that were relatively long-lived, and then the final, lower MW fragment that contains “trimmed” Calmodulin. Graph 2.1 was used to estimate the size of the fragments formed. The Calmodulin fragment had a calculated MW of 16,900 D, a value similar to the expected value. Although not identical to the Calmodulin peptide engineered into CCLP, it is likely Calmodulin with excess C-terminal, and N-Terminal CLS residues that remained following collagenase cleavage. The intermediates appear at MW 36,000 D and 20,300 D. Immediate cleavage occurs forming an intermediate, when either N, or C terminal Calmodulin is cleaved off, resulting in an intermediate CCLP3 construct that has only two Calmodulins in the construct, as opposed to the original CCLP3 with three Calmodulins, and corresponds to the transient protein band at ~ 36,000 in Figure 2.3. This pattern implies an initial cleavage event, likely loss of the C-terminal fragment, and then subsequent internal cleavage to create two fragments that are then trimmed to the final Calmodulin unit at ~ 17,000 D. The exact same intermediates are formed in the digest at pH 7.4 (Figure 2.4), but they persist longer. Taken together, this pH dependent study of proteolytic activity indicates that CCLP3 is a substrate for proteolysis and that the protease has highest activity at pH 6.5.
Figure 2.2. Proteolysis of CCLP3 by Collagenase in MES Buffer pH 5.7. Lane 1 contains the protein ladder, lane 2 contains collagenase, lane 3 contains CCLP3 stock, and lanes 4-11 contain CCLP3 digests times 0 min-640 min.

Figure 2.3. Proteolysis of CCLP3 by Collagenase in MES Buffer pH 6.5. Lane 1 contains the protein ladder, lane 2 contains collagenase, lane 3 contains CCLP3 stock, and lanes 4-12 contain CCLP3 digests times 0 min-1280 min.
Figure 2.4. Proteolysis of CCLP3 by Collagenase in MES Buffer pH 7.4. Lane 1 contains the protein ladder, lane 2 contains collagenase, lane 3 contains CCLP3 stock, and lanes 4-12 contain CCLP3 digests times 0min-1280min.

Graph 2.1: The trend-line formed from the Gel migration patterns of the protein ladder for examination of CCLP3 fragment identification in proteolysis. The logarithm base 10 of the molecular weight plotted against the relative mobility of the MW standard protein ladder to produce a trend-line. The equation for the trend-line is then used to determine molecular weight identities for major bands. Major bands were determined to be 16,900 kD, and 20,300 kD.

Figures 2.5 and 2.6 show the results of pH dependence of the proteolysis of CCLP2. This construct has only two Calmodulin sequences and each sequence is
followed by 3 repeats of CLS. As such, proteolysis of this construct creates a feathered look to the low MW fragments, and no high MW fragment as observed in the CCLP3 digest. This indicated that the cleavage at one of the CLS sites between the two Calmodulin sequences cleaves CCLP2 in half and then subsequently there is cleavage of the other CLS sites, creating the feathered, higher MW intermediates as the CLS sites are cleaved. Catalytic efficiency at pH 6.5 is difficult to observe due to cleavage of CCLP2 main band forming band fragments that are indistinguishable.

Figure 2.5. Proteolysis of CCLP2 by Collagenase in MES Buffer pH 6.5. Lane 1 contains the protein ladder, lane 2 contains CCLP2 stock, lane 3 contains collagenase, and lanes 4-12 contain CCLP2 digests times 0 min-1280 min, and lane 13 contains 1280min digest with EDTA added.
Figure 2.6. Proteolysis of CCLP2 by Collagenase in Tris Buffer pH 7.4. Lane 1 contains the protein ladder, lane 2 contains CCLP2 stock, lane 3 contains collagenase and lanes 4-12 contain CCLP2 digests times 0 min-1280 min, and lane 13 contains 1280 min digest with EDTA added.

