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Abstract

Throughout this spring, I worked in Dr. Spencer Lake's Musculoskeletal Soft Tissue Laboratory to begin testing bovine tendon to study the effects of elastin on the mechanical properties of tendon. To study the effects of elastin, the mechanical properties of tendon were compared with and without elastin. This process was completed by utilizing elastase, which enzymatically degrades elastin and renders it non-functional. Once the elastin is degraded, the mechanical properties of the tendon without elastin was tested using an Instron. More specifically, I utilized a method that was created in my previous semester's work, which consisted of a repeatable testing method as well as a minimal change in mechanical properties of the tendon. Overall, 30 tendons were tested which included the long digital extensor tendon (LDET) and superficial digital flexor tendon (SDFT). It was found the LDET seemed to have a more aggressive relaxation and other mechanical properties changed, while the SDFT data showed inconclusive results. Although the results might be initially frustrating, we are working to better analyze to look at different parameters and creating a more consistent protocol.

Introduction

Tendons act as one of the most important parts of a human's musculoskeletal system, as they are the connectors between bone and muscle. The compositional and organizational properties of tendons determine their ability to provide adequate mechanical function. While the collagen matrix is known to provide high tensile strength, relatively little is known about how the non-collagenous components of a tendon, and more specifically elastic fibers, influence its mechanical properties [1-3]. Elastic fibers are composed of elastin deposited onto a microfibrillar scaffold and exist predominantly in the interfascicular matrix (IFM), which is located between

collagen fascicles and may link them together [1, 4]. A schematic of the tendon structure can be seen in fig. 1 below.
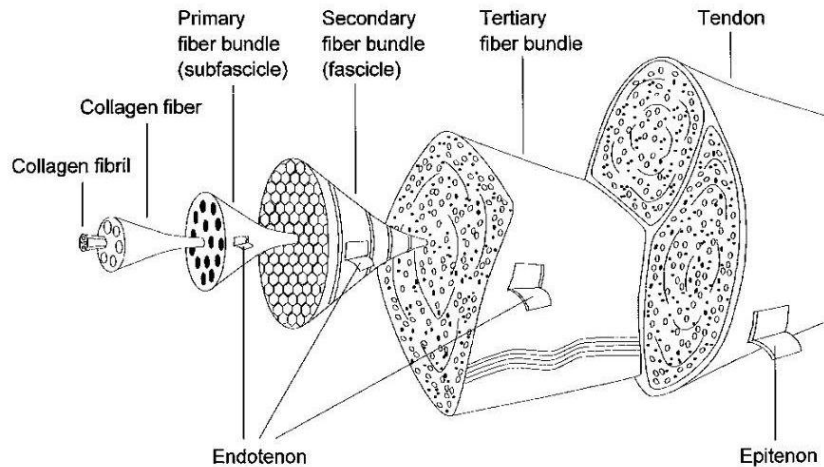

Figure 1: Composition of a tendon with more than one fascicle [5].

Elastic fibers have been suggested to provide important mechanical roles in tendon; for example, they may allow a tendon to recoil after being loaded. Clinically, understanding the effect of elastic fibers on the mechanical properties of tendon could facilitate future research on diseases such as Marfan Syndrome and Williams Syndrome, both of which stem from mutations to elastic fiber-related genes [5]. More in-depth understanding would allow clinicians to help patients with elastic fiber deficiencies in a more informed and effective manner.

However, there is little experimental evidence on how elastic fibers affect the tensile mechanics of tendon. Previous research using genetically modified mouse models has suggested that elastin has an effect on a tendon's mechanical properties, but the effect was less than initially anticipated. Because of the vast difference in tendon size between mice and larger animals, the distribution of the elastin within the tendon and IFM may also be quite different [3]. This is mostly due to the fact that larger tendons are made up of many fascicles while mouse tendons are similar to a single fascicle. This means the larger tendons will have more elastin, and

therefore will have a larger potential for mechanical properties to change. . Therefore, comparing the contribution of elastin in larger tendons with a prevalent IFM may better elucidate how elastin functions under tensile load in tendon.

