Deformation-Dependent Enzyme Mechanokinetic Cleavage of Type I Collagen

Collagen is a key structural protein in the extracellular matrix of many tissues. It provides biological tissues with tensile mechanical strength and is enzymatically cleaved by a class of matrix metalloproteinases known as collagenases. Collagen enzymatic kinetics has been well characterized in solubilized, gel, and reconstituted forms. However, limited information exists on enzyme degradation of structurally intact collagen fibers and, more importantly, on the effect of mechanical deformation on collagen cleavage. We studied the degradation of native rat tail tendon fibers by collagenase after the fibers were mechanically elongated to strains of ε = 1–10%. After the fibers were elongated and the stress was allowed to relax, the fiber was immersed in Clostridium histolyticum collagenase and the decrease in stress (σ) was monitored as a means of calculating the rate of enzyme cleavage of the fiber. An enzyme mechanokinetic (EMK) relaxation function T_E(ε) in s⁻¹ was calculated from the linear stress-time response during fiber cleavage, where T_E(ε) corresponds to the zero order Michaelis–Menten enzyme-substrate kinetic response. The EMK relaxation function T_E(ε) was found to decrease with applied strain at a rate of ~9% per percent strain, with complete inhibition of collagen cleavage predicted to occur at a strain of ~11%. However, comparison of the EMK response (T_E versus ε) to collagen’s stress-strain response (σ versus ε) suggested the possibility of three different EMK responses: (1) constant T_E(ε) within the toe region (ε < 3%), (2) a rapid decrease (~50%) in the transition of the toe-to-heel region (ε = 3%) followed by (3) a constant value throughout the heel (ε = 3–5%) and linear (ε = 5–10%) regions. This observation suggests that the mechanism for the strain-dependent inhibition of enzyme cleavage of the collagen triple helix may be by a conformational change in the triple helix since the decrease in T_E(ε) appeared concomitant with stretching of the collagen molecule. [DOI: 10.1115/1.3078177]

Keywords: collagen, cleavage, deformation, enzyme mechanokinetics, stress relaxation

1 Introduction

Collagen degradation is a mechanism for extracellular matrix (ECM) remodeling and maintenance, and a response to trauma, disease, and inflammation. Collagenases 1, 2, and 3 are the primary enzymes that act to degrade interstitial collagens (types I, II, and III) in humans and animals. These collagenases are part of a larger family of enzymes (matrix metalloproteinases (MMPs)) characterized by a zinc dependency for catalytic activity [1]. MMPs are secreted by the cell as inert zymogens in response to the cell being activated by inflammatory cytokines, such as growth factors (interleukin-1) [2] and mechanical loads [3]. In order for collagen cleavage to occur, the collagenase (MMPs 1, 8, 13, respectively) gains access to the collagen triple helix by binding to the enzyme’s attachment domain along the α-chains, followed by separation (unwinding) of the α-chains to expose the cleavage site, and then cleavage of the α-chain by the enzyme’s catalytic domain [1,4]. Collagenases contain two protein domains joined by a linker (hinge), a hemopexin C domain to which the collagen molecule attaches, and a catalytic domain responsible for the α-chain cleavage [1,4]. MMPs 1, 8, and 13 will cleave all three α-chains of interstitial collagens by a single scission at a specific site, located 3/4 from the N terminal and 1/4 from the C terminal, which is characterized by a Gly775–Ile776 or Gly775–Leu776 peptide bond, resulting in two fragments of the collagen molecule [1]. Following this initial cleavage other MMPs (mainly gelatinases and stromelysins) can collectively further degrade the collagen fragments. However, the mechanism of the initial cleavage of the collagen molecule must originate with collagen binding, triple-helix unfolding, and 3/2 scissoring.

Like mammalian collagenases, Clostridium histolyticum collagenase (CHC or bacterial collagenase) is both Zn²⁺ and Ca²⁺ dependent. However, it differs from mammalian collagenases in that it can cleave collagen at multiple sites [5,6]. The bacterial collagenases are a group of highly related metalloproteinases that yield several subdivisions of distinct collagenases, Classes 1 and 2. Lin et al. [7] demonstrated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis that purified CHC consists of two independent but comparable collagenases, type-β and type-δ collagenases. Class 1 enzymes initially cleave collagen near the periphery of the triple-helix domain, while class 2 enzymes cleave near the interior, fragmenting the collagen into nine segments [5].

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Our focus in this study was on collagenases and fibrillar type collagens. We are particularly interested in the ability of collagenases to cleave fibrillar collagens of the musculoskeletal system (ligaments, tendons, cartilage, and meniscus). Diarthrodial joints are subjected to a lifetime of mechanical loading. One mechanism associated with the initiation of collagen degradation is cleavage of the collagen network in the extracellular matrix by collagenases, primarily MMPs 1 and 13 [3,8]. Mechanical factors are important when considering rates and mechanisms of enzymatic activity, and how a mechanical deformation of the substrate, enzyme, or both (i.e., the mechanochemical interaction [9,10]) will affect the enzymatic activity is applicable to many tissues and enzymes. We refer to this physical mechanism as *enzyme mechanokinetics* (EMK). In our study we utilized rat tail tendon to model the collagen-enzyme mechanokinetic interactions. The linear orientation of type I collagen in rat tail tendon allows us to easily extract collagen fibers and uniaxially deform these fibers in tension while exposing them to enzymatic degradation.

The phenomenon of deformation-dependent cleavage of collagen by bacterial collagenase was previously reported in three earlier studies. Using a thin collagen tape formed from reconstituted bovine tendon (1.3 mm × 12 cm × 32 μm thick), Huang and Yannas [9] found that increasing tensile strain up to 4% (grip-to-grip) resulted in a decrease in the rate of enzymatic degradation, while strains above this (up to 7%) caused an increase in the rate. They speculated that these phenomena were a result of restricted enzyme transport to the cleavage site and the opening of new cleavage sites, respectively. However, the potentially disrupted and unknown structural organization of reconstituted collagen limits the interpretation of their results on effect of axial strain on the interaction between the collagen molecule and collagenase. Nabeshima et al. [10] applied a single uniaxial tensile strain of 4% (grip-to-grip) to intact rabbit patellar tendon, and after 20 h of incubation with enzyme found that the tendon’s tensile failure strength was significantly greater compared with the tensile strength of similarly incubated unloaded tendon. They reported that mass transport of the enzyme into the tendon was not responsible for this difference, and attributed the inhibition in enzyme cleavage of the tendon to several possible mechanisms associated with altered collagenase-matrix interaction, including stretching of the triple helix and local pH changes from redistribution of free water. More recently, Ruberti and Hallab [11] uniaxially loaded (1–2 N) strips of bovine cornea (3 mm wide × 20 mm long × 0.8 mm thick) in Clostridiopeptidase A for 120 h and reported a loss in collagen birefringence only in the collagen fibers that were not aligned to the tensile load. The authors attributed this result to a “strain-stabilization” mechanism making enzyme binding and cleavage more difficult, though tissue strains were not reported.

