Influence of Mechanical Load on Enzymatic Cleavage of Corneal Collagen

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Abstract

Previous studies have verified that mechanical load aids the development and adaption of load-bearing collagenous matrices. It has been hypothesized that collagen fibrils in native tissue are “strain-stabilized” such that they are protected (to some extent) from enzymatic attack by application of mechanical load. If true, collagen and its complement native enzymes (MMPs) could comprise the basis of “smart” structure which is intrinsically load-adaptive. The objective of this thesis is to test the hypotheses that strain/load controls the pattern of enzymatic degradation in corneal tissue. In this investigation strips of bovine corneal tissue are placed under fixed uniaxial load in a custom, miniature incubated mechanical testing system in both load and strain control modes. The strips are then exposed to bacterial collagenase (Clostridium Histolyticum) to investigate strains and strain-rates throughout the degradation process. This system “Bioreactor” is capable of producing in vivo environmental conditions while recording either strain or load. Also, Transmission electron microscopy and polarization optical microscopy were employed to determine if the degradation of the tissue is preferential. The result indicates a reduction in cutting rate when load decreases from 1.0N to 0.25N, while further decreasing of load (to 0.1N) will result in an increase in cutting rate. This result suggests that loads of approximately 0.25N, which is close to the in vivo loads, the degradation reaches a minimum rate. However, the data demonstrates that the fibrils are not completely protected from enzyme attack. Thus, more investigation is needed to indicate if the application of mechanical load actually enhances the molecular stability or it just delays the diffusion of the enzyme molecules into the tissue.
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Chapter 1: Introduction

1.1: Collagen

Collagens are one of the most important structural proteins and load-bearing molecules in vertebrate animals. The word collagen is derived from Greek word “kallagene”, which means “glue forming” regarding gelatin’s form and they are categorized by Roman numerals (collagen type I, II, III…). There are over 25 different types of collagen in animals (for comprehensive compilation see Canty and Kadler 2005 [1]) which are a major component in Extra-Cellular Matrices (ECMs). Of these, five “fibril forming” collagens have been detected (I, II, III, V, XI) in connective tissue such as Ligaments, Tendons and Cornea. Different connective tissues have their own specific microscopic and macroscopic structural appearances which are completely adapted with their functions and performances (i.e. transparencies in cornea, tensile bearing in tendon and load-bearing in bone). Fibroblast cells, the common cell in connective tissue, synthesize and maintain the ECMs and provide a structural framework for connective tissues. In other words, Fibroblasts are thought to play a major role in maintaining the integrity of connective tissue by synthesizing, secreting and organizing collagen in ECMs. Furthermore, Collagens are very important in ECMs since they provide mechanical strength and flexibility of tissue and also promote cell growth. Collagen molecules are adapted to carry tensile loads along their principal axis and are thus anisotropically arranged into aligned fibrils in load-bearing ECMs. There is little information available about how collagenous tissue organization is generated and maintained during development and growth [2, 3]. However it has been presumed that mechanical load plays a critical role in epigenetic regulation of load-bearing collagenous ECMs [2-5].
Collagen type I is the most abundant of all types in connective tissue (and is consequently the most observed) [6]. It forms over 80% of collagenous structures in all ECMs (most connective tissue). It is the principal determinant of load-bearing matrix mechanical properties and plays a crucial role in cartilage mechanical behavior [7]. The basic structure of collagens at the supramolecular level is a right-handed, linear, triple helix comprising three left-handed helical alpha chains to form a rope-like molecule (Figure 1A). The chemical structure of each collagen molecule is named αA, αB and αC and consists of sequential chains of the Gly-X-Y which are repeated in the special form of (Gly-Xaa-Yaa) in which, Xaa and Yaa are typically Proline and Hydroxyproline. Gly is the smallest amino acid found and is every third residue (Figure 1B) [8].

Figure 1: A) Triple-helix of three polypeptide chains – B) The polypeptide chains comprise Glysine (Gly-X-Y repeats where X and Y are generally pro- or hypro-) repeating triplet of amino acid residues [8].
Pro-collagen is 300 nm long, 1.5 nm wide and is comprised of three α chains. Pro-collagen chains are synthesized by cells and are brought together by C-propeptides and generate a triple-helical molecule as pro-collagens trimer. This molecule is comprised of three parts (N-proteinase, collagen and C-proteinase). The C-propeptide is one side of the pro-collagen molecule and the N-propeptide is the other side. Procollagen is subjected to proteinase enzymes which cleave propeptides from pro-collagen. In other words, they cleave N- and C- terminals from procollagen and convert it to tropocollagen. Without these enzymes the formation of fibrils would not occur. Procollagen N-proteinase cleaves N-propeptide and procollagen C-proteinase cleaves C-propeptide. After this process, collagen monomer is ready to combine with other collagen monomers. (Figure 2)

![Collagen Fibril Production Diagram](image)

Figure 2: Overview of collagen fibrils production by fibroblast. Procollagen chains are synthesized in the endoplasmic reticulum(ER) to form a rod-like triple helical domain. Then, N- and C- propeptides are removed from the procollagen in order to assemble into fibrils (Canty and Kadler et al [1])
The assembly of collagen molecules into fibrils is an entropy driven process [9]. In other words, the change of energy of collagen molecules and their associated water plays an important role in this process. These processes are driven by the loss of solvent molecules from the surface of proteins and result in assembled fibrils with a circular cross-section. The following specific model for the assembly of collagen type I (fibril fusion) is assumed by Kadler either in vivo or in vitro [8]. Each side of collagen fibrils has a different shape and affects how collagen fibrils are assembled together. Growth of collagen fibrils is from the pointed tip (C-telopeptides) while the other side, rounded end (N-telopeptides), is growing a new pointed tip. At the end of the growth process, the collagen fibril has two pointed tips (N-termini) which are ready to merge with other collagen monomers. (Figure 3) The assembly of fibrils is from either N-end to N-end or one N-end to C-end.

Figure 3: The assumed model for fibrils fusion (two N-ends or one N- and one C-) Kadler et al [9].
Native collagen fibrils have a distinctive 67 nm banding pattern which is the same as correctly assembled fibrils in vitro. All collagen molecules are not parallel; rather, they are staggered to produce a gap between collagen molecules (42 nm) in one part and an overlap (25nm) in another part in the longitudinal direction [8]. This gap and overlap are clearly observable in TEM (Transmission Electron Micrograph) pictures (Figure 4). The dark sides are gap areas between the collagen molecules in contrast to the white areas showing overlap. This length is repeated every 67nm and is called the D-period which is divided in 0.6D (gap) and 0.4D (overlap).

Figure 4: Schematic representation of the axial packing arrangement of triple-helical collagen molecules in fibrils. Dark and white areas demonstrate D-periodic of self assembly fibrils into gap and overlap assembly. [8]

Self-assembly is not just limited to end-to-end fusion, monomers are also able to have lateral fusion formed by cross-linking. There are many models for covalent cross-linking assembly but the accepted structure is the five stranded Smith micro-fibril (Figure 5). In this model five collagen type I micro-fibrils are assembled to each other to form a
collagen fibril. The diameter of micro-fibril is 4 nm which is the minimum diameter for filamentous structure that possesses an axial D repeat. This assembly of collagen micro-fibrils generates the formation of larger fibrils [10]. The diameter of fibrils ranges from 30nm to 500nm and depends on tissue, age and genetics. In other words, the diameter and formation of fibrils from micro-fibril are completely different in different kinds of tissue.

Figure 5: A) Longitudinal view of collagen molecules (five molecule segment 1 to 5) in D staged array B) The section of radial packing Holmes model C) Enlarged view of boxed area which indicates the fibril comprised of five individual micro-fibrils D) Schematic of assembly of micro-fibrils into the fibril [10]

Connective tissues are categorized not only by different fibril diameters (self-assembly) but also by the organization of fibrils in tissue. In cornea, for example, fibrils are
arranged in multiple layers or lamella. Each layer is comprised of aligned (parallel or anti parallel) fibrils. Between layers, there is a constant angular twist (often 90°) which results in a plywood-like arrangement [10]. In contrast, fibrils of bone are formed in a concentric cylinder shape and fibrils of tendon are parallel but wavy crimped. Also, fibrils of skin have complex three-dimensional weave. Figure 6 demonstrates different suprafibrillar architectures similar to different tissues which arise naturally from liquid crystalline collagen monomer. Studies verify that the structure of cornea represents a cholesteric organization and tendon has a precholesteric arrangement [11-13].

![Figure 6: Cholesteric liquid crystalline textures of collagen fibrils from four different connective tissues A) Scale B) Bone osteon C) Tendon and D) Skin [11-13]](image)

### 1.2: Cornea

Cornea is the connective tissue which is explored in this study. The main part of this tissue contains of collagen type I fibril with smaller amount of collagen type V and more than 66% water surrounding collagen fibrils [14, 15]. Also, it comprises approximately 200 flattened plates or lamella of collagen fibrils (Figure 7A) which transmit about 99% of the incident light into the eye [26]. Corneal transparency has been well-studied and is thought to depend on corneal collagen fibril diameter mono-dispersity, diameter size and
spacing [25]. This transparency is because of size and concentration of fibrils at each flattened plate (Figure 7D). Factors which control the growth and arrangement of collagen fibrils are not known yet but it has been proposed that collagen fibril diameter (type I) may be controlled by minor collagen types (such as type V) [16]. Interfibrils (space between collagen fibril) comprise of collagen type VI [17] and glycosaminoglycans (GAGs) which provide an osmotic pressure, drawing fluid into the ECM [18]. The GAGs are typically bound to core protein to form lumican and decorin proteoglycans [19]. It has been demonstrated that there is a structural relationship between these proteoglycan and collagen fibrils [20]. X-ray diffractions from cornea provide detailed structural information about corneal collagen fibrils (Figure 7C). By using this method it is possible to calculate the number of fibrils, micro-fibrils and monomers and also the diameter of fibrils in a specific cornea. Meek and Leonard et al [15] assumed four fibrils as a unit in cornea stroma (Figure 7B). This assumption makes it easy to separate collagen fibrils from each other. The volume per length (U) is calculated by $U = i \times p$, where $i$, the center to center distance, is $i = 1.12p$ therefore $U = 1.12p^2$. By this definition it is possible to calculate interfibril space on a specific plate ($v_i$). For example in bovine cornea, $p=58.2 \pm 4.5$, $D_{fibril}=38.2 \pm 0.08$ and $N_{\text{number molecules per fibril}} = 405$ are estimated by X-ray method.
Figure 7: A) Schematic of cornea lamella B) Schematic interfibril (four collagen fibrils in cross section) C) Electron micrograph of collagen fibrils in the corneal stroma of a rabbit [15]. D) TEM pictures from Bovine Cornea (Extracellular Matrix Engineering Research Laboratory, May 2007).
1.3: Mechanical Properties of Collagen

A significant characteristic of collagen type I is its ability to form stress-bearing structures in connective tissue. It is important to know the mechanical properties of collagen at the molecular and fibrillar level. At the fibrillar level, the function of tissue on stress-strain curve is modeled as a worm-like chain. The stress-strain curve of tendon as a collagen-rich tissue was investigated by the application of X-ray scattering. Figure 8 illustrates the stress-strain curve which is obviously comprised of three parts [23].

![Stress-Strain Curve](image)

Figure 8: Three different regimes of loading collagen fibrils [23]

In the toe region, the tissue (fibrils) is extended under very low load and the D-period shape of fibril changes to straight shape. The small strain removes all macroscopic crimp in collagen fibrils [22]. After passing toe region (more than 3% strain), fibrils scatter from each other because of straightening of kinks in collagen molecules (beyond 3%). At heel region, this scattering causes that fibrils have a transition from toe to linear...
region. In the linear region, most kinks are straightened and the collagen molecules (cross links) glides past each other in the longitudinal direction.

![Image of collagen molecule](image)

Figure 9: A) Real stretching of a single collagen molecule by beads B) The stress-strain curve for stretching a single collagen I monomer [24].

