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Glycation cross-linking induced mechanical–enzymatic cleavage of microscale tendon fibers

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ABSTRACT

Recent molecular modeling data using collagen peptides predicted that mechanical force transmitted through intermolecular cross-links resulted in collagen triple helix unwinding. These simulations further predicted that this unwinding, referred to as triple helical microunfolding, occurred at forces well below canonical collagen damage mechanisms. Based in large part on these data, we hypothesized that mechanical loading of glycation cross-linked tendon microfibers would result in accelerated collagenolytic enzyme damage. This hypothesis is in stark contrast to reports in literature that indicated that individually mechanical loading or cross-linking each retards enzymatic degradation of collagen substrates. Using our Collagen Enzyme Mechano-Kinetic Automated Testing (CEMKAT) System we mechanically loaded collagen-rich tendon microfibers that had been chemically cross-linked with sugar and tested for degrading enzyme susceptibility. Our results indicated that cross-linked fibers were >5 times more resistant to enzymatic degradation while unloaded but became highly susceptible to enzyme cleavage when they were stretched by an applied mechanical deformation.

1. Introduction

Fibrillar collagens are structural proteins that assemble into a complex ordered structure of molecules cross-linked together to form an interconnected supramolecular fibril structure (Ottani et al., 2002). Collagens contribute to the mechanical properties of almost all tissues throughout the body including skin, tendon, ligament, bone, and cartilage (Bailey et al., 1998; Ottani et al., 2002).

Collagen is highly resistant to enzymatic breakdown, but is susceptible to a small number of specialized collagenolytic enzymes or collagenases. In part due to this resistance to enzymatic cleavage, collagen has a very slow turnover rate in many tissues of the body. The half-life for collagen has been reported on the order of decades in healthy tissues (Maroudas et al., 1992; Bank et al., 1999; Verzijl et al., 2000b). Due to the long protein half-life *in vivo*, collagen is one of the proteins that undergo spontaneous glycation and the formation of measurable amounts of Advanced Glycation End products (AGEs) during aging (Verzijl et al., 2000a, 2000b; Sell and Monnier, 2004; Choudhary et al., 2011).

Glycation, also called non-enzymatic glycosylation, is a spontaneous, non-enzymatic process in which a reducing sugar such as glucose or fructose reacts with a free amino group (e.g. lysine or arginine) to form a reactive Schiff base. The Schiff base then rearranges to form an Amadori product, which undergoes further reactions, collectively known as a Maillard reaction, to form AGEs (Bailey et al., 1998; Aronson, 2003). Of interest is that these reactions can result in stable covalent cross-links between two amine groups of amino acids such as lysine or arginine (Bailey et al., 1998; Verzijl et al., 2002; Sell and Monnier, 2004).

AGE accumulation in soft tissues is a function of tissue aging and accelerated by diabetes due to hyperglycemia (Bai et al., 1992; Reddy et al., 2002; Reddy, 2003; Freemont and Hoyland, 2007). AGE cross-linking results in changes in the mechanical properties of soft tissues, which include increased Young's modulus, maximum failure load and toughness, while in mineralized tissue there are minimal changes in these properties after glycation (Reddy et al., 2002; Reddy, 2003). In addition AGE cross-linking has been implicated in a variety of pathological aging-related changes, including vascular (Aronson, 2003) and articular cartilage stiffening (Verzijl et al., 2000a; Chen et al., 2002) which might contribute to arteriosclerosis and osteoarthritis, respectively.

Computational molecular modeling was previously performed using steered molecular dynamics to simulate mechanical loading of a collagen covalent cross-link (Bourne and Torzilli, 2011). These loading conditions approximated mechanical force transmitted through covalent intermolecular cross-links, such as those caused by AGEs. Computational results predicted that force transmitted via cross-links would result in local disruption and microunfolding of the collagen triple helix at approximately 350 pN (minor microunfolding) and 900 pN (major microunfolding) (Bourne and Torzilli, 2011), and well below previously described collagen failure mechanisms (Buehler, 2006; Tang et al., 2010; Bourne and Torzilli, 2011).

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Due to the hierarchical structure of collagen within the extracellular matrix of tissues, tensile forces applied at the macro and micro scales are transmitted via intermolecular cross-links (Puxkandl et al., 2002). At the molecular level (nanoscale) this results in forces being transmitted across the amino acid side chains that form bridging intramolecular cross-links (Tang et al., 2010). Recent molecular modeling results now indicate that the transmission of tensile load via these cross-links can cause disruption and microunfolding of the triple helix and that these microunfolding events occur at force levels (<1000 pN) below previously reported damage mechanisms (Bourne and Torzilli, 2011).

Based on our molecular modeling results we hypothesized that mechanical forces on cross-linked collagen substrates would accelerate enzyme degradation. We tested this by applying tensile deformation to glycation cross-linked microscale tendon fibers (fascicles of ~300 μm diameter) and quantified the rate of collagenase enzymatic degradation.

2. Results

2.1. Relative fluorescence measurements

Ribose incorporation into fibers over time was assessed by fluorescence of solubilized fibers following 0 to 10 days of incubation (Verzijl et al., 2002). Maximum fluorescence was estimated from measurements of fibers incubated in ribose for 5 weeks, and data were well described by a 2 parameter exponential rise-to-max equation ($R^2 = 0.99$) (Fig. 1). 3-day and 7-day ribose incubation results in approximately 20% and 40% fluorescence relative to maximum, respectively.