Unfortunately the use of reconstituted, unorganized, and gross collagen specimens, and imprecise and indirect measures of collagen strain and degradation, makes interpretation of these results difficult when trying to determine the mechanisms of enzyme-substrate interaction. Our study was designed to avoid these shortcomings by using a single collagen fiber (rat tail tendon) in its native state, measuring the applied strain directly on the fiber (longitudinal and transverse), measuring the degradation of single fibers both mechanically and optically. We utilized a similar *mechanochemical model* as Huang and Yannas [9] to test whether uniaxial tensile deformation of type I collagen would result in a change in the collagen-enzyme cleavage process. We also chose to use Clostridium histolyticum collagenase as opposed to mammalian collagenase because CHC is readily available, inexpensive, and biochemically active at room temperature [12], the latter critical to our mechanochemical testing system. We hypothesized that a mechanically applied elongation of the rat tail tendon fiber would result in inhibition of the enzymatic degradation, and that the inhibition would be proportional to the amount of fiber elongation. To test this hypothesis we stretched (loaded) rat tail tendon fibers to different strain levels, immersed the fibers in bacterial collagenase, and measured the rate of enzyme cleavage by measuring the decrease in fiber load. We found that as the strain applied to the collagen fibers increased, the rate of enzyme degradation was significantly reduced, though not completely inhibited.

2 Materials and Methods

2.1 Chemicals. Dulbecco’s phosphate buffered saline (PBS) with calcium (CaCl₂, 0.133 mg/ml) and magnesium (MgCl₂, 0.1 mg/ml), dimethylmethyylene blue (DMMB), and dimethylaminozobenzaldehyde (DMAB) reagents were obtained from Sigma-Aldrich (St. Louis, MO). Type II bacterial collagenase (*Clostridium histolyticum*, ≥125 units/mg dry weight (DW)) was obtained from Worthington Biochemical Corporation (No. CLS-2, Lakewood, NJ). All other reagents were obtained from Sigma-Aldrich and were reagent grade unless otherwise noted.

2.2 Rat Tail Tendons. In accordance with institutional animal care and use procedures, type I collagen fibers from the tails of three 6 month old Lewis rats, euthanized for other unrelated studies, were used in this investigation. Using a sterile scalpel blade the tail was sectioned between the coccygeal vertebrae at the base and distal tip of the tail for a total length of approximately 150 mm. The tail was removed immediately after euthanasia and placed in a −20°C storage freezer. Upon freeze-thaw (~60 min at room temperature 23–25°C) type I collagen fibers (i.e., fascicle of diameter ~300 μm) were teased from the tail, sectioned into 70 mm length specimens, and soaked in 15 ml 1× PBS. The fibers were divided into four groups: (1) water, collagen, and proteoglycan contents (n=12), (2) mechanical testing (n=6), (3) osmotic stress effect (n=4), and (4) EMK testing (n=24).

2.3 Collagen-to-Enzyme Ratio. An estimate for the total number of collagen molecules (substrate concentration [S]) in the collagen fiber was calculated using the surface area of the tendon fiber exposed to the aqueous environment, divided by the approximate collagen molecule size, assuming complete packing. The enzyme concentration [E] was derived from the total collagenase weight, divided by the approximate molecular weight of a bacterial collagenase enzyme, suspended uniformly in a fixed volume. We modeled the collagenase-collagen interaction as a solid two-dimensional sheet of collagen substrate contacted by a single layer of collagenase in suspension. Since the reaction of interest, [E]×[S], is restricted to a two-dimensional area, we approximated a volume per molecule of collagenase enzyme in uniform suspension and then calculated a face surface area by considering the volume as a cube. It is important to note that these simplifications in this model include no consideration of potential noncollagenous substrates at the surface of the fibril (e.g., proteoglycan), possible steric hindrance between the collagen and collagenase molecules, and as stated above, assume a uniform suspension. Using the molecular weight for Rattus norvegicus (rat) collagen obtained from the National Center for Biotechnology Information protein database and for bacterial collagenase of 116 kDa [6,13], we estimated the maximum substrate-to-enzyme concentration ratio [S]:[E] >100:1. Thus the collagen fiber cleavage could be assumed to follow a zero order kinetic reaction during the early collagenase degradation phase [14].

2.4 Water, Proteoglycan and Collagen Contents. Tendon fibers (n=12) were incubated in buffered saline for 1 h, weighed (wet) on a microbalance (25 Electrobalance, Cerritos, CA), lyophilized overnight for 24 h, and weighed (dry). The fiber water content (percent wet weight) was calculated from the wet and dry weights. Dehydrated samples were then solubilized with papain,

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and aliquots were taken for further analysis of collagen and proteoglycan content.

The collagen content of the tendon fibers was assayed by determination of hydroxyproline content. Hydroxyproline was measured by chromophore formation with 4-dimethylaminobenzaldehyde against standards and reported as a percent of fiber DW (modified from Refs. [15,16]. The solubilized samples were hydrolyzed under acidic conditions at 110°C for 3 h, oxidized with chloramine-T, and then exposed to DMAB dissolved in 1-propanol and perchloric acid (Baker, Phillipsburg, NJ) at 65°C. Chromophore absorption was then measured with a spectrophotometer (340 ATTC, Tecan U.S., Chapel Hill, NC) at 550 nm and compared with a standard curve of pure hydroxyproline.

Proteoglycan content was assayed by determination of the glycosaminoglycan (GAG) content. GAG was measured colorimetrically by reaction with 1, 9-dimethylmethylene blue dye and reported as a percent of fiber dry weight (modified from Refs. [17,18]). Solubilized samples were diluted with distilled water, exposed to DMMB dye, absorbance measured at 540 nm, and compared against a standard curve of chondroitin-4-sulfate.