Moreover, recently due to modern technology at the molecular level, optical tweezers and atomic force microscopes can stretch collagen molecules and fibrils in order to find mechanical properties of them. The flexibility of collagen was tested at the molecular level by Sun et al [24] using optical tweezers. They determined the mechanical properties of type I collagen monomers by optical tweezers and measured the persistence length of collagen type I monomer (14.5 nm). In this method, the pro-collagen molecule is attached to two polystyrene beads (Figure 9A) while one of them is fixed and the other is moveable. The results determining the stress-strain curve (highly non-linear stress-strain curve) of stretching single collagen molecule are matched with the worm-like chain (Figure 9B) elasticity model which confirms that collagen molecule is a flexible molecule.
Chapter 2: Bacterial Collagenase

In addition to collagen self-assembly and load-bearing characteristics, collagen degradation is an important step in the development, remodeling, homeostasis and pathology of load-bearing ECM [27]. Matrix Metalloproteinase (MMP), Bacterial Collagenase (Clostridium Histolyticum) and Cathepsin are the best known enzymes capable of directly degrading the collagen triple helix [28-31]. Collagenase recognizes the sequence -R-Pro-8-X-Gly-Pro-R- where X is most often a neutral amino acid. It breaks the peptide bonds (CO-NH) in the collagen molecule and normally targets the connective tissue [32]. In other words, a chemical bond is formed between two molecules when the carboxyl group of the collagenase molecules reacts with the amino group of collagen (dehydration synthesis) and then releases a molecule of water. These enzymes attach to the collagen triple helix (amino acids groups) by binding to the enzyme’s attachment domain (carboxyl group) along the α-chains and then separate (unwind) the α-chains to the ¾-¼ cleavage site when exposed to MMPs or unwind it to a random cleavage site when exposed to Bacterial Collagenase [33,34].

Clostridium Histolyticum is one of the pathogenic clostridia which can produce various proteases, such as a collagenase (clostridiopeptidase A) and clostridia (clostridiopeptidase B) and is highly proteolytic. Although this enzyme has been used in biological experiments, the detailed biochemical properties are not yet fully understood. The enzyme can cleave both native and denatured collagen fibrils (Figure 10) because of zinc-metalloprotease, unlike eukaryotic collagenase (as MMP) which cleaves only specific native collagen as mentioned above [35]. Clostridium Histolyticum has two different collagenase genes (colG and colH) in its chromosome which will be encoded
into two distinct enzymes; class I collagenase (ColH) and class II collagenase (ColG).

There is no significant similarity between these two types of amino acids and MMP’s structure. The full-length class I collagenase (ColG) has two collagen-binding domains (CBDs) at its C-terminus, while the class II collagenase (ColH) contains only one CBD. These three domains are similar to each other and comprise almost 110 amino acid residues and bind to various types of insoluble collagen. It was assumed that these CBDs which contain C-terminus (as anchor peptide-signaling molecule) bind to insoluble collagen molecules and then cleave this molecule. Calcium ions (as catalytic facilitators) enhance the binding of CBD to insoluble collagen and collagenous peptides. This type of collagenase is activated by four gram atom calcium (Ca2+) per mole enzyme.

Although the term collagenase implies that there is a single enzyme produced by Clostridium histolyticum, this is not the case. Crude collagenase is a mixture of enzymes (mostly proteases) secreted by C. histolyticum. All may contain 10 to 18 components (by electrophoresis), only 8 of which have been identified. Figure 11 demonstrates the sequence of events which lead to degradation of collagen fibrils by collagenase.

Figure 10: Collagenase binds to and locally unwinds collagen before it cleaves the triple-helical interstitial collagen [36]
First, connective tissue cells synthesis collagenase and neutral proteases using several factors (1). Then mucopolysaccharides which surround collagen fibril are removed by the proteoglycan-degrading enzymes to expose the fibrils to collagenase (2). Inactive collagenase is secreted (3) which is usually found in the Extracellular Matrix (ECM) bound to an inhibitor (4). Then an activating enzyme removes the inhibitors (5). Glycosidases complete the degradation of the proteoglycans afterward (6). The active collagenase binds to fibrillar collagen (7) and splits the first collagen molecules into two fragments (TCA and TCB) which denature and begin to unfold at body temperature 37°C. The enzyme now moves on to an adjacent molecule (8). The denatured collagen fragments are then subject to other proteases (9). Finally non-specific neutral proteases degrade the collagen polypeptides (10) [35]

Figure 11: Sequence of events leading to degradation of collagen fibrils [35]
Chapter 3: Hypothesis

Our investigation for this study is based on the Hypothesis which we refer to as “strain-stabilization” when collagen is exposed to specific or non-specific enzymes during degradation process. Recently it has been hypothesized that the adaption and reorganization of load-bearing ECM is directed by strain at the level of the matrix molecules (as opposed to being directly cell-mediated) [37]. This hypothesis requires that collagen monomers are preferentially removed from load-bearing ECM if they are not sufficiently strained. In other words, the collagen matrix is a smart, load-adapting biomaterial in the presence of the enzyme when subject to mechanical load. Figure 12 is a schematic demonstrating the hypothesis in anisotropic tissue where loaded collagen fibrils (red rods) aligned with the load axis survive during the degradation, while the unloaded fibrils (blue rods) are removed preferentially by the enzyme. There have been two studies which suggest that mechanical loads/strains can influence the enzymatic degradation of collagen.

![Figure 12: Schematic of corneal fibrils (A) before and (B) after enzymatic cleavage. Ruberti et al (2005) [31]](image-url)
Hunag and Yannas [38] indicated that 1) there is a linear relationship between the degradation rate and concentration of bacterial collagenase 2) the activity of bacterial collagenase is maximum at PH 7-8 3) degradation rate is dependent on temperature 4) bacterial collagenase is activated by CA++ and inactivated by EDTA 5) degradation rate increases significantly by denaturation 6) degradation rate significantly decreases around 4% strain on collagen fibrils. They quantified that the degradation rate of bovine tendon strips at range 1-7% strain reaches the lowest possible rate at 4% strain (Figure 13). They justified that unfolding process in low strain (1-4%) will reduce the enzyme flux rate into the substrate which leads to a decrease in degradation rate. On the other hand, high strain (4-7%) may cause changes in the structure of the molecules which will result in opening of new sites of enzymatic attacks or rupturing the collagen molecules.

Figure 13: Variation of degradation with strain (——) the equilibrium stress-strain curve for the collagen substrate (-----), temperature 37°C, concentration 2 units/cm³, pH 7.4 Molecular weight 29000. (huang and yannas et al [38])
In another study, Nabeshima [39] investigated the stability of loaded collagen in tendon against enzymatic degradation. The results captured from their own device demonstrated that degradation process is slow at 4% strain on tendon strips from rabbit in the presence of 60 u/ml purified bacterial collagenase for 20 hours. This result corroborates the Huang and Yannas study which shows that 4% strain is the lowest possible degradation rate in collagen fibril in native tissue. Figure 14 demonstrates the effect of collagenase on the loaded specimens with and without the delayed addition load. The addition of load after 4 hours on specimens made a significantly different load on loaded and slack specimens while the force was the same on the control and loaded specimens. Nabeshima’s study suggests “strain inhibition” of collagen degradation. They discussed two different mechanisms responsible for this observation. 1- The collagenase accessibility to matrix collagen is reduced because of a decrease in matrix permeability caused by strain. 2- Chemical kinetics of enzyme binding has changed. However, their further investigation shows that the diffusion rate of enzyme was not significantly affected by strain.
Both studies reviewed above suggest that the degradation of collagen fibrils can be inhibited by application of mechanical load. The first investigation by Huang and Yannas et al attributed the stability of collagen against the degradation to changes in the diffusion rate of the enzyme through the loaded matrix. In the second investigation, Nabeshima et al. compared the degradation rate of loaded tissue with unloaded specimens and then studied diffusion to show that the diffusion rate of the enzyme is not significantly different in the loaded specimens. However brilliant results from both investigations on the mechanism of the degradation of collagen fibril, especially the diffusion, provide opportunity for further work. This theory was investigated in more details by Ruberti and

Figure 14: Maximum force (mean ± SD) for each group. NS = Not Significant Nebeshima et al [39]
Hallab [37] in a new study to test the hypothesis that strains/loads on corneal collagen fibrils alter the mechanism of degradation.

In Ruberti and Hallab study [37], several experiments were done on bovine corneal strips as native collagen fibrils under microscope. They examined the effect of load on the degradation process by application of Transmission Electron Microcopy images and birefringence signals. Three different experiments were done on corneal strips; loaded and unloaded specimens in the presence of 0.1 mM bacterial collagenase without calcium and loaded specimens into only PBS (without enzymes, sham). All loaded specimens with or without the enzyme sustain the applied load and maintain the mechanical integrity for up to 120 hours while the unloaded/reloaded specimens lost their mechanical integrity very quickly. Furthermore, the birefringence signal from loaded and unloaded specimens during degradation process is significantly different while there is no change in loaded undigested specimens (Figure 15).

To observe the degradation of specimens with the birefringence signal, polarizers were orientated at 0°-90° and aligned with load axis to illuminate the fibrils which are not aligned in the direction of the applied load. The pictures captured from polarizers at 0°-90° show that collagen fibrils disappear (dark) after complete degradation while the pictures from polarizers at 45°-135° demonstrate that collagen fibrils remained intact. Also, the polarizers illuminate the loaded fibrils at 0°-90° and 45°-135° orientation on loaded, undigested specimens. (Figure 15)
Figure 15: The birefringence images from loaded and unloaded specimens before and after the degradation Ruberti et al [37].

TEM images from normal and digested corneal lamella (cross section) confirm that loaded fibrils survive throughout the enzymatic degradation (Figure 16). The TEM image (A) demonstrates that the classic multilayered lamellar structure of cornea can readily be appreciated with its alternating layers of lines and dots (see section A). The magnified image (see section C) from undigested corneal lamella shows that some fibrils are aligned with the load axis (dots) and some fibrils are not (lines). In contrast to images A and C, the normal orientation of loaded, digested corneal lamella is disrupted in the image (B). Most of the remaining fibrils appear aligned with the load (dots) while the unloaded fibrils (lines) have disappeared.

The results captured by Ruberti and Hallab (et al 2005) [37] support the hypothesis that collagen fibrils are smart, load-adopting biomaterials. In other words, the degradation of collagen fibrils is modulated by application of strain/load. The purpose of this study is to test this hypothesis and investigate the degradation of collagen in more detail on a custom
designed miniature bioreactor. The miniature bioreactor [40] is able to create desired accurate load and high resolution strain at physiological conditions (37°C temperature). This device helped us to investigate the degradation kinetics of corneal collagen fibrils along with time lapse imaging through the birefringence signal.

Figure 16: TEM images from (A) normal corneal lamella (B) loaded, digested cornea (C) magnified image of normal cornea (D) magnified image of loaded, digested corneal Ruberti et al [37]
Chapter 4: Bioreactor

4.1: Structural Design

To investigate the effect of enzymes on collagen fibrils during degradation, it is necessary to prepare suitable conditions for various experimental protocols. Excellent controlled temperature at 37°C, isolated fluid environment from outside and high accuracy measurements on both load and displacement during testing have been produced by a custom-built miniature bioreactor. The basic structure of this instrument was designed and built by Northeastern University's Mechanical and Industrial Engineering, capstone design students under advisement of professor Ruberti. [40] (Figure 17A).

