Relationships between the tensile strength and diameter of collagen fibrils isolated from mouse tail tendons

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Abstract
The purpose of the present study is to determine the tensile properties of the single collagen fibrils with various diameters using our original tensile test method. The fibrils were directly isolated from mouse tail tendons. The both ends of the fibril were wound onto the tips of microneedles several times using micromanipulators. The fibril and tips were immersed in physiological saline solution. Then, the fibril was stretched to failure by moving the microneedle. The tensile properties of the 26 fibrils with various diameters ranging from 140 to 490 nm were determined. The stress-strain curves of these fibrils were almost linear or upward convex at the large strain region. These fibrils showed the tensile strength of 47-310 MPa, the strain at failure of 7.3-81 %, and the tangent modulus of 160-1600 MPa. The tensile strength and tangent modulus decreased significantly with increasing the diameter. On the other hand, the strain at failure increased significantly with increasing the diameter.

Key words: Mechanical properties, Tensile test, Tensile strength, Collagen, Fibril, Tendon, Diameter

1. Introduction

Tendons have such hierarchical structures as collagen fascicles, fibers, fibrils, subfibrils, microfibrils, and molecules (Kastelic et al., 1978, Ottani et al., 2002, Silver et al., 2003). The smallest component is a molecule (~1.5 nm diameter and ~300 nm length), and five molecules are aggregated into a microfibril (~3.5 nm diameter). Microfibrils are packed into a tetragonal lattice to form a subfibril (10-20 nm diameter). Subfibrils are bundled to build a fibril (50-500 nm diameter). The fibril with large diameter is composed of more subfibrils than that with small diameter. A bundle of fibrils is called a fiber (~1 μm diameter). A fascicle (80-320 μm diameter) consists of the fibrils with various diameters embedded in proteoglycans. The distribution of the fibril diameters is changed by remodeling and aging (Tsuchida et al. 1997, Lavagnino et al. 2005, Derwin and Soslowsky 1999). The basic knowledge of their structures and mechanical properties is essential to the biomechanics of tendons.

Many studies have been performed on the mechanical properties of tendons, their fascicles, and their fibers (for example, Yamamoto et al., 1992, Yamamoto et al., 1999a, Miyazaki and Hayashi, 1999). At the fibrillar level, direct mechanical measurements have only recently become possible by atomic force microscopy (AFM) and microelectromechanical systems (MEMS). The mechanical properties of single collagen fibrils have been measured using AFM-based tensile (Svensson et al., 2010, 2013, van der Rijt et al., 2006, Yang et al., 2012), nanoindentation (Grant et al., 2008, 2009, Heim et al., 2006, Wenger et al., 2007), and bending (Heim et al., 2007, Yang et al., 2007, 2008) tests, and MEMS-based tensile (Eppell et al., 2006, Shen et al., 2008, 2010) tests.

The primary role of tendons is the transmission of contractile forces from muscle to bone. The large tensile forces are directly applied to the tendons (for example, Mow et al. 1990, Nigg and Herzog, 1994, Yamamoto and Ota 2009). Therefore, in many previous studies for the mechanical properties of tendons and their fascicles, tensile tests have been performed and the stress at failure (tensile strength) has been determined (for example, Yamamoto et al., 1993, Yamamoto et al., 1999b). On the study for the fibrils, it is important to determine their tensile strength by tensile testing. However, there were only a few investigations for the tensile strength of fibrils (Svensson et al., 2013, Yang et al.,...
2012). To our knowledge, there have been no studies on the relationships between the tensile strength and diameter of fibrils.

The purpose of the present study is to determine the tensile stress-strain properties of the single collagen fibrils with various diameters. Using our original tensile test method (Yamamoto and Toda, 2013), the collagen fibrils directly isolated from mouse tail tendons were stretched to failure, and their tensile strength and strain at failure were determined.

2. Materials and methods

2.1 Isolation of fibrils

A tail of a male DD-y mouse age 4 weeks weighing 23.0 g was wrapped in gauze moistened with physiological saline solution, covered with thin plastic film, and stored at −4°C. Tissue collection was approved by the animal care committee of Ritsumeikan University. Before tensile testing, the tail was thawed at room temperature. Fascicles with the diameter of ~100 μm were resected from the tail tendons with a surgical knife and split along the longitudinal axis with two pairs of forceps. These fascicles were immersed in distilled water in a test tube and stirred for 1 hour with a test tube mixer to be loosened into a cottony state. A small amount of the cottony fibrils was picked up and spread on a glass slide siliconized with Sigmacote (Sigma-Aldrich, USA).