**2.5 Mechanical Test System.** All mechanical and chemical testing procedures were performed at room temperature (23–25°C). A novel collagen-enzyme mechanokinetic automated test system (CEMKATS) was developed to elongate the type I collagen fiber (mechanical-elongation phase) and to measure the change in load (1) after the elongation was applied (mechanical-relaxation phase), and (2) after the fiber was submerged in enzyme (enzyme-cleavage phase). The CEMKATS (Fig. 1) consisted of two stepper motors (range=12.5 mm, resolution=2 µm) (SteppeMike, Oriel, Stratford, CT) aligned axially on linear stages (Parker Hannifin, Cleveland, OH), positioned approximately 15 cm apart, which were mounted onto an optical vibration isolation table (Newport, Stratford, CT) (Fig. 1). The motors were connected to a stepper controller (Oriel, Stratford, CT) interfaced to a personal computer (Pentium 4, Gateway, Irvine, CA) via an RS-232 terminal data link. LabVIEW software (National Instruments, Austin, TX) was used to drive each stepper motor in full-step mode (2 µm/step) at a maximum velocity of 1 mm/s. Both motors were programmed to operate simultaneously to elongate the collagen fiber at a constant 2 mm/s.

Custom-made grips to hold the ends of the collagen fiber were attached to each stepper motor (Fig. 1(b)). Each grip consisted of a flat surface with a ~0.5 mm deep V-groove extending to a small hole. One of the grips was attached to a 250 g load cell (resolution=0.24 mg, Sensotec Model 31, Honeywell, Columbus, OH), which was interfaced with the computer via a signal conditioner (Model GM) and data acquisition board (National Instruments). At the time of testing, the grip ends were positioned ~27 mm apart, the ends of the collagen fiber (wet) placed into the groove and through the hole, and the fiber secured in the groove and hole using ethyl cyanoacrylate glue (Elmer’s, Columbus, OH). Using the motors, the fiber was slowly tensed to ~1–2 g and then unloaded to remove any slack in the fiber. Measurement of the initial grip-to-grip fiber length, Lg, was made using a Vernier caliper (Lg ~ 25–27 mm), and this length used to determine the amount of fiber elongation, ΔL, required to achieve the desired fiber strain, Eo = (ΔL/Lg). The fiber was then suspended into a 1 mm wide by 25 mm long channel containing PBS at room temperature. A continuous PBS drip (~1 drop/s) was applied to the fiber through a syringe needle positioned near the end of the channel, such that the fiber remained hydrated (immersed) throughout the duration of the load-relaxation phase of the test (Fig. 1(b)).

In this test configuration, about ~1 mm of the fiber length was exposed to the air between the ends of each grip and the channel. The portion of the collagen fiber exposed to air (~8% of the fiber) will shrink and stiffen as it dries and could possibly affect the final strain applied to the fiber within the channel at the end of the
mechanical-relaxation phase (1–8 h). To correct for possible drying artifacts when using the grip-to-grip length to calculate the fiber strain, two black gage marks (~0.2 mm thick, inkjet printer ink) were placed approximately 2–5 mm apart in the center of the fiber, equidistant from each of the grips, and digitally imaged through a light microscope (Wild, Heerbrugg, Switzerland) using a digital camera (DMCLe, Polaroid, Waltham, MA) (Figs. 1(b) and 2). The camera was interfaced to a second computer, where it recorded time-captured images of the gage marks and the fiber’s diameter during the tests (Fig. 2). Images were recorded at four time points: (1) prestrain \( t_p \), (2) poststrain \( t_p \), (3) poststress (load) relaxation \( t_r \), and (4) postenzyme cleavage \( t_c \) (Figs. 2 and 3). The poststrain and poststress relaxation images were analyzed using NIH IMAGE J software to measure the distance (resolution of ~1 \( \mu \text{m} \)) between the center of the gage marks and outer edges of the fiber’s diameter, and these were used to calculate the axial and radial strains within the fiber at \( t_p \) and \( t_r \), respectively. The fiber’s axial strain \( e_a \) was calculated from the change in distance \( \Delta l \) between the gage marks, \( e_a = \Delta l / l_0 \), and the radial strain \( e_r \) calculated from the change in fiber diameter \( \Delta d = d_p - d_r \), where \( l_0 \) and \( d_0 \) are the prestrain distance and diameter, respectively. The fiber stress, \( \sigma \), was calculated from the load, \( F \), and fiber cross-sectional area, \( A \), where \( \sigma = F / A \) in N/m² (Pascal), assuming the fiber cross section was circular [19].

2.6 Mechanical-Relaxation Tests. Preliminary mechanical tests were performed to determine the time required for stress relaxation (load equilibrium) after the application of the applied strain \( E_w \). Collagen fibers were strained to 2% or 4% (grip-to-grip, 2 mm/s) in PBS (pH=7.2), and the strain held constant while monitoring the decrease in stress (load) as a function of time (1 Hz sampling frequency) until an equilibrium-stress relaxation stress, \( \sigma_r \), was reached at \( t_r \). The mechanical stress-relaxation response \( \sigma_m(t) \) was analyzed using a three-parameter power function of time [20], given by

\[
\sigma_m(t) = \sigma_p - \alpha t^\beta
\]

where \( \sigma_p \) is the time-independent peak stress at the end of the mechanical-elongation phase at time \( t_p \), \( t \) is time, and \( \alpha \) and \( \beta \) are relaxation constants, and the stresses \( \sigma_m(t) \) are calculated using the prestrain fiber cross-sectional diameter, \( D_o \), and area, \( A_o \). The relaxation constants \( \alpha \) and \( \beta \) can be easily determined for each fiber from the least-squares best-fit of Eq. (1) to the experimental data.

2.7 Effect of Fiber Strain on Collagen Cleavage. Fibers \((n=24)\) were stretched to 1%, 2%, 3%, or 4% in PBS (pH=7.2), allowed to reach stress equilibrium \((\sigma_r \rightarrow \sigma_r)\), and 1% bacterial collagenase (10 mg/ml) in PBS (pH=7.2) added to the entire channel (~1 s). The enzyme-degradation phase was continuously monitored by recording the decrease in the fiber’s enzyme-degradation stress, \( \sigma_e(t) \), as the collagen in the fiber was cleaved by the bacterial collagenase (Fig. 3). The fiber stress \( \sigma_e(t) \) was calculated from the load and \( A_o \) and monitored until \( \sigma_e(t) \rightarrow 0 \).