In order to isolate the mechanical effect of elastic fibers, comparisons can be made by testing tendons before and after enzymatic elastin degradation treatment under the same loading conditions. Previous studies have used elastase to determine the effects of elastin digestion on the quasi-static mechanical properties of tendon and ligament; however, no studies to date have investigated changes to viscoelastic properties in tension with elastase treatment [3,7]. In this study, bovine long digital extensor tendon (LDET) and superficial digital flexor tendon (SDFT), which are functionally analogous to the human tibialis anterior tendon and the Achilles tendon respectively, were used as representative tendons for large species. A figure of their anatomy inside a cow hoof can be shown below in fig. 2 where the blue shows the LDET and the red shows the SDFT.
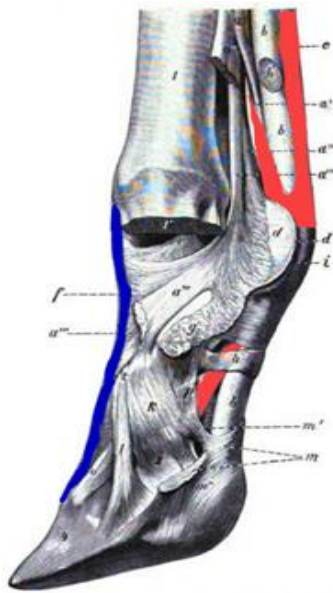

Figure 2: A picture of the anatomy of a bovine hoof with blue highlights showing the LDET and red showing the SDFT.

These two tendons were chosen because of their function in the cow and our predicted role of elastin in functionally distinct tendons. The LDET is a positional tendon while the SDFT is an energy storing tendon, which means the SDFT would be subject to much higher loads and strains. Due to the function of elastin, it is predicted that SDFTs would utilize elastin more, and therefore show a larger change in mechanical properties when elastin is degraded. This would give us a more comprehensive analysis of the function of elastin in tendon. Overall, the purpose of this study was to utilize mechanical testing before and after elastase incubation to determine the role of elastin on the viscoelastic mechanical properties of tendon.

Methods

Bovine LDETs and SDFTs were acquired from a local abattoir and frozen at -20° C until use. Before testing, samples were thawed at room temperature for 30-60 minutes. Cross-sectional area of each tendon was measured using a non-contact laser scanning device. Samples were loaded in custom clamps gripped between pieces of sandpaper to ensure minimal slipping occurred. Clamps were placed in an Instron 5542 Testing Machine, a 0.5 MPa preload was applied, and the gauge length was then measured using calipers. Once the gauge length and preload were determined, samples were preconditioned using ten cycles to 6% strain and subsequently tested in stress relaxation for ten minutes at 6% strain followed by triangular loading waveforms to 2, 4, and 6% strain to assess hysteresis (Fig. 3). Following testing, the clamps were removed and incubated in either PBS (controls) or 3.5-4 units/mL elastase solution for 24 hours while maintaining a constant gauge length. Both control and test solutions contained 0.1 mg/mL soybean trypsin inhibitor to prevent digestion of collagen. Following incubation, the clamps were reloaded into the Instron and the tendon was loaded using the same

protocol as the pre-incubation test. Changes in mechanical parameters after PBS or elastase
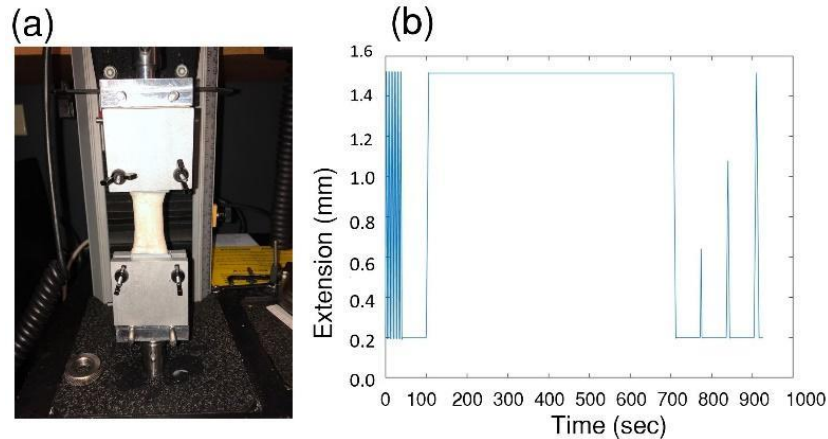
incubation are reported.