In order to grip a bovine cornea strip (6mm thickness × 17.5±2.5mm length) under controlled load or strain at 37°C controlled temperature, a miniature chamber is needed which allows direct optical visualization by polarizers during enzymatic degradation. All loaded samples should be maintained at physiological temperature 37°C and in nutrient medium (DMEM) which are isolated from outside. A load cell with high accuracy (0.001 lbs, Honeywell Sensotec Columbus OH) and Linear motor with high accuracy (0.1 um, zaber technologies Vancouver, BC) were chosen for Bioreactor. Figure 17A, B demonstrates the specifications for the major instruments used in the bioreactor construction. [40]
Figure 17: A) Collagen Bioreactor, features include load cell (1) Zaber Actuator (2) peristaltic pump (3) cam grips (4) polarizers (5) and high speed camera (7) B) Bioreactor mechanism’s schematic C) polarizers D) the miniature Chamber [40] (Church et al)
The volume of chamber is around 7 ml which is heated by two cartridges; there are two inlets to allow circulation of fluid inside the tissue chamber (Figure 17D). The inside diameter of chamber is 10mm and its length is 70mm. The main body of chamber is made from stain steel; quartz glass is installed and sealed at the back and front of chamber in order to make it easy to optically assess the samples during experiments. Two copper blocks through which silicon tubes are passed and attached to each side of chamber in order to preheat the fluid and to stabilize tissue temperature during enzymatic degradation. The copper blocks also serve to uniformly distribute the heat produced by heater cartridges. The grips can move smoothly inside the chamber while producing a uniaxial strain in the corneal tissue samples.

The heating system of bioreactor is comprised of AC SSR (Omega, Stamford, CT), Temperature controller (Omega, Stamford, CT), two cartridge heaters and a thermocouple. Temperature controller modulates the AC SSR based on feedback from the thermocouple to maintain the temperature in the chamber within 1 degree of target (usually 37ºC). Two heater cartridges are embedded in conductive copper blocks which are fixed on both sides of chamber which conduct the heat to the fluid in the chamber. To control the temperature of the reaction chamber the PID values on temperature controller (Proportional, Integral, and Derivative) are adjusted in order to maintain the temperature to 37ºC inside the chamber. The two cartridges heat the copper blocks on each side of chamber which in turn heats the internal media and the fluid inside the tubes. The Proportional, Integral and Derivative values determine the amount of set point offset (variance) over time and evaluate the rate of change on heater and power on cartridges. [40]
4.2: Birefringence- Time Lapse Imaging

In order to observe the orientation of fibrils during each experiment, two polarizers were located and set in the front and back of chamber (Figure 17C). The birefringence is measured by polarizers and the tissue specimen will absorb and transmit light through the lenses depending on the polarization angles. If the orientation of polarizers is set at 45° and 135°, the fibrils in layers of corneal strips with orientations of 0° and 90° will be illuminated. Figure 18 demonstrates the orientation of fibrils and polarizers and the form of observed corneal strips in two positions. The black dashed line is the orientation of the polarizers and red arrows represent the orientation of the preferentially illuminated fibrils on corneal strips. A digital camera was used to take pictures at each position. To make a movie from degradation the camera takes pictures every 10 seconds by the software Fire-i (version 3.0, The IEEE 1394 Digital Camera Control software). This software is capable of taking pictures every one millisecond using high speed camera. All pictures took are merged into a movie by media maker.

Figure 18: The diagram of orientation of fibrils and polarizers, the black dashed lines shows the direction of polarizer and the red arrows demonstrate the direction of fibrils on corneal strips. (Church et al) [34]
4.3: Computer programming:

The bioreactor was built and programmed by Capstone Lab Project and improved by Kelli Church [40]. For this project, the control system was modified to improve the force accuracy to (± 0.01 N) in load control. The strain control mode was also programmed. Labview programming was used to operate the bioreactor in both load and strain control. This programming is able to control the load between 0 to 5N with high accuracy (± 0.01 N) in load control and is able to measure and record load and displacement in both load and strain control modes. The goal of programming the bioreactor is to mechanically stimulate thin corneal strips in either constant force or constant displacement mode. The PID (Proportional, Integrals and Derivative) values on controller obtain the constant force in load control mode.

Program is comprised of three parts in load control mode. The first part moves the actuator to zero position which is the lowest position in the chamber. The second part moves the actuator to absolute position in order to install the corneal strips on both grips inside the chamber. In the third part, the program is set to a desired force for stretching the sample in load control mode. Figure 19 shows the main panel of program which is used to control the load and displacement in bioreactor. The Labview block diagram of the load control mode can be viewed in appendix A.

Furthermore, the program is comprised of three parts in strain control mode. The first and second parts move the actuator to zero and absolute position respectively. Third part then moves the sample to desired strain % (Figure 20). The Labview block diagram of strain control mode can be viewed in appendix B.
Figure 19: Labview front Diagram in Load Control Mode
Figure 20: Labview front diagram in Strain Control Mode
Chapter 5: Materials and Methods

5.1: Specimen Preparation

Uniaxial tensile specimens were generated from right and left eyes of two weeks old Bovine (40-100 pounds) obtained from Research 87 Inc. (Boylston, MA). After cleaning the globes, some corneas were gently removed with epithelium and endothelium layers (protocol 1 and 2), while some corneas were removed and epithelium and endothelium layers were scraped off by razor blade (Protocol 3 and 4). After this step samples were stored at -80°C for future use. Before testing, the samples were removed from freezer and thawed at room temperature for 10 minutes. A custom made cutting die was used to generate accurate repeatable strips from the tissue. The central region of each dissected cornea was used to produce one vertically oriented tensile specimen from Superior to Inferior (approximately 0.7 mm thick x 17.5 ± 2.5 mm length x 6 mm wide- Figure 21 including 5 mm sclera at either end of the strip which were attached to the grips by glue and cams throughout the experiment. Before digesting, all tensile specimens were kept moistened with 37°C DMEM- Dulbecco's Modified Eagle's Medium-(Mediatech Inc., Manassas, VA) inside a miniature bioreactor chamber.

5.2: Digestion Protocol

The collagenase used for all protocols (1, 2, 3 and 4) is crude collagenase from Clostridium Histolyticum (Clostridiopeptidase A, Sigma-Aldrich- No.C130). The molecular weight of collagenase ranges from 68kDa to 125kDa [41]. This enzyme is assayed by sigma at 7.4 pH without Ca++ while optimum pH changes from 6.3 to 8.8 [42]. In order to make 0.05mM active
collagenase solution for all protocols (1, 2, 3 and 4), the average molecular weight chosen was almost 95kDa and 4.75mg/ml collagenase was mixed with DMEM (Dulbecco's Modified Eagle's Medium). Each mole collagenase needs four gram atoms calcium Ca\(^{++}\) to prepare active bacterial collagenase \[^{[42]}\]. DMEM comprises of 200mg/ml Calcium Chloride and gives 1.7mM Ca\(^{++}\) to the solution which is enough calcium to activate the enzyme. In summary, each collagenase solution with a concentration 0.05mM comprises almost \(3 \times 10^{19}\) active collagenase molecules along with \(1.24 \times 10^{21}\) calcium (Ca\(^{++}\)) molecules.

The concentration of bacterial collagenase solutions in all protocols (1, 2, 3 and 4) was 0.05mM (4.75mg/ml) which is mixed with 200mg/ml of calcium chloride in DMEM. All experiments in protocols 1, 2 and 3 were tested with Bacterial collagenase with 0.31 units/mg while all experiments in protocol 4 were tested with bacterial collagenase with 0.26 units/mg.

In summary, materials used in different protocols are classified in four categories:

1- Protocols 1 and 2: corneal strips with epithelium and endothelium layers in 0.05mM BC (0.31 units/mg).

2- Protocol 3: corneal strips without epithelium and endothelium layers in 0.05mM BC (0.31 units/mg).

3- Protocol 4: corneal strips without epithelium and endothelium layers in 0.05mM BC (0.26 units/mg).

Bacterial Collagenase solutions were made before testing and stored in -80°C until the beginning of experiments to be stable and active for each experiment. The collagenase solution is stable for at least one year when stored at -80°C; however freeze-thaw cycles damage the enzyme solution. In order to protect the enzyme in constant activity, the whole solution was divided into 7ml collagenase solutions in different batches for each experiment. In aqueous
solutions, bacterial collagenase at 4°C loses its activity in 3 hours while in one hour there is no
loss in its activity at 40°C with pH 7 and 1 mM Ca$^{++}$ [43].

Loaded tissue specimens were positioned and stretched between two grips at 37°C in DMEM,
Sigma-Aldrich). After 15 minutes (creep in) the DMEM was changed to 0.05 mM preheat
Bacterial Collagenase together with 200 mg/ml calcium chloride mixed with DMEM. Before
injection into the chamber, collagenase solution was kept in a water bath (37°C) for 30 minutes
in order to preheat the enzyme.

5.3: Methods

To demonstrate the effect of the active Bacterial Collagenase solution on bovine cornea,
different testing protocols (1, 2, 3, and 4) were performed in the Bioreactor.

5.3.1: Protocol 1

All experiments in this protocol were performed in load-control mode while strain was
recorded. In Load control Test (Creep Test) a specific Load is held constant and the strain
response to this Load is determined. Uniaxial tensile specimens with the epithelium and
endothelium layers were removed from freezer and thawed at room temperature for 10 minutes
and targeted and cut with custom made cutting die to generate accurate repeatable strips from the
tissue (6mm width, 17.5±2.5mm length and 0.7±0.1 thickness). Each corneal strip was
positioned between two grips inside the chamber, immersed in 37°C DMEM and uniaxially
stretched. A preload of 0.01N was applied to the specimens before commencing testing and the
resulting strain was called “zero strain”. Also, appropriate PID values (for cornea) were set in
order to provide small oscillations in load control system (± 0.01N) during experiments.
Following a short preload, the experimental load of 0.25N, 0.5N and 1N were applied for one
hour to allow “creep-in”. Four different tests where performed:
1- Control Test with Load (No Digestion)
2- Control Test with No Load (No Digestion)
3- Digestion Test with No Load
4- Digestion Test with Load

1- Control Test with Load (no digestion): For some specimens (N=16), at the end of “creep in” the tissue remained at 0.5N and 1N for an additional 2 hours without injecting collagenase solution while strain was recorded.

2- Control Test without Load (no digestion): For some specimens (N=10), at the end of creep in all specimens were unloaded and then reloaded after 1 hour until failure.

3- Digestion Test without Load: some specimens (N=18) were unloaded at the end of “creep-in” and exposed to 0.05mM Crude Bacterial Collagenase. The specimens were then reloaded at each level loading (0.5N and 1.0N) after one hour until failure. Load and strain were recorded throughout the experiment.

4- Digestion Test with Load: some specimens (N=27) remained loaded (0.25, 0.5 and 1.0N) at the end of “creep-in” and the DMEM was replaced by preheated 0.05mM active Crude Bacterial Collagenase (Sigma Aldrich No.C0130) for 2 more hours.

5.3.2: Protocol 2

In the Strain Control Test, the displacement is held constant and the stress response is measured over time. This type of test is another method to interpret the degradation rate of corneal specimens. This method is less dynamic than the load control test and provides a limited sampling of the effect of strain or load on degradation rate. All corneal strips with the epithelium and endothelium layers were removed from freezer and thawed at room temperature room for 10
minutes and targeted and cut with custom made cutting die to generate accurate repeatable strips from the tissue (6mm width, 17.5±2.5mm length and 0.7±0.1 thickness). Samples (N=30) were then positioned between two grips inside the chamber. In this experiment the specimens were exposed to fixed-loads of 0.5N and 1.0N for one hour (Load control setup). Then a standard stress relaxation test was applied to the specimens along with injection of 37ºC, 0.05 mM Crude Bacterial Collagenase and two different oils. The heavy silicon base oil was placed in the bottom of the chamber and the light vacuum pump oil was placed at the top of the chamber to create a small reaction zone in the middle of the specimen. Volumes of enzyme solution were applied to the sample to test the effect of bacterial collagenase during strain control experiments were 0.5ml (5mm Height on samples), 0.1ml (10mm Height on samples) and the whole chamber. Load response data was collected in Labview during enzymatic degradation.

In summary, protocol 2 was performed in three different tests:

1- Digestion Test in 0.5ml collagenase
2- Digestion Test in 1.0ml collagenase
3- Digestion Test in whole chamber

5.3.3: Protocols 3 and 4:

All experiments in both protocols 3 and 4 were performed in load-control mode while strain was recorded. All corneal samples without the epithelium and endothelium layers at -80ºC were removed from the freezer and thawed at room temperature room for 10 minutes and targeted and cut with custom made cutting die to generate accurate repeatable strips from the tissue (6mm width, 17.5±2.5mm length and 0.7±0.1 thickness). After all specimens were positioned inside the chamber, a preload of 0.01N was applied to them before commencing testing. The displacement
at this small load was assumed to be the position of “zero” strain. Also, appropriate PID values (for cornea) were set in order to provide small oscillations in the load control system (± 0.01N) during experiments. At the beginning of the test, the experimental load was applied to all samples. Then we classified the experiments in one of three categories. (Figure 22)

**Test 1-Control Test with Load:** After preloading 0.01N, some specimens (N=6) were loaded at 0.1N, 0.25N and 0.5N for 90 minutes. This test was designed to determine the expected normal creep response to demonstrate the patency of grip design at the various applied loads.