In some cases, the fiber’s mechanical-relaxation stress \( \sigma_m(t) \) did not reach equilibrium before the enzyme was introduced. Thus, Eq. (1) was used to calculate (predict) the mechanical stress relaxation \( \sigma_m^n(t) \) occurring during the enzyme-degradation phase (Fig. 3). The decrease in fiber stress due only to the enzyme cleavage of the fiber, \( \sigma_e^n(t) \), was calculated from the measured enzyme-degradation stress, \( \sigma_e(t) \), by removing the mechanical stress relaxation occurring after the enzyme was introduced at time \( t_r \), such that

Fig. 2 The local fiber axial strain (change in distance between gage marks) and radial strain (change in diameter) at the center of the fiber were recorded using a microscope and digital camera image capture at (a) time \( t=0 \), unstrained initial lengths, \( l_0 \) and \( d_0 \) (b) time=\( t_p \), at the end of the elongation phase at peak stress, \( l_p \) and \( d_p \) (c) time=\( t_r \), after mechanical stress relaxation, \( l_r \) and \( d_r \) (d) time=\( t_e \) after collagenase cleavage, \( l_e \) and \( d_e \). The initial, elongation, and relaxation lengths ((a), (b), and (c)), respectively, were used to determine the fiber’s axial and radial strains. Fiber images (right) are for an initial 3% grip-to-grip strain.

Fig. 3 Typical mechanical-elongation (a), mechanical stress-relaxation (c), and enzyme-cleavage (e) responses for the rat tail tendon fibers subjected to a constant applied strain (3%) and degradation by bacterial collagenase, respectively. The peak stress occurs at the end of the mechanical-elongation phase (b). Immediately after the fiber is exposed to the collagenase (d) there is a small transient increase in fiber stress due to the osmotic stress. The mechanical stress-relaxation response (c) was curve-fit using Eq. (1) (dotted line) and extended beyond (d) to predict the continued mechanical stress relaxation (b). This was used to correct the enzyme-cleavage response (g). The linear portion of the corrected enzyme-cleavage response (h) was used to calculate the EMK relaxation function. For clarity the data shown in (a), (e), and (g) are plotted at 1 s intervals, and in (c) at every 100 s.
\[ \sigma_e(t) = \sigma_f(t) + \sigma_r - \sigma_m(t) \]  
for \( t \geq t_r \) (2)

According to the Michaelis–Menten model for simple steady-state enzyme-substrate kinetics, the rate of enzyme degradation of collagen will be linear (zeroth order) with respect to time under the condition that \([S] \geq [E]\) (concentration of substrate \(\geq\) enzyme). Thus, we defined the slope of the linear (initial) portion of the enzyme-cleavage response (\(\sigma_e(t)\) versus time) to be the EMK relaxation function \(T_E(\varepsilon)\) in \(s^{-1}\), given by

\[ T_E(\varepsilon) = \left[ \frac{d\sigma_e(\varepsilon)}{d\varepsilon} \right]_{\sigma_e} \]  
where \(\sigma_m(t)\) has been normalized to the equilibrium-relaxation stress \(\sigma_e(t)\) at time \(t_r\). The EMK relaxation function \(T_E(\varepsilon)\) is a measure of the collagenolytic activity for fiber degradation, which is assumed to start on the outside of the fiber, as this layer is most accessible, and dependent on the applied strain \(\varepsilon\) (axial and radial). The EMK relaxation function was determined for each fiber from the best-fit straight line to the initial portion of the enzyme-cleavage phase after osmotic stress relaxation (Fig. 3).

2.8 Effect of Osmotic Stress. Solvents, such as water, that are capable of forming intramolecular hydrogen-bonds with the triple helices will cause the collagen fibers to swell, whereas solvents containing large molecules (e.g., C. histolyticum collag enase, 116 kDa [6]) will draw water out of the triple helices [21]. As a result of the interaction of water and solutes with the collagen molecule, a significant intramolecular hydration force is possible if the fiber is subjected to an osmotic stress. This hydration force could affect the axial load while the fiber is held at a constant elongation. To evaluate the effect of an osmotic stress on the fiber when the collag enase is introduced into the channel, separate tests were performed using dextran (MW=79.4 kDa) and inactivated bacterial collagenase. In the former, stress-relaxation tests were performed on fibers stretched to 2% or 4% strain in PBS containing Ca\(^{2+}\) (pH=7.2). Once stress equilibrium was reached, dextran (10 mg/ml) in PBS solution was added to the channel, and the change in the stress \(\sigma_m\) was monitored until stress equilibrium was again reached. In the latter case, tests were performed in which the collagenase activity was inhibited by omitting the Ca\(^{2+}\) from the PBS (CHC is Ca\(^{2+}\) dependent). Similar to the dextran tests, stress-relaxation tests were first performed in Ca\(^{2+}\) and Mg\(^{2+}\) free PBS, then bacterial collagenase (10 mg/ml in Ca\(^{2+}\) and Mg\(^{2+}\) free PBS) was added once stress equilibrium was reached, and \(\sigma_m(t)\) again monitored until stress equilibrium was again reached.

2.9 Statistical Data Analysis. The parameters \(\alpha\), \(\beta\), and \(T_E(\varepsilon)\) were calculated from the experimental data for the mechanical-relaxation and enzyme-cleavage phases using best-fit linear least-squares regression analysis (SYSTAT software, Richmond, CA) to Eqs. (1) and (3), respectively. Means ± standard deviations were calculated for all measured parameters unless indicated otherwise. Statistical significance was determined using Analysis of Variance (ANOVA) and the two-tailed student’s t-test at the \(\alpha=0.05\) level. Multiple linear regression analysis of the collagenolytic activity, \(T_E(\varepsilon)\), was performed to determine its dependency on the axial and radial strains, fiber diameter, and the peak and equilibrium-relaxation stresses [22,23].