Figure 3: (a) A LDET sample loaded in the custom-made clamps. Samples were loaded in tension in the Instron 5542. (b) A time versus displacement graph for the testing protocol. The protocol for the pre and post incubation tensile testing protocol included 10 cycles of preconditioning at 6% strain, followed by a 10-minute stress relaxation at 6% strain and hysteresis curves to 2, 4 and 6% strain.

Between experiments, the enzyme solution was filtered using a hollow fiber filter with a

maximum pore size of 0.1 μm to remove released tendon fragments and prevent bacterial growth

in the solution. For elastase-treated samples, the activity of the elastase solution was measured

daily [8] and additional elastase was added as necessary to ensure constant activity across all

samples. This entire process was repeated for 20 total tendons used in this analysis. The

breakdown of these groups includes LDET control (n=5), LDET elastase incubated (n=5), SDFT

control (n=5), and SDFT elastase incubated (n=5) samples.

Results

For all of the following results, the data shows the change in the given parameter between

pre and post incubation, whether it be PBS or elastase. This means that change in mechanical

properties is analyzed rather than just the magnitude of the parameter. It was found that elastase

treatment altered the viscoelastic and quasi-static mechanical properties of LDETs compared to
PBS treated controls, and SDFT data was inconclusive. Overall, the SDFT data was very
consistent, but also did not elucidate the effects of elastin on the mechanical properties of tendon
as expected. The first set of results compared the transition stress and strain, as well as the linear
modulus for the control and elastase samples of each tendon type.. Note that transition stress and
strain is defined as the value of the stress and strain when the sample transitions from the toe
region to the linear region of the loading curve. A figure of this concept can be seen below in fig.
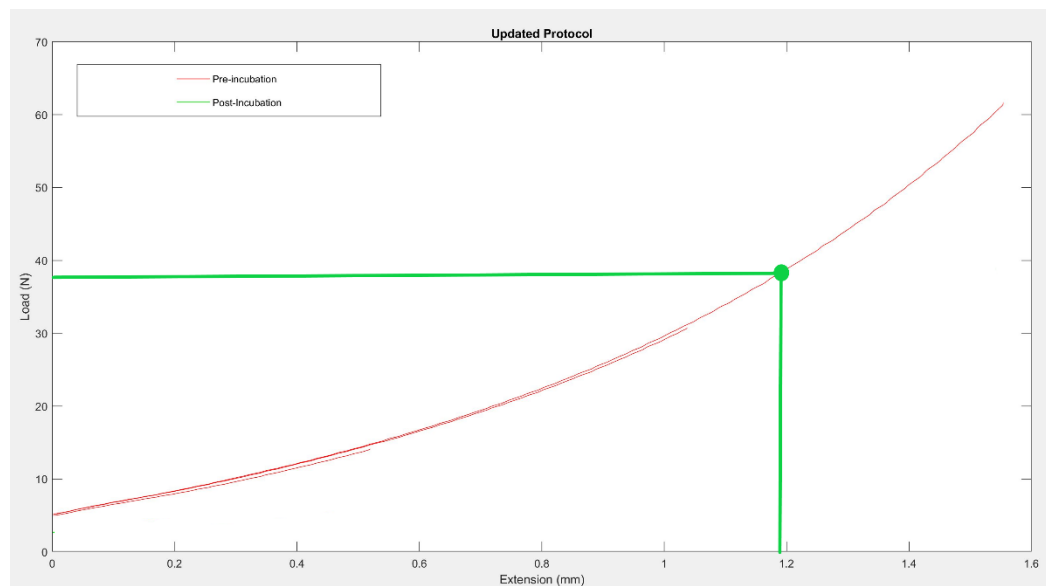4.