Graph 2.2. The trend-line formed from the Gel migration patterns of the protein ladder for examination of CCLP2 fragment identification. The logarithm base 10 of the molecular weight plotted against the relative mobility of the MW standard protein ladder to produce a trend-line. The equation for the trend-line is then used to determine molecular weight identities for major bands. Major band formed from digest was determined to be 21,000 kD.
Once we observed pH- and time- dependent cleavage by collagenase, we decided to test pH- and time- dependence of Matrix-Metalloproteinases that are involved in the human inflammatory response. MMPs require activation, which entails cleaving a cysteine residue that interferes with the active site, before being catalytically active. Upon observation of Figure 2.7, and 2.8, notable similarities in molecular weights of Active, and inactive MMP-1, and MMP-8 imply either that the enzymes that we received were not MMP-1, and MMP-8, or that the activation protocol is incorrect. Lane 1 of each Gel is the standard protein ladder, and lanes 2-5 of Figure 2.7, and lanes 2-6 of Figure 2.8, are either active or inactive MMP-1, or active or inactive MMP-8 (exact identities are detailed in the figure legends). Despite the differences in identity of protease in each of these wells, the bands are strikingly similar in each of these wells, alluding to the fact that their actual identities are unknown.

![Figure 2.7](image_url)

**Figure 2.7.** CCLP3 proteolysis by activated MMP-1. Apparent activation of MMP-1, and MMP-8 yielded no change in molecular weight when compared to the inactive, pro-peptide form of the enzymes. Despite this, CCLP3 cleavage by MMP-1 was apparent but
minimal, which warrants further investigation into MMP activity. Lane 1 contains the protein ladder, lane 2 contains inactive MMP-1, lane 3 contains active MMP-1, lane 4 contains inactive MMP-8, lane 5 contains active MMP-8, lane 6, and 7 contain CCLP3 digest by MMP-1 0hr, and 16hr respectively, and lane 8, and 9 contain CCLP3 digest by MMP-8 0hr and 16hr respectively.

Figure 2.8. CCLP3 proteolysis by activated MMP-1. Activation of MMP-1 and MMP-8 yielded no change in molecular weight when compared to the inactive, pro-peptide forms of this enzyme, however cleavage of CCLP3 by MMP-1 is apparent, which warrants further investigation into MMP activity. Lane 1 contains the protein ladder, lane 2 contains the MMP-1 inactive pro-enzyme, lane 3-6 contain activated MMP 0hr, 1hr, 2hr and 4hr respectively, lane 7 contains CCLP3 stock, Lane 8-11 contain CCLP3 digest by MMP-1 0hr, 16hr, 48hr, and 72hr respectively.*

*Experimental errors in loading lanes 3,8,9, and 12 resulted in lower concentrations than other lanes, and can be disregarded.
CHAPTER 4

CONCLUSION

Following extensive experimentation with collagenase at physiological temperature and differing physiological pHs, it can be concluded that the enzyme does exhibit pH dependence in activity. SDS-PAGE of CCLP3 digest by collagenase reveals that at 5.7 pH, the enzyme exhibited minimal activity, whereas proteolysis experiments carried out at 6.5 pH produced the optimal results, cleaving the specified site in the shortest amount of time, with proteolysis at pH 7.4 as a control. The proteolysis experiments of CCLP2 by collagenase at different pHs yielded results that are less clear. With CCLP2, it is difficult to make absolute statements regarding pH dependence due to main band CCLP2 fragments disappearing in both digests after approximately 80 minutes, and secondary band formation being too close together to distinguish between bands. In order to optimize a gradual, pH dependent drug delivery, the CCLP3 construct is likely the best of our current construct for a hydrogel-based drug delivery system.