3 Results

3.1 Water, Proteoglycan, and Collagen Contents. Analyses were performed on individual tendon fibers (\(N=12\)) to determine the water content (free water per fiber wet weight) and the amounts of proteoglycan (GAG per dry weight) and collagen (hydroxyproline per dry weight). Water content of the tendon fibers was 65.2 ± 1.8%. GAG content was 0.28 ± 0.07% per dry weight, in agreement with the 0.24% reported by others [24]. The GAGs are located on the proteoglycan monomer (aggrecan), which contains approximately 93% GAGs (87% chondroitin sulfate and 6% keratan sulfate) and 7% protein. Thus the amount of proteoglycan was approximately 0.49% per dry weight and 0.17% per wet weight, similar to previously reported values for tendon [25]. Hydroxyproline content was 8.5 ± 0.5% per dry weight, in agreement with 8.2% reported by Marsolais et al. [26] and 8.9% reported by Scott et al. [27]. If the content of hydroxyproline is assumed to be 12.5% by weight of collagen [16], the collagen content was approximately 68% and 24% of the fiber’s dry and wet weights, respectively.

3.2 Stress-Relaxation Tests. Preliminary mechanical stress-relaxation tests were performed on collagen fibers to determine the time required to reach stress equilibrium prior to the introduction of the bacterial collagenase. Typical stress-time responses were observed for all fibers, with a rapid increase in stress during the mechanical-elongation phase followed by a slow decrease in stress once the grip-to-grip elongation ceased (mechanical-relaxation phase) (Fig. 3). Fibers were strained to 2% or 4% (\(\varepsilon_f\), \(n=3\) each) based on their initial fiber grip-to-grip lengths (\(L_o\) =25.3 ± 0.5 mm). However, the actual axial strain measured directly on the fiber at the end of the elongation phase, \(\varepsilon(t_f)\), was 2.09 ± 0.06% and 4.55 ± 0.26%, respectively. The corresponding peak stresses \(\sigma_f\) were 5.8 ± 2.3 MPa and 15.2 ± 11.2 MPa, and these decreased by 67% and 91% at equilibrium. The stress-relaxation response \(\sigma_m(t)\) was fit to the three-parameter power function (Eq. (1)) yielding \(\alpha\) and \(\beta\) values of 1.271 ± 0.589 MPa and 0.138 ± 0.024 for the 2% applied strain, respectively, and 0.641 ± 0.252 MPa and 0.292 ± 0.106 for the 4% strain. Finally, the fibers reached stress equilibrium in 1.6 ± 1.3 h and 4.7 ± 1.8 h, respectively, determined when the load-relaxation rate was <1 mg/s or ~0.001 \(\sigma_f/s\).

For the enzyme studies collagen fibers were stretched to 1%, 2%, 3%, or 4% axial (grip-to-grip) strain and allowed to reach stress equilibrium (\(t_r~2-4~h\), mean 3.3 ± 0.9 h, Table 1) before the introduction of the collagenase. Twenty-four fibers were tested, with initial diameters \(D_o\) of 324 ± 86 \(\mu \)m (range of 177–582 \(\mu \)m) and grip-to-grip lengths \(L_o\) of 25.3 ± 0.6 mm (Table 1). The stress-relaxation response for all 24 samples was similar to those reported above. Values for the fiber peak and equilibrium stresses and strains, time to reach mechanical equilibrium, and the relaxation constants \(\alpha\) and \(\beta\) are given in Table 1 for each applied strain \(\varepsilon_f\). The fiber peak axial strain \(\varepsilon(t_f)\) ranged...
from 0.7% to 4.8%, while the peak radial strain $\varepsilon_r(t_p)$ was significantly greater, ranging from 2.9% to 9.5% (Fig. 4). At stress equilibrium the axial and radial strains increased to approximately twice that of their peak values, increasing by 1.71±0.37 and 2.04±0.63, respectively, even though the grip-to-grip strain was held constant (Table 1, Fig. 4). The increase in strain may be from drying of the exposed fiber ends or uneven fibril elongations, though the exact mechanism for this was not determined (see Sec. 4).

We also found that the radial strain was almost twice that of the axial strain at both times. Poisson’s ratio ($\nu = \varepsilon_r/\varepsilon_a$=radial strain/axial strain) at stress equilibrium $\nu(t_e)$ was linearly correlated (equal) to Poisson’s ratio at the peak stress $\nu(t_p)$ ($\nu(t_e) = 0.972 \pm 0.085$, $r = 0.758$). The mean peak and equilibrium Poisson’s ratios were $\nu(t_p) = 2.23 \pm 1.83$ and $\nu(t_e) = 2.64 \pm 2.41$, respectively, which were not statistically different ($p > 0.05$, pooled mean $\nu = 2.43 \pm 2.16, n = 48$), again indicating equivalence at both times. These values for collagen fibers are similar to those for whole tendon [28] and indicate that interstitial fluid (water) is being exuded as the fiber is elongated. When Poisson’s ratio was plotted against the axial strain at the peak and equilibrium stresses (times $t_p$ and $t_e$, respectively, Fig. 5), it was found that Poisson’s ratio decreased rapidly for small axial strains (~2% for peak and ~4% for equilibrium), and then remained relatively constant for higher axial strains. The data fit best to an exponential-linear model ($r = 0.945$), as shown in Fig. 5. This model indicates that when initially loaded, the fiber’s diameter rapidly decreases as the fiber begins to elongate (exponential decay), but with increasing load and axial strain it becomes more difficult to decrease the fiber’s diameter (linear decay).

Finally, analysis of the peak and equilibrium stresses as a function of their respective peak and equilibrium strains found that the peak stress decreased linearly with peak strain (slope $= 0.28 \pm 0.41$ MPa/mm/mm, $p < 0.004$) while the equilibrium stress was independent of the equilibrium strain (mean $= 1.8 \pm 1.4$ MPa, $p > 0.5$) (Fig. 6). The decrease in the peak stress with stress relaxation (peak/equilibrium $= 7.3 \pm 7.1$) was significantly greater than the increase in the peak stress with stress relaxation (equilibrium/peak $= 1.7 \pm 0.08$), suggesting these are independent phenomena.