2.2 Tensile test

Two micromanipulators (MM-89, MMO-202N, Narishige, Japan) were mounted on an inverted dark-field microscope (IX71, Olympus, Japan) (Fig. 1). For gripping a single fibril on the glass slide, two microneedles were fabricated of a glass rod (1 mm diameter; G-1000, Narishige) using a micropipette puller (PC-10, Narishige). Their tips were tapered down to the diameter of about 2 μm. These microneedles were connected to the micromanipulators.

![Diagram of tensile test setup](Fig. 1 Schematic diagram of tensile tester (Yamamoto and Toda, 2013). Two micromanipulators were mounted on an inverted dark-field microscope. For gripping a single fibril on the glass slide, two microneedles were connected to the micromanipulators. The microscopic images were recorded on a DVD recorder and analyzed using an image analyzer.)
One end of a single fibril was picked up from the glass slide with the tip of one microneedle. The tip of the other microneedle was attached to the other end of the fibril (Fig. 2(a)). The end of the fibril was wound onto the tip of the microneedle several times. Care was taken to overlap the fibril wound at the next time on the fibril wound at the first time. And also, the other end of the fibril was wound onto the tip of the other microneedle. The fibril and tips were immersed in physiological saline solution for 3 minutes. The initial length of the fibril ($L_0$) was measured (Fig. 2(b)). Then, the fibril was stretched to failure by moving the microneedle (Fig. 2(c)). During tensile testing, the fibril was firmly attached to the tips of the microneedles, and broken between the tips with no slippage observed. The microscopic images were recorded on a DVD recorder. The deflection of the microneedle ($\delta$) and the length of the fibril ($L$) were determined using an image analyzer (XL-20, Olympus).

Fig. 2 Schematic diagram of the procedure of tensile test. (a) One end of a single fibril was picked up from the glass slide with the tip of one microneedle. The tip of the other microneedle was attached to the other end of the fibril. (b) The end of the fibril was wound to overlap each other onto the tip of the microneedle several times. The other end of the fibril was also wound onto the tip of the other microneedle. The fibril and tips were immersed in physiological saline solution for 3 minutes. The initial length of the fibril ($L_0$) was measured. (c) The fibril was stretched to failure by moving the microneedle. During tensile testing, the fibril was firmly attached to the tips of the microneedles, and broken between the tips with no slippage observed. The deflection of the microneedle ($\delta$) and the length of the fibril ($L$) were determined using an image analyzer.
2.3 Calibration of the spring constant of microneedles

The load applied to the fibril \( F \) was determined from the deflection of the microneedle. After the tensile test, the tip of the microneedle was pressed on the weighing pan of an ultra-microbalance (UMX2, Mettler-Toledo, Switzerland) using a micromanipulator. The deflection of the microneedle was measured using the image analyzer and the applied load was determined from the output of the ultra-microbalance. The load-deflection relation was linear, and the slope of the line (the spring constant of the microneedle) was 0.46-0.80 N/m.

2.4 Measurement of the cross-sectional area of fibrils

After the tensile test, the failed fibril hanging down from the microneedle was attached on the microneedle. The microneedle with the failed fibril was observed with a scanning electron microscope (SEM) (Fig. 3). The diameter of the fibril \( d \) was measured. The cross-sectional area was determined from the diameter, assuming that the cross section was circular. The average of cross-sectional areas at the ten positions was calculated as the representative of the cross-sectional area of the fibril \( A \).

2.5 Calculation of tensile properties

Stress \( \sigma \) was calculated from the applied load and the cross-sectional area of the fibril, as given by \( \sigma = F / A \). Strain \( \varepsilon \) was determined from the deformation of the fibril, as given by \( \varepsilon = (L - L_0) / L_0 \). From these data, the stress-strain relation was obtained. Tensile strength \( \sigma_B \) and strain at failure \( \varepsilon_B \) were defined as the stress and strain at the failure point of the fibril, respectively. Tangent modulus \( E_T \) was defined as the slope of the linear portion of the stress-strain curve.

2.6 Statistical analysis

A simple regression analysis was used to evaluate statistical correlations between the tensile properties and diameter. The significance level for these analyses was set at \( p = 0.05 \).

Fig. 3 Typical SEM image of a collagen fibril after tensile testing. The diameter of the fibril \( d \) was measured. The cross-sectional area was determined from the diameter, assuming that the cross section was circular. The average of cross-sectional areas at the ten positions was calculated as the representative of the cross-sectional area of the fibril \( A \).
3. Results

The tensile properties of the 26 fibrils with various diameters ranging from 140 to 490 nm were determined. The initial length of these fibrils was 19-64 μm. The typical stress-strain curves of these fibrils were almost linear or upward convex at the large strain region (Fig. 4). On the all stress-strain curves, an initial toe region was not observed. The tensile strength and tangent modulus decreased with increasing the diameter. On the other hand, the strain at failure increased with increasing the diameter.