**Test 2-Digestion Test with Load:** After preloading 0.01N, some specimens (N=62) were loaded at 0.1N, 0.25N and 0.5N for 15 minutes in order to creep-in. Then the DMEM was replaced by preheated active bacterial collagenase solution 0.05mM for 90 minutes.

**Test 3-Digestion Test without Load:** After preloading 0.01N, some specimens (N=10) were loaded at 0.25N for 15 minutes in order to reach stabilization phase. All specimens were unloaded and bacterial collagenase solution was injected for 35 and 45 minutes and then reloaded at 0.25N loading.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Type</th>
<th>Load</th>
<th>Collagenase Concentration</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 1</td>
<td>Control Test with Load</td>
<td>0.1N, 0.25N, 0.5N</td>
<td>DMEM+ CaCL</td>
<td>6</td>
</tr>
<tr>
<td>Test 2</td>
<td>Digestion Test with Load</td>
<td>0.1N, 0.25N, 0.5N</td>
<td>0.05mM+DMEM+ CaCL</td>
<td>62</td>
</tr>
<tr>
<td>Test 3</td>
<td>Digestion Test without Load</td>
<td>0.25N</td>
<td>0.05mM+DMEM+ CaCL</td>
<td>15</td>
</tr>
</tbody>
</table>

Figure 22: The testing protocol chosen for Bioreactor

### 5.4: Polarization Microscope (Birefringence) – Time Lapse Imaging:

The corneal stroma is a transparent array of aligned collagen fibrils which alternate in direction. They are also effectively mechanically isolated (with regard to tension) from one another. Thus, uniaxial tension preferentially loads one set of fibrils, while others remain unloaded. In other
words, if the polarization lenses (43mm Diameter, Prinz, Japan) are set to 45°-135°, they display the corneal fibrils orientated at 0°-90° which align with load axis. Arrays of corneal fibrils will become dark through the polarizers if fibrils orientation is not the same as the load axis while, the aligned fibrils with load axis exhibit a significantly brighter birefringent signal through the polarizers. The specimens were digitally imaged by high speed camera (Prosillica, Canada) every 10 seconds for up 90 minutes through crossed polarizers during each experiment and then the final movies were generated from these pictures.

5.5: Transmission Electron Microscopy (TEM) - Post Processing:

All specimens were removed from bioreactor after 60 minutes and processed for TEM [44]. Briefly, the specimens were immersed in 1/2 strength Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.1M Cacodylate buffer, pH 7.4) for 1 hour, post-fixed in 2% osmium tetroxide and serially dehydrated in graded ethanol. Following dehydration, specimens were embedded in Epon-Araldite and thin-sectioned perpendicular to the applied load in the center of the tensile specimen. Specimens were stained with Uranyl Acetate and Lead Citrate in methanol and viewed on the JEOL 1000 Transmission Electron Microscope (JOEL, Tokyo, Japan).
Chapter 6: Result

6.1: Protocol 1 – Load Control Test

Figures 23, 24 show the strain% vs. time for all experiments in the control test with 0.5 and 1.0N Load. The data captured demonstrate that after “creep in” the increase in strain is below 5% during 2 hours in both 0.5N and 1N loading levels. It confirms that the grips generally do not slip during the experiment. A few experiments were eliminated from analysis because of slipping of specimens during the experiments. Figure 25A, B display the data captured from control test without load and then reloaded with 0.5N and 1.0N load respectively. The digestion test without load and reloaded with 0.5N and 1.0N can be viewed in figures 26 and 27.

Time dependent strain and strain rate obtained from protocol 1 are shown in figures 28-32. Figure 28-30 demonstrate strain% vs. time in the digestion tests in 0.25N, 0.5N and 1.0N Load respectively. The strain rate data can be viewed in figure 31 for all the three different loading levels. Figure 32 also shows the strain rate in the digestion tests without load and then reloaded with 0.5N and 1.0N load. Some experiments were eliminated from analysis in both digestion tests with and without load because their strain was not stabilized during “creep in” (one hour after applying load) or specimens came out from the grips. More than five samples are selected to calculate the average, standard deviation and strain rate. The data shows that strains in digestion tests with load (0.5N and 1.0N) are higher than strain% in digestion tests without load after 2 hours.
Figure 23: Protocol 1- A) all experiments in Control Test with 0.5N Load B) selected Control Test experiments with 0.5N Load and their average.
Figure 24: Protocol 1 - A) all experiments in Control Test with 1.0N Load B) selected Control Test experiments with 1.0N Load and their average
Figure 25: Protocol 1 - A) all experiments in Control Test without load (Reloaded with 0.5N) B) all Experiments in Control Test without Load (Reloaded with 1.0N).
Figure 26: Protocol 1 - A) all experiment in Digestion Test without load (No load, reloaded with 0.5N) 
B) selected experiments in Digestion (No load, reloaded with 0.5N).
Figure 27: Protocol 1 - A) all experiment in Digestion Test without load (No load, reloaded with 1.0N) B) selected experiments in Digestion Test (No load, reloaded with
Figure 28: Protocol 1 - A) all experiment in Digestion Test with 0.25N Load B) selected experiments in Digestion Test with 0.25N Load.
Figure 29: Protocol 1 - A) all experiment in Digestion Test with 0.5N Load B) selected experiments in Digestion Test with 0.5N Load.
Figure 30: Protocol 1 - A) all experiment in Digestion Test with 1N Load B) selected experiments in Digestion Test with 1.0N Load.
Figure 31: Protocol 1 - Strain rate as function of time in Digestion Test with 0.25N (A), 0.5N (B) and 1N(C) Load.
Figure 32: Protocol 1- The strain rate of digestion test without load for 0.5N (A) and 1N (B)
6.2: Protocol 2 - Strain Control Test

Figure 33 shows all standard stress relaxation test results for the three different experiments (protocol 2) with initial loads of 0.5N and 1.0N. The volume of collagenase at each of the three experiments is 0.5 ml (A,B), 1.0 ml (C,D) and whole chamber (E,F) respectively. Strain % in all graphs comprises two parts. First strain increase up to 5% in load control mode (before one hour). Then strain is maintained constant for one hour and load decrease in the presence of pre-heated bacterial collagenase. The data demonstrate that load changes from 0.5±0.02 to 0.09±0.03 and from 1.0±0.02 to 0.16±0.05 after one hour respectively. The data in all three experiments show that the residual stress is marginally higher for the more highly strained samples (1N loaded) but that difference was not statically significant.
Load 1N Load Control - 1 ml collagenase 0.05 mM - Strain Control

Load 0.5N Load Control - Collagenase 0.05 mM - Strain Control
Figure 33: Protocol 2 - Strain Control Test; Stress Relaxation Test with A) 0.5N and 0.5ml of collagenase B) 1.0N and 0.5ml of collagenase C) 0.5N and 1ml of collagenase D) 1.0N and 1ml of collagenase E) 0.5N and whole chamber filled with collagenase F) 1.0N and whole chamber filled with collagenase.
6.3: Protocol 3 – Load Control Test

Figure 34 demonstrates strain % vs. time in control tests with 0.1N and 0.25N (A and B respectively). The change in strain % after more than one hour is less than 2%.
Load and strain were recorded during loading (figure 35) for the digestion test without load and then reloaded with 0.25N after 35 and 45 minutes (A, B respectively). The average of load and strain can be viewed in figure 35C. Specimens that were unloaded in the presence of bacterial collagenase for 35 minutes, upon reloading, experienced a sharp rise in strain and could not sustain the 0.25N Load. The specimens reloaded after 45 minutes similarly could not sustain the 0.25N load.

Figure 34: Protocol 3 - A) Control Test with 0.1N Load B) Control Test with 0.25N Load C) Average strain and load in Control Test with 0.25N Load
Figure 35: Protocol 3 - A) Digestion Test without Load – Reloaded specimens after 35 min B) Digestion Test without Load – Reloaded specimens after 45 min C) Average strain and load in Digestion Test without Load.
Figures 36, 37 and 38 show the results of the digestion test with 0.1N, 0.25N and 0.5N Load respectively. These graphs comprise three stages;

1- Loading: all samples were loaded to reach the specific load in DMEM buffer
2- Stabilization: all samples were maintained at the specific load for 15 minutes more in DMEM buffer to reach stable strain. (Creep in)
3- Digestion: all specimens were maintained at the specific load in the presence of collagenase.

Some experiments were not considered in the statistical analysis because of experimental problems. Some experiments did not stabilize during 15 minutes (“creep in” before injecting the collagenase or they were outside of the standard deviation of the average strain.
Figure 36: Protocol 1 - A) all experiments in Digestion Test with 0.1N Load B) selected and average strain % vs. time in Digestion Test with 0.1N Load
Figure 37: Protocol 3 - A) all experiments in Digestion Test with 0.25N Load B) selected and average strain % vs. time in Digestion Test with 0.25N Load
Figure 38: Protocol 3 - A) all experiments in Digestion Test with 0.5N Load B) selected and average strain % vs. time in Digestion Test with 0.5N Load
6.4: Protocol 4 – Load Control Test

Figure 39 demonstrates the strain % vs. time and load vs. time for 0.1N loading in protocol 4. Some experiments did not stabilize during 15 minutes before injecting the collagenase or they were out of standard deviation of the average strain graph therefore were not considered. Load vs. time for five experiments and the average can be viewed in figure 39C. Figure 39D clearly shows that the samples can not sustain the 0.1N load after 60 minutes in average.

Figure 40 demonstrates the strain % vs. time and load vs. time for 0.25N loading. It indicates that the samples can sustain the 0.25N load up to 70 minutes in average. (Figure 40D)

Graphs of TEM samples in each loading level are shown to compare with average graph. Strain % captured from TEM samples in 0.25N Load is outside of the standard deviation of average strain graph. This suggest that this sample was degraded faster compared to the other samples, while TEM samples in 0.1 and 0.5N load are reasonably close to the strain average graph.
Figure 39: Protocol 4 - A) all experiments in Digestion Test with 0.1N Load B) selected and average strain % vs. time C) selected load vs. time in digestion Test with 0.1N Load D) Final graph of strain and load vs. time in digested loaded 0.1N
Figure 40: Protocol 4 - A) all experiments in Digestion Test with 0.25N Load B) selected and average strain % vs. time in Digestion Test with 0.25N Load C) selected load vs. time in digestion Test with 0.25N Load D) Final graph of strain and load vs. time in digestion Test with 0.1N Load
Figure 41: Protocol 4 - Graphs of strain % vs. time in Digestion Test with 0.5N Load.
6.5: Strain Rate