### Table 1 Mechanical and enzyme mechanokinetic stress-relaxation parameters (means±standard deviation; $n=6, 7, 5, \text{and } 6$ fibers for 1%, 2%, 3%, and 4% strains, respectively)

<table>
<thead>
<tr>
<th>Applied strain (grip-to-grip), $E_o$ (%)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber diameter, $d_o$ ($\mu$m)</td>
<td>324±67</td>
<td>303±65</td>
<td>380±139</td>
<td>304±68</td>
</tr>
<tr>
<td>Mechanical stress relaxation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak axial strain, $\varepsilon_a(t_p)$ (%)</td>
<td>1.01±0.27</td>
<td>2.49±3.9</td>
<td>3.25±0.16</td>
<td>4.38±0.42</td>
</tr>
<tr>
<td>Equilibrium axial strain, $\varepsilon_a(t_e)$ (%)</td>
<td>1.90±0.74</td>
<td>4.54±0.91</td>
<td>5.13±1.16</td>
<td>6.86±1.98</td>
</tr>
<tr>
<td>Peak radial strain, $\varepsilon_r(t_p)$ (%)</td>
<td>4.3±1.24</td>
<td>4.15±1.1</td>
<td>4.34±0.85</td>
<td>5.16±2.39</td>
</tr>
<tr>
<td>Equilibrium radial strain, $\varepsilon_r(t_e)$ (%)</td>
<td>8.72±3.53</td>
<td>7.43±1.62</td>
<td>9.94±2.2</td>
<td>8.93±3.95</td>
</tr>
<tr>
<td>Peak stress, $\sigma_p$ (MPa)</td>
<td>3.96±1.89</td>
<td>8.40±2.73</td>
<td>6.04±3.86</td>
<td>14.55±8.96</td>
</tr>
<tr>
<td>Equilibrium stress, $\sigma_r$ (MPa)</td>
<td>1.51±1.39</td>
<td>2.76±1.32</td>
<td>1.59±1.69</td>
<td>1.17±0.56</td>
</tr>
<tr>
<td>Mechanical equilibrium time, $t_e$ (h)</td>
<td>2.1±0.9</td>
<td>3.1±1.1</td>
<td>3.9±1.2</td>
<td>4.2±1.5</td>
</tr>
<tr>
<td>Relaxation constant, $\alpha$ (MPa)</td>
<td>0.44±0.32</td>
<td>1.22±0.66</td>
<td>0.90±0.95</td>
<td>2.34±2.86</td>
</tr>
<tr>
<td>Relaxation constant, $\beta$</td>
<td>0.21±0.05</td>
<td>0.16±0.04</td>
<td>0.18±0.09</td>
<td>0.21±0.08</td>
</tr>
<tr>
<td>EMK stress relaxation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmotic equilibrium time (min)</td>
<td>5.2±5.0</td>
<td>17.2±8.9</td>
<td>3.9±4.1</td>
<td>17.4±22.2</td>
</tr>
<tr>
<td>Zero order time (min)</td>
<td>8.4±3.3</td>
<td>16.8±5.7</td>
<td>27.9±16.1</td>
<td>18.9±18.9</td>
</tr>
<tr>
<td>Relaxation function, $T_p(\varepsilon_r)$ ($10^{-4}$ s$^{-1}$)</td>
<td>2.49±0.89</td>
<td>1.40±0.49</td>
<td>1.07±0.33</td>
<td>0.72±0.33</td>
</tr>
</tbody>
</table>

Fig. 5  The Poisson’s ratio ($\varepsilon_r/\varepsilon_a$) at the end of the mechanical-elongation (peak, gray squares) and mechanical stress-relaxation (equilibrium, black squares) phases decreased with increasing axial strain ($\varepsilon_a$) at the peak and equilibrium times, $t_p$ and $t_e$, respectively. For both phases the data fit best to an exponential-linear model (gray and black lines, respectively). Note that the greatest decrease in the Poisson’s ratio occurs for $\varepsilon_a(t_p)\leq3\%$.  

Fig. 6  The peak stress at the end of the mechanical-elongation phase (peak ■), at time $t_p$, linearly increased with increasing axial peak strain. However, the equilibrium stress at the end of the stress-relaxation phase (equilibrium ▽), time $t_e$, was independent of the equilibrium stress. A best-fit linear model ±95% confidence intervals is shown for the peak and equilibrium phases. Note that after stress-relaxation the equilibrium strains increased by 1.71±0.37, as shown in Fig. 4.
molecule and the collagen's intramolecular space. This osmotic stress has been shown to change the triple-helical spacing between the individual $\alpha$ chains [21,29]. To evaluate how an osmotic stress would affect the equilibrium stress $\sigma_{e}$ (magnitude and time), dextran or inactive bacterial collagenase in PBS solution was introduced into the bath channel after stress equilibrium was reached in fibers strained to 2% or 4%, the latter inactivated by the absence of Ca$^{2+}$ from the PBS. For fibers strained to 2%, the dextran and collagenase caused an immediate increase (0.6% and 1.5%, respectively) in the axial stress $\sigma_{m}$ and then a return (decrease) to the initial equilibrium stress over a period of several minutes. Fibers stretched to 4% had no change in their equilibrium stress following introduction of the dextran or collagenase. For both strains and molecules, the equilibrium stress did not change for an additional 45 min period, indicating the absence of any long-term osmotic effect and verifying the collagenase inactivity.

3.4 Enzyme Mechanokinetic Tests. After the fibers were elongated and allowed to reach stress equilibrium, 1% bacterial collagenase was added to the bath channel, and the stress was continuously monitored. As the collagenase cleaved the collagen molecules, the decrease in the fiber’s enzyme-cleavage stress, $\sigma_{c}(t)$, was recorded until $\sigma_{c}(t)=0$. The EMK relaxation function $T_E(e)$, Eq. (3), was calculated from the slope of a best-fit straight line to the experimental data for $\sigma_{m}(t)$ versus time by least-squares regression analysis (Fig. 3). Only the initial linear portion of the response (zero order) was used, and to avoid any osmotic effects the zero order response interval (pooled mean $\pm 17.5 \pm 12.7$ min) was always taken after osmotic stress equilibrium (11.5 $\pm 12.8$ min) was reached (Table 1). Mean values for $T_E(e)$ at each applied strain $E_{c}$ are given in Table 1. Overall there was a 73% decrease in enzymatic activity ($T_E$) as the strain increased from 1% to 4%. When $T_E(e)$ was plotted versus the fiber’s axial peak and equilibrium strains (Fig. 7), $T_E(e)$ was found to linearly decrease with increasing axial strain at a rate of 17.0% linearly decrease with increasing axial strain at a rate of 17.0% and 10.9%, respectively.