**Figure 4: The green dot shows a sample transition point on a load-extension curve
and the green lines show the corresponding load and extension**

Note that in fig. 4, the stress is analogous to the stress and the extension is the same as the
strain. Now that the transition point is properly defined, the data which compared LDETs and
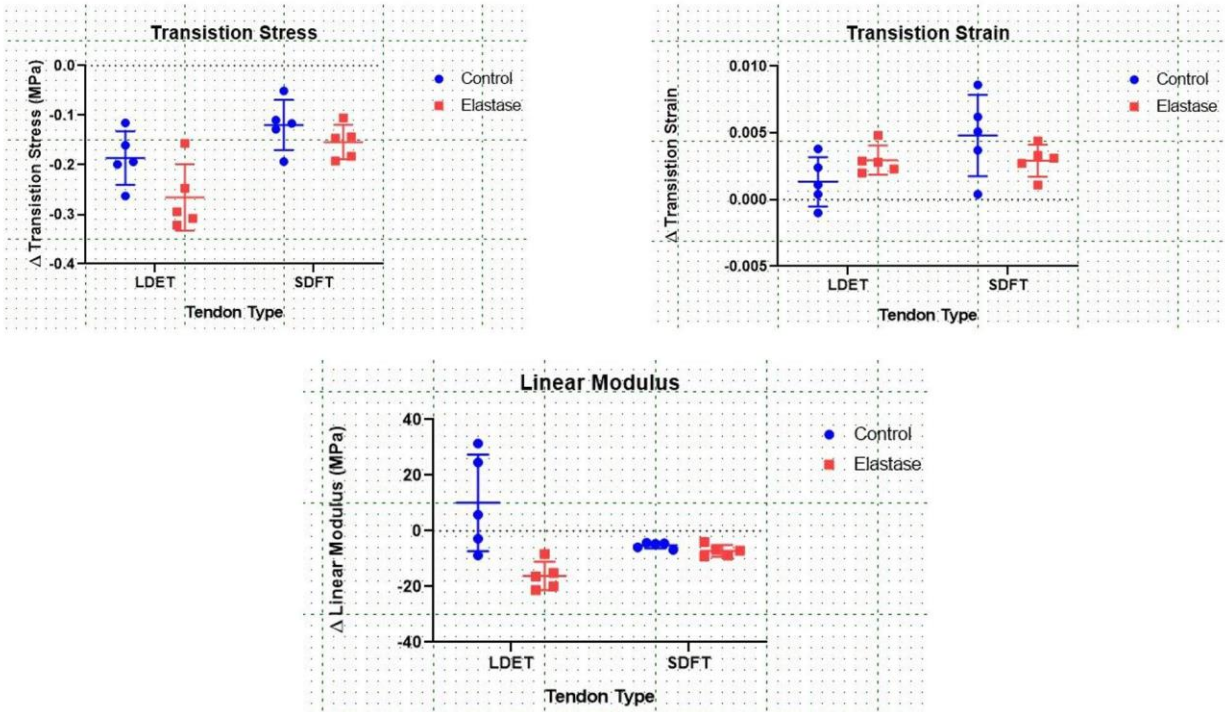SDFTs can be seen below in fig. 5.

Figure 5: (top left) The change of transition stress before and after incubation for each tendon type, (top right) The change of transition strain before and after incubation for each tendon type, (bottom) The change of linear modulus before and after incubation for each tendon type

As shown before, it is clear the LDET has changes in all three parameters, while the SDFT has slight changes in the transition stress and strain, while the linear modulus is remarkably consistent. It is also interesting to note that for transition strain, the LDET showed an increase in the difference between pre and post incubation tests, while the SDFT showed a decrease. Overall, the LDET showed expected results, but were fairly variable, while the SDFT showed incredibly consistent results, but did not show the expected results. Next the stress relaxation between the LDET and SDFT samples will be analyzed.