6.5.1 Protocol 3

Time dependent strain rate data obtained from all experiments in protocol 3 are shown in figures 42, 43 and 44. The strain rate which may be interpreted loosely as a measure of the enzymatic cleavage rate is employed to investigate the effect of mechanical load on degradation rate. Figure 42 demonstrates that strain rate as a function of time and strain rate as a function of strain % for digestion test with 0.1N Load. After injecting collagenase into the chamber, the graph comprises two sections. First, strain rate is constant for approximately 40 minutes and then it goes up very sharply until failure. Also, the strain rate as function of time shows that strain goes up very sharply after 15% strain (Figure 42). In other words, strain rate is almost constant before 15% strain while it is increasing very fast during degradation process until failure. Figure 43 demonstrates the strain rate as a function of time and strain rate as a function of strain % from digestion tests with 0.25N Load. The graph 43A shows that strain rate is constant for 40 minutes as well and it slowly increases before 60 minutes. Also, the strain rate is almost constant before 15% strain and then the rate goes up very slowly. The data from the digestion test with 0.25N shows that the strain rate reaches 0.06 strain/s after 70 minutes while in 0.1N test it reaches this point after 60 minutes. Figure 44 shows the strain rate of digestion test with 0.5N load. Both graphs (A and B) show that strain rate is constant before 40 minutes while the rate increases very sharply after this time. Moreover, the strain rate in the digestion test with 0.5N load is more than the digestion test with 0.1N and 0.25N Load before 15% strain while after 15% strain, this rate increases to failure more slowly than the digestion test with 0.1N. The strain rate reaches 0.06 strain/s after 65 minutes during degradation process while 0.1N test reaches this point before 60 minutes.
Figure 42: Protocol 3 - A) Strain Rate vs. Time from Digestion Test with 0.1N Load B) Strain Rate vs. Strain % from Digestion Test with 0.1N Load
Figure 43: Protocol 3 - A) Strain Rate vs. Time from Digestion Test with 0.25N Load B) Strain Rate vs. Strain % from Digestion Test with 0.25N Load
Figure 44: Protocol 3 - A) Strain Rate vs. Time from Digestion Test with 0.5N Load B) Strain Rate vs. Strain % from Digestion Test with 0.5N Load
6.5.2: Protocol 4

Figures 45, 46 and 47 demonstrate the strain rate as functions of time and strain % at each digestion test with load. The data from new bacterial collagenase solutions with the same concentration (0.05mM) and method (protocols 3 and 4) as explained before are shown in graphs A and B of each figure. Moreover, TEM data from digestion tests with 0.1N, 0.25N and 0.5N Load are shown in each strain rate graph. Average graphs and TEM samples will be compared together to investigate the degradation process on TEM pictures. Unfortunately the TEM sample obtained from a digestion test with 0.25N Load during degradation process is far from the average (standard deviation, error bar). In other words, the strain rate is much larger than the average at this loading level. It demonstrates that this sample was degrading faster than its typical rate and its rate of degradation is very close to digestion test with 0.1N load.

Strain rate from digestion test with 0.1N load is constant for 40 minutes and then it sharply goes up after this time. The data in this graph is very close to data obtained from old bacterial collagenase solutions. Strain rate as function of strain shows that the rate is almost constant before 15% strain which is exactly the same as the data obtained from old bacterial collagenase solutions. Also, the strain rate on digestion test with 0.25N Load is a little larger than strain rate from old bacterial collagenase solutions. The strain rate in digestion test with 0.5N Load is a little larger compared to the one with old bacterial collagenase solutions as well (protocol 4).
Figure 45: Protocol 4 - A) Strain Rate vs. Time from Digestion Test with 0.1N Load B) Strain Rate vs. Strain % from Digestion Test with 0.1N Load
Figure 46: Protocol 4 - A) Strain Rate vs. Time from Digestion Test with 0.25N Load B) Strain Rate vs. Strain % from Digestion Test with 0.25N Load
Figure 47: Protocol 4 - A) Strain Rate vs. Time from Digestion Test with 0.5N Load B) Strain Rate vs. Strain % from Digestion Test with 0.5N Load
6.6: Effective Loaded Area

Effective loaded area on specimens is a good method to examine how fast load-bearing material is being removed. To calculate the effective area of each sample, the modulus of specimens were calculated assuming 25% of the sample cross-sectional area is load-bearing (6mm width X 0.7mm thickness) during the stabilization stage (creep in stage, before 15 minutes). Then effective loaded areas a function of strain were calculated at each digestion test with load. Figure 48 A, B and C demonstrate the effective loaded areas as functions of time during the digestion test with 0.1N, 0.25N and 0.5N Loads respectively. Graph 48A shows that the average effective loaded area at 0.1N Loading reaches 0.2 mm\(^2\) after 50 minutes and then less than 0.05 mm\(^2\) when graph 48B shows that average effective loaded area reaches 0.2 mm\(^2\) after more than 60 minutes and 0.05 mm\(^2\) after more than 75 minutes when loading is 0.25N. These areas reach 0.2 mm\(^2\) after 60 minutes and then 0.1 mm\(^2\) after less than 70 minutes with 0.5N Load level.

Figure 49 A, B and C demonstrate that the effective loaded areas at each digestion test with 0.1 and 0.25N and 0.5N Load respectively in new bacterial collagenase solutions (same 0.05mM concentration and method on corneal specimens) is very close to the ones with old bacterial collagenase solutions. In other words, the effective loaded areas at 0.1N load is rapidly decreasing to less than 0.05 mm\(^2\) after 65 minutes while these areas at 0.25N and 0.5N loadings reach 0.05 mm\(^2\) after more than 75 minutes.
Figure 48: Protocol 3 - Effective Loaded Area vs. Time from Digestion Test (old bacterial collagenase solutions) with 0.1N Load (A) 0.25N Load (B) and 0.5N(C)
Figure 49: Protocol 4 - Effective Loaded Area vs. Time from Digestion Test (new bacterial collagenase) with 0.1N Load (A) 0.25N Load (B) and 0.5N (C)
TEM sample at 0.1N Load is very close to the experimental average while TEM samples taken at 0.25N and 0.5N Load are far from their experimental average (standard deviation, error bars). In both cases these samples were degraded faster than the other specimens under 0.25N and 0.5N. The degradation rates are close to the digestion test with 0.1N load. As the graph shows, other specimens at 0.25N load reach the effective area of 0.2 mm² after 60 minutes while the TEM sample reaches this point before 60 minutes.
6.7: Polarization Microscopy (Birefringence) Results – Time Lapse Imaging

Polarization microscopy (with crossed polarizers) permits selective observation of the birefringence associated with fibrils oriented at 45° to either of the polarizer axes. Thus, the fate of aligned arrays of corneal fibrils in particular direction may be selected and followed via their birefringence signal. During degradation experiments, fibrils parallel (loaded) to or at a 45° angle relative to the load direction (unloaded) were imaged every 10 seconds for up 90 minutes through crossed polarizers. Images were taken from the center of the specimen strip. The polarization imaging also allowed us to examine the specimen for anomalous behavior (premature tearing or evidence of grip slippage).

Figures 50-53 show polarization sequences (images captured every 10 minutes after “creep-in” crossed-polarizer axes 0°-90° relative to load direction) for all protocols after initial loading (stabilization stage). Control Test with Load 0.25N: The Birefringence signal does not change appreciably with time. Images confirm limited creep following initial loading and confirm that the grips do not slip (Figure 52). Digestion Test without Load: Image sequences from unloaded specimens exposed to bacterial collagenase demonstrated a marked change over time (Figure 53). During degradation, the birefringence signal diminished uniformly suggesting a uniform attack on the fibrils in the tissue. After reloading the signal is lost very fast. Digestion Test with Load: Birefringence signal is lost quite rapidly indicting disruption of unloaded fibrils aligned at 45° relative to the applied force (Figures 50, 51). These pictures suggest strain-based preferential degradation of fibrils in the corneal strip. The signal loss in digestion test with 0.1N is faster than in digestion test with 0.25N load.
Figure 50 - Time lapse imaging in Digestion Test with 0.1N Load (0° - 90°)

<table>
<thead>
<tr>
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</tr>
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</tr>
<tr>
<td>65mins</td>
<td>82.64%</td>
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Figure 51 - Time lapse imaging in Digestion Test with 0.25N Load (0° - 90°)

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<td>65mins</td>
<td>32.94%</td>
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<tr>
<td>75mins</td>
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</table>
Figure 52 - Time lapse imaging in Control Test with 0.25N Load (0°-90°)

<table>
<thead>
<tr>
<th>15mins</th>
<th>25mins</th>
<th>35mins</th>
<th>45mins</th>
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<th>75mins</th>
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<td>Strain 5.32%</td>
<td>Strain 5.41%</td>
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<td>Strain 5.67%</td>
<td>Strain 5.72%</td>
<td>Strain 5.82%</td>
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Figure 53 - Time lapse imaging in Digestion Test without Load - Reload with 0.25N Load (0°-90°)

<table>
<thead>
<tr>
<th>15mins</th>
<th>25mins</th>
<th>35mins</th>
<th>45mins</th>
<th>(Reloading) 55mins</th>
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</thead>
<tbody>
<tr>
<td>Strain 5.42%</td>
<td>Strain 0.00%</td>
<td>Strain 0.00%</td>
<td>Strain 0.00%</td>
<td>Strain 31.00%</td>
<td>Strain 93.24%</td>
</tr>
</tbody>
</table>
Figures (54-57) display images captured from specimens at 0°-90° and 45°-135° relative to load direction in control and digestion test. Figure 54 demonstrates that the signals from specimens do not change appreciably with time for the control experiment. The difference in brightness between the two directions suggests that the birefringent signal is enhanced along the load line. Figures 55, 56 show the loaded specimens in the presence of bacterial collagenase during the degradation. Images captured from 0°-90° on both tests (0.25N and 0.1N) lose intensity while the images from 45°-135° remain bright (Digestion Test with Load). This result suggests that loaded collagen fibrils are not degraded or are degraded later in both tests. The picture from loaded specimens at 0.25N Load shows that the degradation process takes longer compared to 0.1N Load. It also suggests that strain/load protects the collagen fibrils from the enzyme attacks. This approach can be clearly observed in unload/reload specimens pictures (Digestion Test without Load). Images from the unloaded sample suggest the uniform attack of collagenase at 35 minutes (unloading stage) and then pictures taken after reloading (reloading stage) suggest the loss of mechanical integrity in corneal specimen.(Figure 57)
Figure 54: Birefringence pictures captured from Control Test with 0.25N Load

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<tbody>
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</tr>
<tr>
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<td>5.24%</td>
</tr>
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</tr>
<tr>
<td>75mins</td>
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<td>5.82%</td>
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Figure 55: Birefringence pictures captured from Digestion Test with 0.1N Load

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</tr>
<tr>
<td>15mins</td>
<td>45°-135°</td>
<td>4.22%</td>
</tr>
<tr>
<td>65mins</td>
<td>0°-90°</td>
<td>82.64%</td>
</tr>
<tr>
<td>65mins</td>
<td>45°-135°</td>
<td>82.64%</td>
</tr>
</tbody>
</table>
Figure 56: Birefringence pictures captured from Digestion Test with 0.25N Load

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<th>75mins</th>
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<td>Strain 5.16%</td>
<td>Strain 54.54%</td>
<td>Strain 54.54%</td>
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</tbody>
</table>

Figure 57: Birefringence pictures captured from Digestion Test without Load and Reload with 0.25N

<table>
<thead>
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<tr>
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<td>Strain 5.24%</td>
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<td>Strain 0.00%</td>
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</table>

(Reload) 65mins

<table>
<thead>
<tr>
<th>0°-90°</th>
<th>45°-135°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 93.24%</td>
<td>Strain 93.24%</td>
</tr>
</tbody>
</table>
6.8: Transmission Electron Microscopy Pictures

In Figure 58 (A-G) transmission electron micrographs qualitatively demonstrate the effect of degradation on unloaded collagen fibrils. The TEMs are taken perpendicular to the long axis of the tensile specimens. Thus unloaded fibrils will appear in longitudinal section (as lines) and loaded fibrils will appear in cross-section (as dots). Figure 58(A-C) shows the cross-section of a loaded, undigested sample. The classic multilayered lamellar structure of the cornea can readily be appreciated with its alternating layers of lines and dots (see inset). In figure 58D, a loaded digested sample in 0.1N Load indicates significant disruption of the lamellar structure and a relative loss of longitudinal (unloaded) fibrils while loaded digested specimens in 0.25N (Figure 58E) demonstrate the absence of unloaded fibrils and blank spaces in cross-section. This result also is seen in the digestion test with 0.5N Load. The pictures from 0.5N loaded specimen illuminate some loaded fibrils (as dots) among the blank space (figure 58F). The TEM image from unloaded specimen after 35 minutes of degradation is the same as the TEM picture from normal corneal cross-section. Multilayered corneal lamellae are seen in digested unloaded specimens (Figure 58G). It suggests that the collagen fibrils (lines and dots) still exist in all layers but they lack mechanical integrity. The displacement and load data prove that collagen fibrils completely lose the mechanical integrity after reloading.
Figure 58: A) Control test with 0.1N Load

Figure 58: B) Control test with 0.25N Load
Figure 58: C) Control test with 0.5N Load

Figure 58: D) Digestion Test with 0.1N Load
Figure 58: E) Digestion Test with 0.25N Load

Figure 58: F) Digestion Test with 0.5N Load
Figure 58: G) Digestion Test without Load – No Reloading
Chapter 7: Discussion

7.1: Protocol 1

The data obtained from the bioreactor using the old protocol were repeatable and suggest an effect of mechanical load on the degradation of corneal collagen in native tissue. Figure 59A demonstrates strain vs. time from digestion tests with 0.25N, 0.5N and 1.0N Load respectively. The graph shows that none of the lines cross each other after injection of bacterial collagenase solution into the chamber.