Separate verification of enzyme cleavage was observed visually as the fiber’s diameter continuously decreased during the degradation process. The strain-induced inhibition was also confirmed by monitoring the fiber’s diameter at the end of the enzyme-cleavage phase ($\sigma_{c}(t)=0$), where the final diameter decreased by 8.4 $\pm$ 8.2%, 4.0 $\pm$ 2.2%, 1.3 $\pm$ 1.8%, and 1.3 $\pm$ 2.0% for each applied strain of 1%, 2%, 3%, and 4%, respectively.

Multiple linear regression analysis was performed to determine what, if any, independent parameters might influence the decrease in $T_E(e)$. The variables most likely to influence the retardation of collagenolytic activity are the fiber’s axial and radial strains at the peak elongation, $e_{p}(t_{p})$ and $e_{d}(t_{p})$, and at stress equilibrium, $e_{p}(t_{e})$ and $e_{d}(t_{e})$, the stress at the peak and equilibrium, $\sigma_{p}$ and $\sigma_{e}$, respectively, and possibly the fiber’s diameter at the beginning and end of the mechanical-relaxation phase. A stepwise multiple linear regression was performed using all the data for each fiber to determine which independent parameters of fiber geometry and mechanical response had a statistically significant effect on $T_E(e)$. Of all the independent variables, the axial strain at peak and equilibrium accounted for 48% and 33% of the decrease in $T_E(e)$, respectively, while only 2–3% of the variation could be accounted for with radius $e_{d}(t_{p})$, $e_{d}(t_{e})$, and $\sigma_{e}$ in the analysis. The other independent parameters, geometric or mechanical, had no effect on the change in the rate of enzyme cleavage of the collagen fibers.

4 Discussion

The major finding of this study was that mechanical deformation of type I collagen fibers caused by an axial strain (elongation) applied to the fiber will result in a significant decrease in the rate of collagen degradation by bacterial collagenase. Our findings are in agreement with previous reports [9–11] that a tensile strain applied to type I collagen was able to reduce the rate of collagen cleavage by bacterial collagenase. From our statistical analyses it is evident that the axial strain had the largest influence ($–33–48\%$) on decreasing the collagenolytic activity as compared with fiber stress and radial strain. We termed this mechanically-induced substrate-enzyme interaction the EMK effect. While the exact mechanism of action is unknown, the phenomenon documented herein suggests that it is the mechanical deformation of the collagen fiber, at the microstructural level, that is the primary factor responsible for the inhibition in bacterial collagenase cleavage of the collagen molecule. However, as discussed below, further investigations will be necessary to determine the exact mechanism for this change in the mechanochemical kinetic interaction.

Monomeric type I collagen is composed of two $\alpha$(1) chains and one $\alpha$(2) chain. Cleavage by mammalian collagenases occurs by triple helix activity at a specific cleavage site, the mechanism of which has been well characterized [1,4]. Mammalian collagenase attaches to a binding region located along the collagen molecule, initiating the loosening of the triple helix and exposure of the $\frac{1}{3}$–cleavage site, followed by a scissoring across the three chains by the collagenase catalytic domain. It is important to note that mammalian collagenase cannot immediately cleave collagen but must first attach via its hemopexinlike C-terminal domain (CTD) to a substrate-binding site (exosite) located toward the N-terminus of the collagen substrate. A protein linker in the collagenase, which connects the CTD to the catalytic domain, together with the catalytic domain was hypothesized to perturb or unwind the catalytic active site on the triple helix enabling $\alpha$-chain cleavage (termed helix activity) [1,4,30], and was later verified that the linker destabilized the secondary structure of the collagen at the cleavage site allowing cleavage of the $\alpha$ chains [31].

With this in mind bacterium CHCs, of which bacterial collagenase is classified, are able to cleave collagen at numerous sites, all of which are distinct from the mammalian cleavage site but re-
quire a similar type of enzyme-substrate interaction. French et al. [32,33] suggested that the CHC hyper-reactive sites are dependent on site-specific localized conformations and amino acid sequences. Along the collagen molecule repeats of the Gly-Pro-Hyp amino acid sequence are believed to account for the rigid and tightly wound sections that may hinder bacterial collagenase accessibility, while areas void of this sequence were common among the classified hyper-reactive sites [32–34]. Although it is unclear whether or not triple helical activity is observed with CHC, the substrate-binding site S3a and S3b (mammalian exosite equivalent) and segmental structure S2 (mammalian linker domain equivalent) motif is conserved for both bacterial and mammalian collagenases [6]. Our results for bacterial collagenase and type I collagen suggest that elongation of the collagen molecule during stress relaxation and/or degradation is a result of any substrate binding site and the substrate catalytic domain, preventing the segmental structure from reaching the active domain (discussed above), (2) elicit a strain-induced conformational change in the structure of the collagen molecule (triple helices) creating a chemical-thermal instability at the hyper-reactive binding sites, or (3) elicit a strain-induced conformational change in the structure of the collagen molecule and rearrange the constituent components (e.g., minor collagens, proteoglycans, and other glycosaminoglycan containing proteins) to inhibit access to the hyper-reactive sites and thus retarding degradation.

Collagen molecules bind uniaxially via N to C-terminal connections. However, these layers of adjacent collagen molecules are then connected to subsequent layers located above and below by hydrogen N–H and O–H bonds [35,36]. Due to the self assembly nature of collagen, there appears to be a long range attraction, which prevents molecules from coming too far apart and which induces the self assembly, where hydrogen water bridges surround the molecules as they aggregate. Any substrate binding site and the substrate catalytic domain, preventing the segmental structure from reaching the active domain (discussed above), (2) elicit a strain-induced conformational change in the structure of the collagen molecule (triple helices) creating a chemical-thermal instability at the hyper-reactive binding sites, or (3) elicit a strain-induced conformational change in the structure of the collagen molecule and rearrange the constituent components (e.g., minor collagens, proteoglycans, and other glycosaminoglycan containing proteins) to inhibit access to the hyper-reactive sites and thus retarding degradation.