The equilibrium stress is defined as the final magnitude of the stress during the stress relaxation period, the peak stress is the highest stress achieved in the stress relaxation period, the stress relaxation time is the time it takes to reach 50% of the samples maximum load, and the

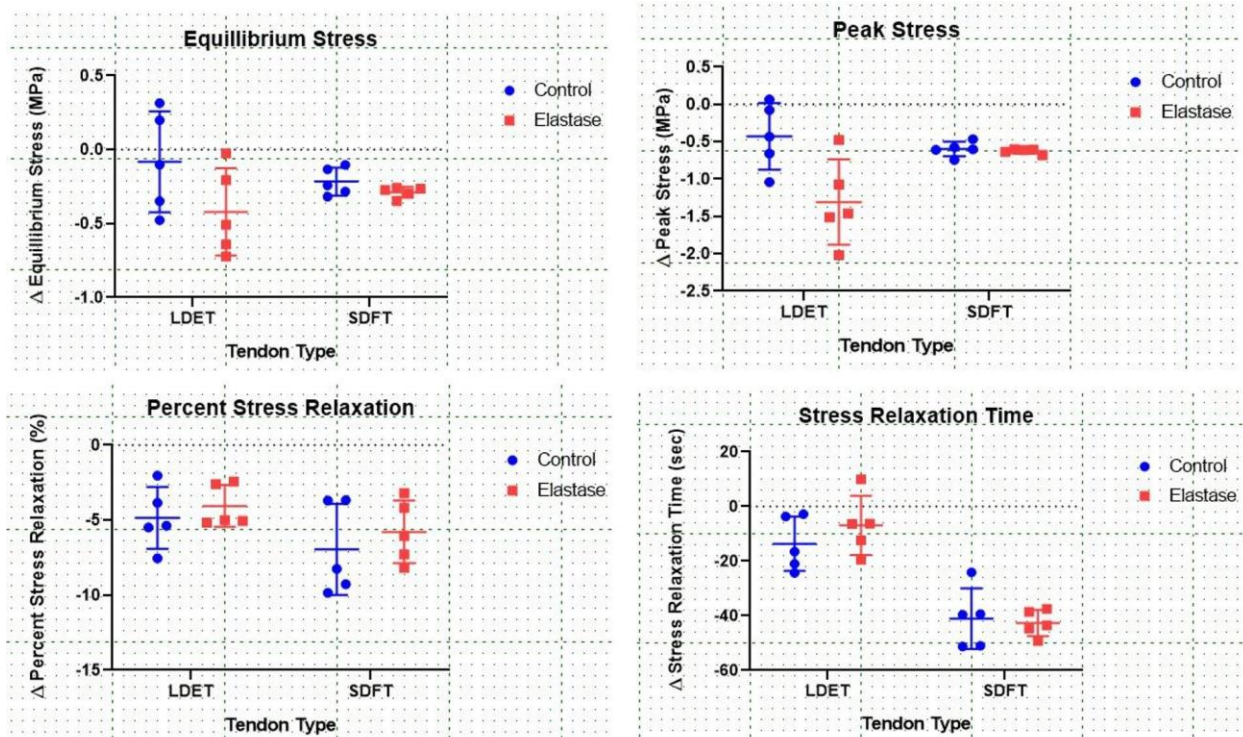percent stress relaxation is a metric used to measure the ratio between the equilibrium and peak stress.

Figure 6: (top left) The change in equilibrium stress before and after incubation for each tendon type, (top right) The change in peak stress before and after incubation for each tendon type, (bottom left) The change in percent relaxation before and after incubation for each tendon type, (bottom right) The change in stress relaxation time before and after incubation for each tendon type.

The SDFT and LDET show similar behavior for all of these parameters, which means the elastase treatment was shown to decrease stresses and increase stress relaxation time and percentage. In general, the LDET was more variable than the SDFT, but was much closer to our expected result, while the SDFT was consistent, but did not show a significant change between control samples and elastase incubation. Our next comparison will be based around hysteresis at multiple clamp strains. Hysteresis is defined as the amount of energy loss between the loading