Figure 59: Protocol 1 - A) Average strain% in digestion test with three different loadings B) Predicted graph of digestion test with 0.1N and 0.25 Load
It also shows that strain is the highest for the largest load (1.0N load) at every instant. The strains are 20%, 25% and 45% in digestion test with 0.25N, 0.5N and 1.0N load respectively after one hour. Also, figure 60B demonstrates that the strain rate as function of time is the highest at digestion test with 1.0N Load and is the lowest at 0.25N Load while figure 60C shows that strain rate as function of strain % is reasonably constant throughout the experiment. The data captured from figures 59 and 60 prove that the cutting rate at same strain % is approximately constant. This hypothesis (constant cutting rate) can predict the digestion test with 0.1N load in old protocol. Figure 59B predicts the digestion test with 0.1N Load in protocol 1. The statistical analysis from the data indicates that the strain captured from the digestion tests in 0.25N, 0.5N and 1.0N load are significantly different (P<0.05) throughout the experiment. (Figure 60A)
Figure 60: Protocol 1 - P value vs. time (A) Average strain rate vs. time (B) vs. strain (C)
Figures 61A and B are comparative graphs for the 1.0 N and 0.5N Load to investigate the effect of load on the degradation process. In both graphs (0.5N and 1N Load) after one hour unloaded/reloaded specimens have smaller strain compared to loaded specimens in the digestion test. After reloading the specimens with 0.5N Load, Strain is around 20% on reloaded specimens while strain on loaded specimens is 30%. Also, figure 61B (1N load) demonstrates the strain on loaded specimens is larger than unloaded/reloaded specimens. The data show that loaded specimens were degraded faster than the unloaded/reloaded specimens and suggest that load did not protect fibrils from the enzyme during degradation. However, this effect could be due to the protection of the stroma by the presence of the limiting membranes (epithelium and endothelium).
Figure 61: Protocol 1 - A) Average strain% for 0.5N Loading B) Average strain% for 1.0N Loading
Protocol 1

Average graphs from Old Protocol

A

- Strain Rate - Digestion Test with 0.5N Load
- Strain Rate - Digestion Test with 1N Load

B

- Strain Rate - Digestion Test with 0.5N Load
- Strain Rate - Digestion Test without 0.5N Load
- Collagenase Added

Time (h:m:s)
Furthermore, the strain rate as function of time on loaded and unloaded specimens during degradation was investigated on figure 62B and C. They demonstrate the strain rate of loaded specimens is much higher than reloaded specimens. Also, figure 62A shows that strain rate with 1N loading is higher than 0.5N Loading at the same time.

Figure 62: Protocol 1 - (A) strain rate vs. time on digestion test without load, strain rate as function of time on 0.5N Loading (B) on 1N Loading (C)
Figure 63: Protocol 2 - Load vs. time in stress relaxation test

7.2: Protocol 2

Figure 63 demonstrates the load vs. time graphs for 0.5 and 1.0 N loaded specimens and followed by a relaxation test. The different regimes of experiment: loading and stress-relaxation are shown in this graph. Stress relaxation tests demonstrate that the load changes from 0.5N±0.02 to 0.09N±0.03 and from 1.0N±0.01 to 0.16N±0.05 after one hour. In the strain control test, the residual stress is marginally higher for the more highly strained samples (1N loaded) but the difference was not statically significant.
7.3: Protocol 3

While the loaded and reloaded specimens from protocol 1 did not support the strain-stabilization hypothesis, we believe that the results obtained were confounded by the presence of epithelial and endothelial membranes which were not removed prior to exposure to the enzyme. Thus actively straining samples would experience more “brakes” in the membranes which would allow for more enzymatic access to the underlying stroma. Protocol 3 corrects this problem and thus produces more accurate results. In addition, this protocol reduces the load to a more physiological value. The data from the new protocol not only support the main hypothesis but also reject the constant cutting rate hypothesis (null hypothesis).

Figure 64 demonstrates the comparative graph from digestion tests with and without load in protocol 3. Figure 64A shows that the average strains from the digestion test with 0.1N vs. 0.25N Load and samples could not sustain 0.1N load after one hour of loading while 0.25N and 0.5N loaded specimens could sustain higher loads after one hour. Also, 0.5N loaded specimens were degraded more slowly than 0.1N loaded specimens after 50 minutes of degradation (Figure 64B). Figure 65A and B demonstrate that strain rate reaches its highest value in 0.1N loaded tissues compared to 0.25N and 0.5N loaded specimens at the same time. In other words, strain rate as function of time on 0.1N loaded specimens is significantly different from 0.25N and 0.5N reloaded specimens (Figure 65A). Furthermore, unloaded specimens in presence bacterial collagenase for 35 minutes totally lost mechanical integrity after reloading (Figure 64A and C). Unloaded/reloaded tissues exhibited much higher strain rate in comparison with 0.1N, 0.25N and 0.5N loaded specimens (Figure 64A and C).
Strain rate as function of strain % in protocol 3 is completely different from protocol 1 (figure 65C). This graph shows that strain rate at same strain % on 0.1N loaded specimens is the highest compared to 0.25N and 0.5N loaded specimens.

The statistical analysis from the data captured protocol 3 indicates that the strain from the digestion tests with 0.25N load and the digestion test without load are significantly different (P<0.05) after reloading (Figure 64D). Also, the difference between the digestion test with 0.1N and 0.25N load is statistically significant after 55 minutes (Figure 64E)
Figure 64: Protocol 3 - Average strain % in digestion test A) with and without 0.1N and 0.25N B) with 0.1, 0.25, 0.5N load C) with and without 0.25 and 0.5N load.
Protocol 3

Average Graphs from The Protocol

- Strain % - Digestion Test with 0.1N Load
- Strain % - Digestion Test with 0.25N Load
- Strain % - Digestion Test with 0.5N Load

Collagenase Added

Protocol 3

Average Graphs and T-Test Strain Results

- P Value
- Strain % - Digestion Test with 0.25N Load
- Strain % - Digestion Test without Load

Collagenase Added

P<0.05
Figure 64 – Protocol 3 - D) P value vs. time strain result in digestion test with or without load E) P Value vs. time result from digestion test in 0.1 and 0.25N
Furthermore, when the strain rates in the digestion test with 0.1 and 0.25N are compared, see Figure 65D, the strain rate from 0.1N loaded specimens is much higher than 0.25N loaded specimens. A T-Test was completed to compare differences in strain rates (Figure 65D). After 45 minutes the P value is almost less than 0.05 which shows strain rates are significantly different. (P<0.05)
Figure 65: Protocol 3 - A) strain rate vs. time for 0.25N, 0.5N Loaded specimens and unloaded/reloaded 0.25N B) strain rate vs. time for 0.1N, 0.5N loaded specimens and 0.25N unloaded C) strain rate as function of strain % for 0.1, 0.25 and 0.5 Loaded specimens D) P value vs. time strain rate result from digestion test with 0.1 N and 0.25 N load
Estimates of effective loaded area loss as function of time indicate that tissue subjected to 0.1N load loses the effective area rapidly the first 20 minutes after injection of collagenase and then completely loses it before one hour while the 0.25N loaded specimens lose the effective area significantly slower and completely lose it 75 minutes after injecting collagenase (Figure 66A). Also, the loss of effective area on 0.5N loaded specimens is slower but not significantly different from 0.1N loaded ones (figure 66B).

A T-Test was ran for the effective loaded area in the digestion tests with 0.1N and 0.25N Load (Figure 66C). The result shows that the difference is statistically significant (P<0.05) between 0.1N and 0.25N loaded specimens throughout the degradation process.
Figure 66: Protocol 3 - The effective loaded area as function of time for 0.1N, 0.25N loaded specimens (A) and 0.1N, 0.5N loaded specimens (B), P value vs. time effective area result from digestion test with 0.1 N and 0.25 N load (C)
7.4: Protocol 4

The data obtained using new bacterial collagenase (first series) is very close to old bacterial collagenase solutions (second series). All graphs from the new series of experiments corroborate data from protocol 3. Figures 67A, B and C indicate that 0.25N loaded specimens were degraded slowly compared to 0.1N loaded specimens and unloaded/reloaded specimens. The degradation process comprises two regimes. In the First section, the strain % and strain rate as function of time is almost the same on 0.1N and 0.25N loaded specimens during 35 minutes after injecting new bacterial collagenase solutions (The null hypothesis states that these should be quite different). After this time the second section starts in which the strain changes with different rates at each loading level (Figures 67A and B). The 0.25N and 0.5N loaded specimens could sustain the applied load 40 minutes post-injection of collagenase while unloaded/reloaded specimens lost mechanical integrity and completely degraded (Figure 67C).

The statistical analysis from the data indicates that the strain captured from the digestion tests in 0.1 N and 0.25N load are significantly different (P<0.05) after one hour (Figure 67D). A T-Test also ran for unloaded/reloaded specimens to compare loaded specimens. The plot (Figure 67E) demonstrates 95% difference (P<0.05) after 50 minutes. Both graphs (strain rates vs. time) support the result captures from protocol 3.
Protocol 4
Average Graphs and T-Test Strain Value

A

- Strain % - Control Test with 0.25N Load
- Strain % - Digestion Test without Load
- Strain % - Digestion Test with 0.1N Load
- Strain % - Digestion Test with 0.25N Load

Collagenase Added

P<0.05

Protocol 4
Average Graphs from The Protocol

D

- P Value
- Strain % - Digestion Test with 0.1N Load
- Strain % - Digestion Test with 0.25N Load

Collagenase Added
Figure 67: Protocol 4 - D) P Value vs. time result in digestion test in 0.1 and 0.25N E) P value vs. time strain result from digestion test with or without load (protocol 3)
Figure 67: Protocol 4 - Average strain % in digestion test A) with and without 0.1N and 0.25N B) with 0.1, 0.25, 0.5N load C) with and without 0.25 and 0.5N load.
Strain rate as function of time in 0.1N, 0.25N and 0.5N loaded specimens is significantly slower (P<0.05) than unloaded/reloaded specimens during degradation (Figure 68A and B). These results are provided as supportive data for the main hypothesis that the collagen fibrils are protected from the collagenase attack by application of mechanical load/strain. Also, the data obtained from the strain rate vs. strain % demonstrate that 0.1N loaded specimens have the highest strain rate compared to 0.25N and 0.5N loaded tissue at any given strain. Also, these data (Figure 68C) generally support the main hypothesis and reject the null hypothesis (constant cutting rate). If the
If the null hypothesis were true and cutting rates were independent of strain, then the strain vs. time curves could not cross.

Statistical analysis from strain rates in digestion test with 0.1N and 0.25N load indicates a 95% difference. Figure 68D demonstrates a T-Test ran for strain rates. After spending 45 minutes in presence of bacterial collagenase, T-Test reveals a 95% difference (P<0.05). This result completely supports the data captured from protocol 3.
Figure 68 – Protocol 4 - A) strain rate vs. time from 0.25N, 0.5N Loaded specimens and unloaded/reloaded 0.25N B) strain rate vs. time for 0.1N, 0.5N loaded specimens and 0.25N unloaded C) strain rate as function of strain % from 0.1, 0.25 and 0.5 Loaded specimens D) P value vs. time strain rate result from digestion test with 0.1 N and 0.25 N load
The effective loaded area calculated from new data demonstrate that the effective loaded area loss as function of time on 0.1N loaded specimens is more rapid during initial degradation (15 minute after injection). The data shows this difference significantly during initial degradation (Figure 69 A, B).