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are also shown that for the strain-independent decrease in the peak stress, this could explain some of the variability in the EMK response if not all of the collagen molecules within the fiber are elongated uniformly.

In our study the axial strains within the fiber at the end of the stress-relaxation phase \(\varepsilon_1(t_1)\) ranged from 1% to 10% (Fig. 6), which spans all three regions of the stress-strain response and is significantly greater than used in all previous studies [9–11]. Thus based on the axial strains at the end of the mechanical-relaxation phase, when the collagenase was introduced, the observed EMK response in our study incorporated all three structural mechanisms involved in fiber elongation (crimp, kinks, cross-links, and triple helix). As can be seen in Fig. 8(b) for \(T_E(\varepsilon)\) versus \(\varepsilon_1(t_1)\), the least significant inhibition of collagenase cleavage occurred in the toe region, where for \(\varepsilon_1(t_1)\leq3\%\) the mean \(T_E(\varepsilon)=2.50 \pm 0.89 \times 10^{-4} \text{ s}^{-1}\) (n = 8). After the toe region there is a significant transition to increased inhibition (decrease in \(T_E(\varepsilon)\)) in the heel region (\(\varepsilon_1(t_1)\) =3–5%), \(T_E(\varepsilon)=1.03 \pm 0.20 \times 10^{-4} \text{ s}^{-1}\), n = 8) and linear region (\(\varepsilon_1(t_1)=5–10\%\), \(T_E(\varepsilon)=1.13 \pm 0.67 \times 10^{-4} \text{ s}^{-1}\), n = 9), where both regions were not significantly different (\(p > 0.6\); \(\varepsilon_1(t_1)>3–10\%\), pooled mean \(T_E(\varepsilon)=1.08 \pm 0.53 \times 10^{-4} \text{ s}^{-1}\), n = 17).

To further analyze the nonlinear characteristic of the \(T_E(\varepsilon)\) versus \(\varepsilon_1(t_1)\) response, a four-parameter logistic function (i.e., a sigmoidal dose response) was fit to the data in Fig. 8(b), given by

\[
T_E(\varepsilon_l) = T_{Emax} + (T_{Emax} - T_{Emin})/(1 + 10^{(\varepsilon_l-o)^B})
\]

where \(T_{Emax} = T_E(0)\), \(T_{Emin} = T_E(\varepsilon_l)\), and the transition slope \(B \geq 1\). Shown in Fig. 8(b) is Eq. (4) fit to the data for \(B=0\) (no slope), yielding \(T_{Emax} = 2.44 \times 10^{-4} \text{ s}^{-1}\), \(T_{Emin} = 1.08 \times 10^{-4} \text{ s}^{-1}\), \(B = 1/\%\), and \(\varepsilon_1 = 3.4\%\), and for a variable slope, yielding \(T_{Emax} = 2.49 \times 10^{-4} \text{ s}^{-1}\), \(T_{Emin} = 1.08 \times 10^{-4} \text{ s}^{-1}\), \(B = 61.3\%\), and \(\varepsilon_1 = 2.9\%\). Note that \(T_{Emax}\) and \(T_{Emin}\) for both cases are equivalent to the means for \(T_E(\varepsilon_l)\) in the toe region and heel/linear regions, respectively, and the strain at the transition between \(T_{Emax}\) and \(T_{Emin}\), \(\varepsilon_1 = 2.9–3.4\%\), is similar to the transition strain between the toe and heel regions (\(3\%\)).

From our results we can speculate on one possible mechanism for the enzyme inhibition indicated by the discontinuity in the EMK function \(T_E\) indicated in Fig. 8, that is, we hypothesize a three-domain model. The above results, taken together with the rapid increase in the fiber stress in the toe region (Poisson’s ratio, Fig. 5), suggests that straightening the crimp in the collagen fiber is insufficient to inhibit enzyme cleavage of the collagen molecule. On the contrary, it suggests that a conformation change in the triple helix is necessary for inhibition. We hypothesize that this can occur by two concurrent mechanisms as the collagen fiber is mechanically loaded, (1) intermolecular triple-helix forces (causing tensile axial strain) as the macromolecular forces are transferred to the \(a\)-chains of the triple helix through the intermolecular kinks and cross-links between the triple helices, and (2) intramolecular triple-helix forces (causing compressive radial strain) as the fiber’s cross-sectional area (diameter) decreases generating increased intramolecular hydration forces. Our data suggest that both these mechanisms would begin in the heel region, after the collagen crimp is straightened, and transition rapidly as the collagen triple helix is deformed.

As stated earlier only \(50\%\) of the reduction in the EMK cleavage of the collagen fiber was attributed to known parameters of which the axial strain accounted for \(48\%\). While the remaining \(\sim50\%\) is currently unknown we presume that the inconsistency between the fiber and molecule axial strains may account for some of this discrepancy. Specifically if every collagen triple-helix molecule within the fiber was not stretched to the same applied strain, as would be expected when the fiber elongation was performed via grip-to-grip attachments, then molecules that were not fully strained would affect the accuracy of the strain-dependent rate of degradation. Those molecules that were not strained or strained to less than the assumed strain would be cleaved as if unloaded or at a different (faster) degradation rate, respectively. Expanding on this concept, if an axial strain is \(>3\%\) it is necessary to alter the collagen molecule’s cleavage/multiple hyper-reactive sites, that is its molecular conformation, and if triple-helix cleavage would be completely inhibited in molecules subjected to an axial strain \(>3\%\), as we hypothesized, then the results found in this study would reflect only those collagen molecules not strained to \(>3\%\). From this perspective, the reduced rate of degradation for strains \(\leq3\%\) could be a result of the time required for enzymes to attach to the exosites and to cleave the collagen molecule, while enzymes attaching to the exosites of those molecules that were strained to \(>3\%\) would not reach the catalytic domain (degradation inhibition).

Other factors in addition to axial strain that might influence the degradation rate of intact solid collagenous structures (fibers) include mass transport (diffusion) of the enzyme into the fibril, steric hindrance of the large enzyme molecules within the fibril, local osmotic and pH effects, and the relative concentrations and interactions of minor collagens (e.g., IX and XI) and glycoproteins (aggrecan, biglycan, and decorin) [14,45]. Enzyme exclusion from the intermolecular space might occur as the fiber is strained,
Acknowledgment

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References