2.2. Unloaded fiber enzyme susceptibility test

Tendon fascicles were tested for susceptibility to enzymatic degradation in an unloaded state by observing fiber digestion to dissolution over a ~24 hour period. Comparable diameter tendon microfibers were obtained from the same animal and incubated unloaded in a 1% by weight bacterial collagenase solution dissolved in Dulbecco's PBS with calcium and magnesium. In all 3 tests, the non-glycated fiber degraded and dissolved fastest, followed by the 3-day glycated fiber. The 7-day glycated fiber showed no visible degradation after prolonged enzyme exposure.

Representative results from one experiment are shown as Fig. 2. In these results the 0-day native non-glycated fiber dissolved after approximately 5 hours of exposure, while the 3-day fiber failed within 20 hours of enzyme exposure. At the conclusion of the test, the 7-day fiber was intact with no visible degradation even after more than 25 hours of enzyme exposure, and had persisted approximately five times longer in the enzyme solution than was needed to completely dissolve a native non-glycated fiber.

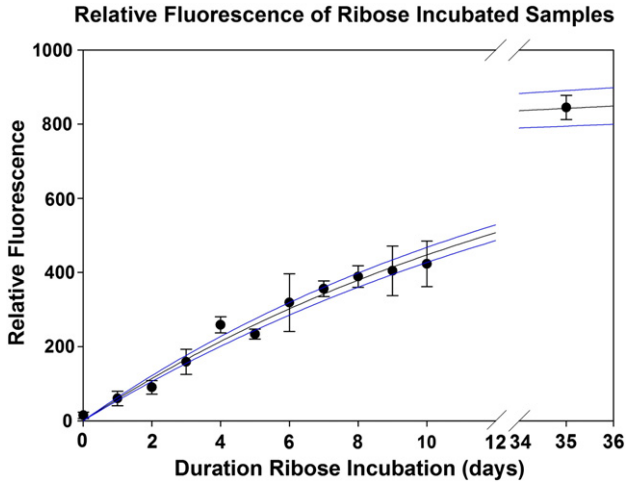

Fig. 1. Relative fluorescence of ribose incubated tail tendon samples. Tail tendon microscale fibers were incubated in ribose for 0–35 days and relative fluorescence was measured daily up to day 10, and a final maximum fluorescence was approximated with a measurement at day 35 (5 weeks). Data was fit with an exponential rise-to-max equation, and shown with 95% confidence bands.

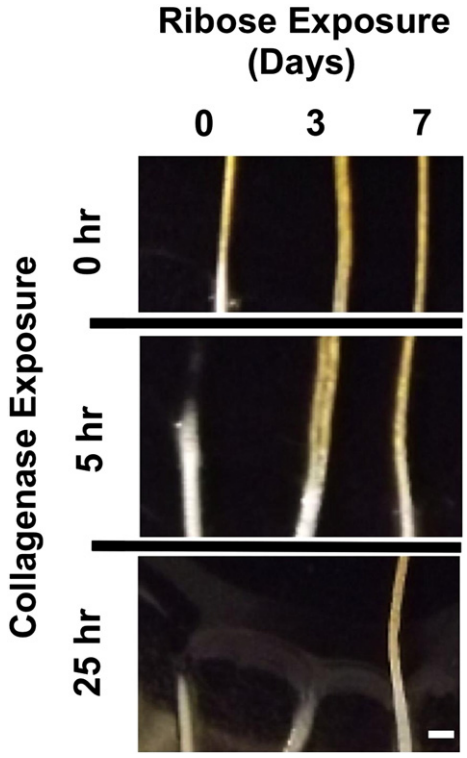

Fig. 2. Unloaded fiber enzyme susceptibility. Fibers of comparable size from the same tail were treated with 0.2 M Ribose for 0, 3 or 7 days and then exposed to a 1% collagenase solution at room temperature. Representative images at the air–collagenase interface of the fibers were digitally recorded at the 0 hours (start) and after 5 and 25 hours of collagenase exposure; the scale bar indicates 1 mm. The native fiber (0-day, no cross-linking) was completely degraded after 5 hours of exposure to collagenase, while the cross-linked fibers were still intact. Between 5 and 25 hours of collagenase exposure the 3-day cross-linked fiber was completely degraded, while the 7-day cross-linked fiber was intact with no visible deterioration after 25 hours of exposure to collagenase.

2.3. Tensile strain at equilibrium

Due to the viscoelastic nature of tendon, an applied peak strain resulted in a peak stress ($t = 0$) that underwent subsequent time-dependent decrease in stress (mechanical stress relaxation) until reaching an equilibrium state at $t> > 0$ (Wyatt et al., 2009). Previous mechanical relaxation tests using native non-glycated rat tail tendon fibers of similar dimension had shown an unusual increase in the tensile strain during stress relaxation, with an equilibrium strain > peak strain for native tail tendon microfibers (Wyatt et al., 2009). Therefore we were interested in comparing the mechanical relaxation strain response between native and cross-linked tissues.

A scatter plot of the peak and equilibrium strains for the native and cross-linked fibers is shown in Fig. 3. A regression analysis was performed for the native and 7-day cross-linked fibers using a linear (straight-line) fit to the equilibrium vs. peak strain data (SigmaPlot 10, Systat Software, Inc., Chicago, IL). The slope of the regression line for the native fibers was 1.45 ± 0.18 (slope ± standard error, r = 0.865, $n = 23$) and was different from unity ($p = 0.02$). The slope of 7-day cross-linked fibers was 0.92 ± 0.09 ($r = 0.904$, $n = 12$), and was not statistically different from unity (i.e., peak = equilibrium, $p = 0.39$). However, the regression slope of the 7-day cross-linked fibers was