and unloading curves of a stress-strain diagram. The hysteresis of the sample was analyzed at 2, 4, and 6% strain and the results can be seen in fig. 7 below.
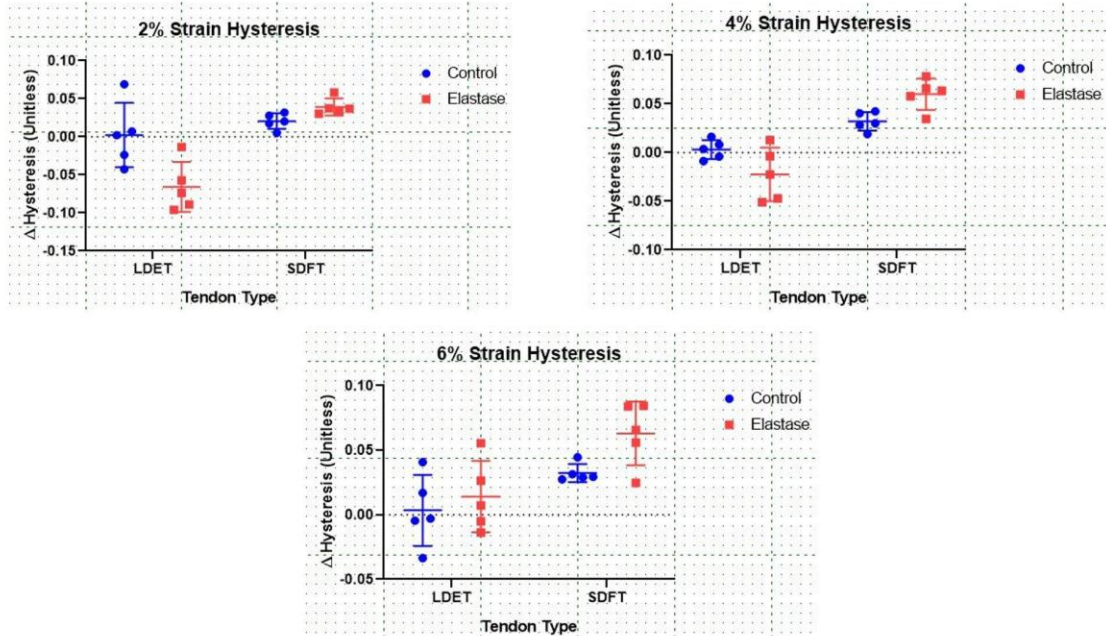

Figure 7: (top left) The change in hysteresis at 2% clamp strain before and after incubation for each tendon type, (top right) The change in hysteresis at 4% clamp strain before and after incubation for each tendon type, (bottom) The change in hysteresis at 6% clamp strain before and after incubation for each tendon type.

The hysteresis results are fairly variable for each specimen at each strain percentage. The SDFT consistently showed an increase in hysteresis, as expected, at each strain percentage. However the LDET showed a decrease in hysteresis at 2% and 4% clamp strain, but an increase at 6% clamp strain. It was again found that the SDFT had more consistent results than the LDET.

Discussion

Our preliminary results show that digestion of elastin significantly altered the viscoelastic properties of tendon in some cases, despite making up only 1-2% of tendon weight [4]. With that being said, the SDFT had several parameters that were unchanged after elastase incubation. These changes were likely due to the fact elastin is a protein in elastic fiber in tendon. However,

the LDET showed more consistent changes after elastase digestion, even though it was a positional tendon. Although it makes up a small percentage of a tendon's dry weight, its function is unique and quite different than collagen, the major component of tendon [4]. When the elastin is digested the tendon's structural mechanisms relax, hence the change in viscoelastic properties. Elastin-dependent results are also intriguing when considering the function of the LDET in bovine hooves.

Like the tibialis anterior tendon in humans, the LDET is a positional tendon, meaning the tendon is not strained with the same magnitude or as frequently as a more energy-storing tendon [8]. This difference in function has previously been shown to relate to differences in mechanical properties and elastin content in functionally distinct tendons [8]. However, significant changes were still observed after elastase incubation, which suggests that elastin plays a significant role in the positional tendon's function. Unexpectedly, the energy storing SDFT did not show similar results, and was often unchanged by elastase incubation. Unfortunately, these results that we were not expecting, and we believe it is not due to the SDFT being unaffected by elastin digestion, but instead the SDFT tests were compromised due to a large loss in load after incubation. So overall, the LDET showed results that were consistent with what was expected, but the individual sample results were quite variable, while the SDFT had consistent results, but the data pre and post incubation was similar.