The P Value calculated from the effective loaded area in digestion test with 0.1N and 0.25N Load (protocol 4) can be viewed on figure 69B. It shows that P value is approximately smaller than 0.05 after injection of bacterial collagenase into the chamber. It suggests significant difference which supports the data captured from protocol 3.
Figure 69: Protocol 4 - A) the effective loaded area as function of time for 0.1N, 0.25N and 0.5N loaded specimens, B) P value vs. time effective area result from digestion test with 0.1 N and 0.25 N load
Chapter 8: Conclusion

This study has demonstrated that our custom, miniature bioreactor is capable of applying loads and measuring displacement with very high temporal, spatial and force resolution. The accuracy of bioreactor in load control system at each load level test (1N to 0.25N) was modified from ±0.1N to ±0.01N load errors. This new resolution helped us to test corneal strips at lower load (0.1N Load) to investigate and test the hypothesis at this load range. There are many sources of error - for example vibrations from the building, temperature from the heating system and friction from the device. The PID controller was able to adjust the applied load on corneal strips with ±0.01N error (high accuracy).

The data captured from protocol 1 although somewhat flawed, demonstrated that strain rate as function of strain is constant during digestion tests at high load level (more than 0.25N load). The protocol was extended to digestion tests with 0.1N load. In other words, the hypothesis predicted the strain rate (cutting rate) as function of strain at digestion test with 0.1N load should be the same as 0.25N and 0.5N cases according to the data captured from high load levels (Figure 60C), however protocols 3,4 altered the result predicted (Figure 65C and 68C).

The data captured from protocols 3, 4 in both series of test by the bioreactor are repeatable and demonstrate that tensile loads similar to in vivo loads reduce the rate of degradation of collagen in native tissue (cornea). The data from load control test are generally supportive of the main hypothesis that collagen in native tissue is protected to some extent from enzymatic attack by the application of mechanical loads (strains). Most striking is the fact that data obtained from completely unloaded specimens clearly
demonstrates total loss of mechanical integrity after 35 minutes (compared to over an hour for loaded samples). The strain rate of unloaded/reloaded specimens as function of time or strain % is the highest possible rate and all specimens could not sustain the applied load after immersing 35 minute in presence of active bacterial collagenase (0.05mM active enzyme) without load. Furthermore, the load control test in protocols 3, 4 dynamically probes multiple strains and suggests a complex relationship between strain, cleavage rate and strain history. By all measurements, the samples under a lower load fail faster than expected (rejecting the null hypothesis of constant cutting rates). Even graphs from the digestion test with 0.25N and 0.5N Load demonstrate that collagen fibrils can maintain the applied load more than 60 minutes while the unloaded/reloaded specimens totally lost mechanical integrity and could not sustain 0.25N load for a few minutes after reloading. Moreover, Estimates of effective loaded area loss as function of time indicate that tissue subjected to 0.1N load loses the effective area rapidly during first 20 minutes of injection of collagenase and then completely fails before one hour while the effective loaded area under 0.25N load in the presence of active bacterial collagenase more slowly loses the effective loaded area throughout the degradation. Taken together, the data from the bioreactor, TEM and polarization images show that degradation process is reduced by application of mechanical load and is dependent on the specific load.

Polarization Microscopy (Birefringence) pictures and TEM pictures from samples qualitatively demonstrate the effect of degradation on unloaded collagen fibrils during degradation process. The qualitative imaging data suggest preferential attack on unloaded fibrils by collagenase molecules. Most images taken during degradation provide evidence
that loaded fibrils aligned with load axis survive and sustain the applied load in the presence of the enzyme. The general loss of birefringence in unloaded specimens suggests uniform attack on the fibrils in the tissue. TEM pictures indicate significant disruption of the lamellar structure and a relative loss of longitudinal (unloaded) fibrils compared to multilayered lamella structure of normal cornea.

Initial fits to the data with a complex reaction-diffusion model indicate that strain may reduce binding affinity but possibly accelerate cleavage of molecules once they are enzyme-bound. Previous studies have shown that strain can stabilize the collagen molecule against enzymatic attack [37, 38, 39]. In this study, we extend the mechanochemical data on bacterial collagenase over multiple strains in a load-controlled experiment. The results clearly show that cleavage rates depend on the applied load/strain. They also show that fibrils are not completely protected by the load and that the specimens will degrade to failure (at these enzyme concentrations). In addition, we are unable to conclude that strain-induced changes in molecular conformation are the protective mechanism. It could be strain-reduced diffusional access as well (Though the TEM and birefringence data suggest that diffusinal access was not impeded by strain).

It should also be noted that crude bacterial collagenase is a non-specific enzyme capable of degrading collagen in addition to many other molecules. This enzyme attacks the collagen molecule at unspecific binding sites while matrix metalloproteinase MMP cleave the single collagen molecule at ¼ and ¾ from the amino-terminus (specific site). Because of this specific binding site for MMP (and because it is in an area that is less stiff), there may be more of an effect on the enzymatic degradation process by application
of load. In addition, bacterial collagenase contains up to 18 different enzymes (8 of them identified) which result in rates and patterns of collagen degradation that are drastically different from \textit{in vivo} digestion (by MMPs or cathepsins). It is clear that moving to a more physiologically relevant enzyme (MMP or cathepsin) should produce results that are more comparable with the \textit{in vivo} remodeling process.
References:


[35] Practical orthopaedic sports medicine and arthroscopy, donald H. johnson, Rober A. pedowitz; page 8-10


Appendix A – Labview Block Diagram (Load Control Mode)

First part – Move the actuator to zero position
Second part – Move the actuator to absolute position
Third part – Set to a desired force for stretching
Appendix B – Labview Block Diagram (Strain Control Mode)

First part – Move the actuator to zero position
Second part – Move the actuator to absolute position
Third part – Set to a desired Strain % for stretching
INFLUENCE OF MECHANICAL LOAD ON ENZYMATIC CLEAVAGE OF NATIVE COLLAGEN

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Abstract

It has been hypothesized that collagen fibrils in native tissue are “strain-stabilized” and thus protected (to some extent) from enzyme attack by the application of mechanical load. If true, collagen and its complement native enzymes (MMPs) could comprise the basis of “smart” structure which is intrinsically load-adaptive. If strain protection of collagen significantly reduces the activity of bacterial collagenase (BC-Clostridium Histolyticum), then benchtop “sculpting” of collagenous matrix for tissue engineering may be practical.

In this investigation, we have quantified the enzymatically-induced creep of uniaxially-loaded (0.25 N) and unloaded control bovine corneal tissue strips (~6.0 mm x 0.7 mm x 17 mm) which were exposed to BC (0.05 mM). Experimental and control strips were loaded for 15 minutes (initial creep-in) then exposed to the BC at which point the controls were unloaded. After 35 minutes of degradation, unloaded controls were re-loaded and the dynamic strain was recorded. All unloaded control specimens were significantly compromised mechanically compared to experimentals and could not hold the applied load for more than a few minutes following reloading. From the data is clear that strain protects the loaded fibrils. How that protection is manifested remains an open question.

Introduction

Collagen is one of the most important structural proteins in vertebrate animals. Over 28 different genetic sequences which could be termed “collagen” have been identified (for comprehensive compilation see Canty and Kadler 2005 [1]). Of these, five “fibril forming” collagens have been detected (I, II, III, V, XI). Type I collagen is the most abundant of the fibril-forming collagens (and is consequently the most investigated [2]). It is the principal determinant of load-bearing matrix mechanical properties (i.e. ligaments, tendons, cornea) and plays a crucial role in cartilage mechanical behavior [3]. The basic structure of type I collagen at the supramolecular level is a right-handed, linear, triple-helix comprising three left-handed helical alpha chains. Collagen molecules are adapted to carry tensile loads along their principal axis and are thus anisotropically arranged into aligned fibrils in load-bearing extracellular matrices (ECMs). There is little information available about how collagenous tissue organization is generated and maintained during development and growth [4, 5]. However, it has been surmised for over a hundred years the mechanical forces play a critical role in the epigenetic regulation of load-bearing collagenous ECMs [4-7]. Recently, it has been hypothesized that the adaptation and reorganization of load-bearing ECM is directed by strain at the level of the matrix molecules (as opposed to being directly cell-mediated) [8]. This hypothesis requires that collagen monomers are preferentially removed from load-bearing ECM if they are not sufficiently strained. Matrix Metalloproteinases (MMPs), Bacterial Collagenase and Cathepsin k are known enzymes capable of directly degrading collagen triple helix [9-12]. These enzymes must first bind
to and then cleave the collagen triple helix. In the case of MMPs, the enzyme must first orient and then unwind the triple helix to perform an effective cleavage event[13, 14]. Thus it is possible that straining collagen fibrils, which change their free energy, could reduce their susceptibility to cleavage. We refer to this concept as “strain-stabilization” which has important implications for our understanding of collagen as an engineering material.

**Materials and Methods**

**Specimen Preparation:**

Corneas, which were chosen for their transparency and unique architecture comprising aligned arrays of non-interacting collagen fibrils, were removed from right or left eyes of two week old cows (40-100 pounds) obtained from Research 87 Inc. (Boylston, MA) and the limiting membranes (epithelium and endothelium) were scraped off by a razor blade. Samples were devitalized by a minimum of three freeze-thaw cycles (to -80°C) and were stored at -80°C until use. Prior to testing, specimens were thawed at room temperature for 10 minutes. A custom made cutting die was used to generate accurate repeatable test strips from the tissue. The central region of each dissected cornea was used to produce one vertically oriented (superior-to-inferior) tensile specimen (approximately 0.7 mm thick x 17.5± 2.5 mm length x 6 mm wide - Figure 1). During excision and mounting into the test chamber, all tensile specimens were kept moistened with 37°C DMEM- Dulbecco's Modified Eagle's Medium- (Mediatech Inc., Manassas, VA).

**Mechanical Loading Apparatus**

To investigate the effect of mechanical forces/strains on the rate of degradation of tissue strips, an environmentally controlled, miniature uniaxial testing device was constructed in conjunction with Northeastern University’s Capstone design course and then modified (Figure 2 [15]). The device comprises a load cell (Honeywell 5Ib or 1Ib, Columbus, OH) and a uniaxial motor (Zaber T-LA60 Richmond, BC, Canada) which are under the control of a custom Labview program. The accuracy of Load cell and uniaxial motor are 0.001N and 0.001mm respectively. The combined system accuracy with PID controller is ±0.01N. The specimen chamber allows optical access for polarization microscopy of the specimen and is equipped with an integrated PID driven temperature control system. Each corneal strip was positioned between two cam grips inside the chamber, immersed in 37°C DMEM and uniaxially stretched. In some cases cyanoacrylate was used to assist in tissue fixation.

**Digestion Protocol:**

Loaded tissue specimens were crept-in for 15 minutes in DMEM. The DMEM was then changed to collagenase solution (DMEM, 0.05mM Bacterial Collagenase (Clostridiopetidase A, Sigma-Aldrich - No.C130) and 200 mg/ml calcium chloride) for the duration of the experiment. Collagenase solutions were made previously and stored in -80°C until use. Before injection into the chamber, collagenase solution was warmed in a water bath (37°C) for 30 minutes.

**Polarization Microscopy (Birefringence) - Live Imaging:**

The corneal stroma comprises transparent arrays of aligned collagen fibrils which alternate in direction. They are also effectively mechanically isolated (with regard to tension) from one another. Thus, uniaxial strain preferentially loads on one set of fibrils, while others remain unloaded. Polarization microscopy (with crossed polarizers) permits selective observation of the birefringence associated with fibrils oriented at 45° to either of the polarizer axes. Thus, the fate of
aligned arrays of corneal fibrils in a particular direction may be selected and followed via their birefringent signal. During degradation experiments, fibrils parallel (loaded) to or at a 45º angle to the load direction (unloaded) were imaged every 10 seconds for up 90 minutes through crossed polarizers. Images were taken in the center of the specimen strip. The polarization imaging also allowed us to examine the specimen for anomalous behavior (premature tearing or evidence of grip slippage).