In order to accurately characterize fragments produced from the cleavage by collagenase, trend lines were formed from the known molecular weight ladder. The calmodulin peptide engineered into CCLP2 and CCLP3 has a molecular weight of 16,837 D. The leader sequence of CCLP3 to the first cleavage site yields a band of 20,233 D. With the trend line, and known molecular weights of expected cleaved constructs, accurate identities of bands were determined. Formation of two distinct bands during CCLP3 digests corresponded to approximately 16,900 D and 20,300 D. The 16,900 D band can be distinguished as Calmodulin, and the band corresponding to 20,300 D is Calmodulin with the leader sequence engineered into the protein for His-Tag purification.
Due to many different cleavage points present in CCLP2, there are several different bands that can form with similar Molecular weights. Also, since single band resolution of the digested construct is poor, it is not possible to conclude which exact construct that includes calmodulin was formed. During CCLP2 SDS-PAGE characterization, final 1280 minute samples were treated with EDTA, in order to chelate the Ca$^{2+}$ from the Calmodulin in case the change in conformation upon calcium binding altered the migration of bands. Upon observation, it can be concluded that Ca2+ did not alter fragment migration by any observable amount.

We began investigation into specific Matrix Metalloproteinase pH dependent catalysis of engineered protein substrate, however due to gel anomalies, the project was put on hold. Activation of MMP-1 occurs through cleavage of the 52-57 kD pro-peptide form, altering the molecular weight by approximately 10 kD for MMP-1, resulting in a 42-47kD active form depending on glycosylation. Activation of MMP-8 occurs through cleavage of the 85kD inactive form to produce an active 65-70kD activated form. Despite a discernable difference in molecular weights between the active and inactive forms of each respective MMP, it is evident by Figure 2.7, and Figure 2.8, the SDS-PAGE of the CCLP3 proteolysis experiments, each band, whether inactive or active, and whether MMP-1 or MMP-8, is strikingly similar in molecular mass. This warrants further research into the activation of MMP-1 and MMP-8, as well as the activity at physiological conditions in vitro.
APPENDIX

The amino acid sequences of the proteins studied in this thesis.

CCLP3 Construct

```
MGSSHHHHHHSSGLVPRGSHMARMDQLTEEQIAEFKEAFSLFDKDGDTTITTK
ELGTVMRSLGQNPTEAELQDMINEVDADNGTIDFPEFLTMMARKMKDTDS
EIREAFRVFDGNGYISAELRHVTNLGEKLTDEEVDDEMIREADIDDGQV
YEEFVQMMTAKGYGGPGQIG/WGQSGYGGSAMADQLTEEQIAEFKEAFSL
FDKDGDTTITTKELGTVMRSLGQNPTEAELQDMINEVDADNGTIDFPEFLT
MMARKMKDTDSIEIREEAFRVFDGNGYISAELRHVTNLGEKLTDEEVDEMI
EADIDDGQVNYEEFVQMMTAKGYGGPGQIG/WGQSGYGGSAMADQLTEEQIAEFKEA
FSLFDKDGDTTITTKELGTVMRSLGQNPTEAELQDMINEVDADNGTIDFPEFLT
MMARKMKDTDSIEIREEAFRVFDGNGYISAELRHVTNLGEKLTDEEVDDEMIREADIDDGQV
```

CCLP2 Construct

```
MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRSGMADQLTEEQIAEFKEAFSL
FDKDGDTTITTKELGTVMRSLGQNPTEAELQDMINEVDADNGTIDFPEFLTMM
ARKMKDTDSIEIREEAFRVFDGNGYISAELRHVTNLGEKLTDEEVDDEMIREADIDDGQV
```

CCLP3 bis-Cys

```
MGSSHHHHHHSSGLVPRGSHMCMADMQLTEEQIAEFKEAFSLFDKDGDTTITTK
ELGTVMRSLGQNPTEAELQDMINEVDADNGTIDFPEFLTMMARKMKDTDS
EIREAFRVFDGNGYISAELRHVTNLGEKLTDEEVDDEMIREADIDDGQV
```

PCLP 3

```
MGSSHHHHHHSSGLVPRGSHMASMTGQGMGRSG
KRRWKKNFIAVASANRFKIISSSGALELDSTQGPQG/IWGQGSRGSGYGS
```

33
A – CLS (collagen like sequence)
A – Calmodulin
A – Leader sequence for His-Tag Purification
/ – Cleavage site