In the SDFT tests, there was a consistent drop in the load for all of the tests, even in the control samples. This is likely due to water loss in the region of tendon compressed in the clamps, thereby decreasing the volume of the sample and effectively loosening the clamp. Therefore, the loss of load overshadows any change in the mechanical properties due to elastase alone. This is corroborated by the LDET not having as dramatic of an effect due to clamp

loosening because it is a much smaller tendon. Another larger issue with the test is that the clamp

strain was not high enough to consistently enter into the linear region. As shown below in fig. 8,

it seems as though the LDET has entered the linear region at 6% clamp strain while the SDFT

could still increase its load before entering the linear region. This means that the clamp strain

was too low to find parameters such as linear modulus. So, when coupled with the fact that the

SDFT has major load drops, it makes sense that the tests were inconclusive.
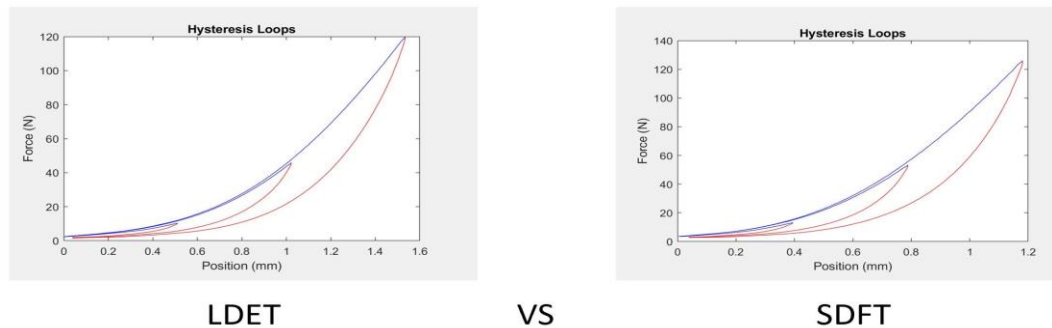

**Figure 8: A comparison of the LDET and SDFT hysteresis curves at 2, 4, and 6%
clamp strain.**

These results are consistent with other publications [7]. It was found that tendons usually

do not start showing a major difference in elastase treatment until after 6% strain. Therefore,

when considering the tendon swelling, the large loss in loads, and the small clamp strain used in

the testing, as well as the geometry of the LDET and SDFT, it makes sense that the LDET

showed somewhat reasonable results while the SDFT showed mostly inconclusive results.

Although this portion of our data collection failed, we are confident that we can modify the test

protocol to find more consistent results for bovine SDFT and LDET.

Conclusion and Future Work

Overall, this study was not wholly successful, but we still learned a fair amount about

elastin's role in the viscoelastic mechanical properties of tendon. As stated previously, it was

found that the LDET data aligns with our predictions, but is variable, while the SDFT shows little difference between control and elastase groups, but is remarkably consistent. This is due to the SDFT size, clamp loosening, and low clamp strain. So although the data isn't ideal, we do know how to change our experiment to be able to create more conclusive data in the future. To start, we will be redoing our tests with a higher clamp strain. The low clamp strain of 6% meant that the linear region was not reached in many SDFT tests. So by increasing the clamp strain to 8% or even 10%, we are confident the linear region will be reached, resulting in higher and more consistent loads between tests. On top of this, we will also use beads on the tendon to measure the tissue strain in the tendon during the test rather than relying solely on clamp strain. Finally, we will also begin incubating the tendons before the testing and tighten the clamps more to ensure the effects of swelling are negligible in the tendon. These changes to the experiment will provide much more useful data points in the future. So although this semester's work didn't yield useable data for the SDFT and potentially the LDET, we did learn exactly what is needed to create the best method possible for testing elastin's effects on the viscoelastic mechanical properties of positional and energy storing tendons.

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