**Transmission Electron Microscopy:**

For each experimental protocol, all specimens were removed from bioreactor after 60 minutes and processed for Transmission Electron Microscopy (TEM) as described in [16]. Briefly, the specimens were immersed in ½ strength Karnovsky’s fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.1M Cacodylate buffer, pH 7.4) for 1 hour, post-fixed in 2% osmium tetroxide and serially dehydrated in graded ethanol. Following dehydration, specimens were embedded in Epon-Araldite and sectioned perpendicular to the applied load in the center of the tensile specimen. Specimens were stained with Uranyl Acetate and Lead Citrate in methanol and viewed on a JEOL 1000 electron microscope (JEOL; Tokyo, Japan).

**Loading protocols:**

All experiments were performed in load-control mode while strain was recorded. Initially, a preload of 0.01N was applied to all specimens and the resulting strain was called “zero strain”. Following a short preload, the experimental load of 0.25N (about normal in vivo load) was applied for 15 minutes to allow “creep-in”. All samples are loaded at 0.25N for 15 minutes to allow dissipation of the majority of the viscoelastic creep. Following this “creep-in”, three test protocols where performed:

![Figure 3- Time sequence live polarization imaging. Crossed-polarizer axes 0º-90º relative to load line. Illumination of off-axis, unloaded fibrils. Series A: Control Test with Load, Series B: Digestion Test without Load and Series C: Digestion Test with Load. Sequences begin at end of “creep-in” period (frequency: 5 minutes; duration 35 minutes).](image-url)
1-Control Test with Load (no digestion): For three specimens (N=3), at the end of “creep in” the tissue remained at 0.25N for an additional 75 minutes while strain was recorded.

2-Digestion Test without Load: Five specimens (N=5) were unloaded at the end of “creep-in” and exposed to collagenase solution. The specimens were then reloaded after 35 minutes until failure. Load and strain were recorded.

3-Digestion Test with Load: Eight specimens (N=8) remained loaded at the end of “creep-in” and the DMEM was replaced by preheated active bacterial collagenase solution for 75 minutes. Load and strain were recorded.

Results

Live polarization imaging (polarization axes at 0º-90º): Figure 3 shows polarization sequences (images captured every 10 minutes after “creep-in”) for all protocols after initial loading (stabilization stage). Control Test with Load: Birefringence signal does not change appreciably with time. Images confirm limited creep following initial loading and confirm that the grips do not slip (Figure 3A). Digestion Test without Load: Image sequence from unloaded specimens exposed to bacterial collagenase demonstrated a marked change over time (Figure 3B). During degradation, the birefringence signal diminished uniformly suggesting a uniform attack on the fibrils in the tissue. Digestion Test with Load: Birefringence signal is lost quite rapidly indicating disruption of unloaded fibrils aligned at a 45º to the applied force (Figure 3C). These pictures suggest strain-based preferential degradation of fibrils in the corneal strip.

Figure 4(A-C) displays the pictures captured from specimens at 0º-90º and 45º-135º relative to load direction in control and digestion test. The crossed-polarizers set at 0º-90º reveal the unloaded collagen fibrils while the crossed-polarizers set at 45º-135º represent the loaded collagen fibrils. Figure 4A (Control Test with Load 0.25N) demonstrates that the signals from specimens do not change with time. The birefringent signal captured with the polarizers at 45º-135º is much brighter than the signal obtained with the polarizers at 0º-90º. The next set of images, Figures 4B (Digestion Test with Load), show the loaded specimens in the presence of bacterial collagenase during the degradation. Images captured from 0º-90º on 0.25N test lose birefringence (Figure 3C demonstrates the sequence imaging every 10 minutes) while the images from 45º-135º remain bright. This suggests that loaded collagen fibrils are not degraded as quickly. For the load/unload/reload tests, the birefringence signal does not change significantly indicating that relative alignment of collagen fibrils is preserved. However, upon reloading, the sample rapidly loses birefringence for fibrils off the load axis.

Dynamic Mechanical Response

Time dependent strain and strain rate data obtained from each protocol are shown in Figures 5, 6 and 7. Figure 5A demonstrates that loaded controls creep steadily for more than
one hour after the DMEM would have been added in the experimental test. However, the total creep during that period is less than 1%. Figure 5B shows strain and load data for the digestion test with no load. Specimens that were unloaded in the presence of bacterial collagenase for 35 minutes, upon reloading, experienced a sharp rise strain and could not sustain the load 0.25N for more than 5-10 minutes. Complete failure occurred soon after reloading for every sample (typically less than an hour following exposure to BC). None of the samples could sustain the load 45 minutes following collagenase exposure. Figure 4C demonstrates that the digestion with load samples could sustain the applied load for over an hour in the presence of collagenase. The strain vs. time graph comprises three stages;

1-Loading; all samples (N=8) were loaded to 0.25N in DMEM buffer 2- Stabilization; all samples were maintained at 0.25N load for 15 minutes more in DMEM buffer to reach stable strain (“creep in”). 3- Digestion; all specimens were maintained at 0.25N load and 0.05mM pre-heated collagenase was injected to the chamber.

Figure 6(A-C) provides a direct comparison of the mechanochemical dynamics of degradation for each of the experiments. In figure 6A, all three runs are plotted as strain vs. time. The figure is a clear demonstration of the differences between each run. The data show that degradation of the unloaded specimens proceeds more quickly than the loaded specimens. Figure 6B includes a T-Test to illuminate significant differences between the strain curves (p<0.05) in strains between digestion tests with and without load. The results for the T-Test indicate that the strains for the two tests are not similar except where they cross. Thus the intersample mechanical behavior is statistically different at every point with the continuously loaded sample resisting failure more than the load/unload/reloaded sample. This is nicely corroborated in figure 6C which shows the early loss of ability to sustain the applied load in the unloaded sample.

Figure 7(A-B) plots the strain-rate as function of time and strain which may be interpreted loosely as a measure of the enzymatic cleavage rate against time. The strain rate in each test was calculated from derivative of strain vs. time curve. Strain rate plots demonstrate that at all time values the unloaded/reloaded sample strains at a much faster rate (likely indicating mechanical integrity loss during the unloaded period). Though we anticipated that the enzymatic cleavage rate (strain-rate) is a function of strain, Figure 7B shows that the unloaded/reloaded specimens strain at a much higher rate at all strain values indicating that unloading the specimen has likely been mechanically compromised extensively. Thus load history during enzymatic exposure is a critical factor in the prediction of enzyme cleavage induced creep.

The effective loaded area loss as function of time in each protocol has been calculated to investigate the effect of load during the degradation in figure 7C. The effective area loss from unloaded specimens rapidly reaches to 0.06 mm² after reloading compared to loaded specimens after 70 minutes. After reloading, these specimens lose more than 60% of their effective loaded area. The data captured from both protocols as the effective area loss shows significant difference between the digestion test with load and without load (p<0.05). It demonstrates that the mechanical integrity of unloaded specimens is compromised.

In Figure 8(A-B) transmission electron micrographs qualitatively demonstrate the effect of degradation on unloaded collagen fibrils. The TEMs are taken perpendicular to the long axis of the tensile specimens and should be representative of the whole cross-section with regard to loaded and unloaded fibrils. Unloaded fibrils will appear in longitudinal section (as lines) and loaded fibrils will appear in cross-section (as dots). Figure 8A is a loaded, undigested control in cross-section. The classic multilayered lamellar structure of the cornea can readily be appreciated with its alternating layers of lines and dots (see inset). In figure 8B, a loaded digested sample indicates significant disruption of the lamellar structure and a relative loss of longitudinal (unloaded) fibrils.
Figure 5- A) Strain and Load vs. Time for control test with load. B) Strain and Load vs. Time for digestion test without load. C) Strain and Load vs. Time for digestion test with load. Load data is representative of one experiment.

Discussion:

The data are generally supportive of our main hypothesis that collagen in native tissue is protected from enzymatic attack by the application of mechanical loads. It is certainly clear that mechanical unloading accelerates the loss of mechanical integrity substantially and significantly. The unloaded, digested samples are essentially “failed” at the time they are reloaded (35 minutes – Figure 5B and 6A). Indeed some of the samples could not support the load upon reloading. The loaded tissue survived the digestion (i.e. could sustain the applied load) for approximately 15 more minutes. Furthermore, the unloaded/reloaded tissue exhibited higher strain rates for all times (Figure 7A) and for all but extreme strains (Figure 7B) compared to the continuously loaded digested sample. Thus the unloaded/reloaded sample, by all measures, failed before the loaded sample. Birefringence images captured from all protocols at both 0°-90° and 45°-135° suggest that unloaded collagen fibrils may be preferentially removed throughout the degradation process compared to loaded collagen fibrils at the same time (Figure 3B). Images from unloaded specimens suggest the uniform attack of bacterial collagenase on fibrils in the specimens during the period of unloading (Figure 3C). The images generally support the data calculated for the effective loaded area loss (Figure 7C) which confirms that the effective loaded area loss is greater for the unloaded specimens (likely as a result of difference between preferential attack on the unloaded collagen and uniform attack on all collagen).

Figure 6: A) Strain vs. Time B) P Value vs. Time C) Load vs. Time
In figure 8, we examine the internal diffusional control fibrils which, in the continuously loaded, digested sample, should be preferentially attacked by the enzyme. If the enzyme preferentially degrades unloaded fibrils, and has diffusional access to the whole sample, then the longitudinal fibrils should be degraded first. The TEMs are generally supportive of the hypothesis that the loaded fibrils are protected because there appear to be few longitudinal fibrils remaining (compared to the unloaded control specimen). However, the matrix is too disrupted in general to reach any real conclusions as to whether the enzyme had access to all of the fibrils. It is thus not possible to determine if the reduction in degradation rate for the loaded sample is due to strain-dependent diffusional access reduction or strain-dependent fibril resistance to enzymatic attack.

Conclusion:

This study has demonstrated that our custom, miniature bioreactor is capable of applying loads and measuring displacement with very high temporal, spatial and force resolution. The data captured from the experiments by the bioreactor are repeatable and demonstrate that tensile loads similar to in vivo loads reduce the rate of degradation of collagen in native tissue (cornea). Previous studies (including our own) have shown that strain can stabilize the collagen molecule against enzymatic attack [8, 17, 18]. In this investigation, we extend the mechanochemical data on bacterial collagenase over multiple strains (loaded and unloaded protocol) in a load-controlled experiment. Supplementary analyses which include calculations of strain rate and effective loaded area in combination with TEM and polarization pictures show that the degradation process is reduced by application of mechanical load. It is clear that BC cleavage rates depend on the applied load/strain. The results also show that fibrils are not completely protected by the load and that the specimens will degrade to failure (at these enzyme concentrations). Though our birefringence data and TEM suggest that fibrils are preferentially attacked if they are not loaded, we are unable to conclude that strain-induced changes in molecular conformation is the protective mechanism. It could be strain-reduced diffusional access as well.

It should also be noted that crude bacterial collagenase is a non-specific enzyme capable of degrading collagen in addition to many other molecules. It contains up to 18 different enzymes which result in rates and patterns of collagen degradation that are drastically different from in vivo digestion (by MMPs or cathepsins). It is clear that moving to a more physiologically relevant enzyme (MMP or cathepsin) should produce results that are more comparable with the in vivo remodeling process.
Figure 8- Transmission Electron Micrographs of corneal strips in cross-section. (A) Inset from low mag image (above): Control test with load shows “normal” distribution of collagen lamellae with unloaded fibrils oriented longitudinally and loaded fibrils seen in cross section (dots). (B) Inset from low mag image above: Digestion test with load shows significant disruption of the normal lamellar architecture and what appears to be preferential loss of unloaded (longitudinal) fibrils. Bars are 10 microns.
References:


15. Church, K., Influence of mechanical load on enzymatic degradation of type 1 collagen in a miniature bioreactor, in Mechanical and Industrial Engineering. 2007, Northeastern University: Boston.