![Stress-strain curves](image)

Fig. 4 Typical stress-strain curves of the fibrils. These curves were almost linear or upward convex at the large strain region. On the all stress-strain curves, an initial toe region was not observed. The tensile strength and tangent modulus decreased with increasing the diameter. The strain at failure increased with increasing the diameter.

The correlations between the tensile properties and diameter of the 26 fibrils are summarized in Fig. 5. The tensile strength and tangent modulus had negative correlations with the diameter. These values decreased significantly with increasing the diameter (Fig. 5(a), (c)). On the other hand, the strain at failure had a positive correlation with the diameter. This value increased significantly with increasing the diameter (Fig. 5(b)). The absolute values of the correlation coefficients of the tensile strength and tangent modulus were larger than that of the strain at failure.
Fig. 5 Correlations between the tensile properties and diameter of the 26 fibrils. (a) The tensile strength decreased significantly with increasing the diameter. (b) The strain at failure increased significantly with increasing the diameter. (c) The tangent modulus decreased significantly with increasing the diameter.
4. Discussion

The present results demonstrated that the tensile strength and tangent modulus decreased significantly and the strain at failure increased significantly with increasing the diameter. Fibrils are composed of subfibrils and the subfibrils are arranged parallelly in the longitudinal direction. Within larger-diameter fibrils, the orientation of subfibrils is disturbed, and the longitudinally aligned subfibrils decrease. Therefore, the subfibrils are not evenly loaded, but the stress on some subfibrils may exceed their failure strength before the overall mean stress does not. Such sequential breakage of the subfibrils would lead to the decrease in tensile strength and the increase in strain at failure.

Wong et al. (2008) performed the tensile test of the fibers (the diameter of 200-900 nm) electrospun from biodegradable polymer (polycaprolactone), and reported that their tensile strength and tangent modulus decreased with increasing their diameter. This result is similar to that of the present study. In the same work by Wong et al. (2008), the disturbance of the molecular orientation in the spun fibers was found to increase with increasing their diameter using wide angle X-ray diffraction. These results indicated that the larger disturbance of the molecular orientation in the larger-diameter fibers decreased their tensile strength and tangent modulus.

The stress-strain curves of tested fibrils were almost linear or upward convex at the large strain region. In the fibrils with linear stress-strain curves, subfibrils are evenly loaded, and all subfibrils may fail at the same time. On the other hand, the upward convex curves may be due to the sequential breakage of subfibrils.

In the previous studies, we performed the tensile test of stress-shielded rabbit patellar tendons and observed the transverse section of these tendons using a transmission electron microscope (TEM) (Yamamoto et al., 1993, Tsuchida et al. 1997). The tensile strength and tangent modulus of the patellar tendons decreased and the number of fibrils with the diameter larger than 360 nm increased. These results indicate that the tensile strength and tangent modulus of large-diameter fibrils may be smaller than those of small-diameter fibrils. This agrees with the results of the present study. On the other hand, Lavagnino et al. (2005) demonstrated that the decrease in the tensile properties measured in in vitro stress-deprived rat tail tendons was not correlated with their fibril diameter distribution. Derwin and Soslowsky (1999) demonstrated that the aging from 3 to 8 weeks increased the tensile strength of mouse tail tendons and the number of their fibrils with the diameter larger than 250 nm. The contradictions in these results may be attributable to the difference of the mechanical interactions between fibrils and proteoglycans in tendon fascicles. Fascicles consist of fibrils embedded in proteoglycans (Kastelic et al., 1978, Ottani et al., 2002, Silver et al., 2003). Therefore, fascicles may fail by the slippage and breakage between fibrils and proteoglycans rather than by the breakage of fibrils. Further studies should be conducted to determine the effects of remodeling and aging on the mechanical interactions between fibrils and proteoglycans.

Svensson et al. (2013) performed the AFM-based tensile test of the fibrils (the diameter of 210±150 nm) isolated from rat tail tendons, and reported that their tensile strength, strain at failure, and tangent modulus were 200±110 MPa, 16±4 %, 1400±700 MPa, respectively. In our previous study for the fibrils (the diameter of 342±45 nm) isolated from rat tail tendons, their tensile strength, strain at failure, and tangent modulus were 111±69 MPa, 32±10 %, 350±119 MPa, respectively (Yamamoto and Toda, 2013). These results are almost similar to those of the present study. These indicate that the effect of animal species (mouse vs. rat) on the tensile properties of fibrils may be small.

Yang et al. (2012) performed the AFM-based tensile test of the fibrils (the diameter of 305±10 nm) reconstituted from bovine Achilles tendon collagen type I, and reported that their tensile strength, strain at failure, and tangent modulus were 60±10 MPa, 13±2 %, 600±200 MPa, respectively. The tensile strength and strain at failure are smaller than those of the present study. These lower values may be due to the lack of cross-links in the reconstituted fibrils as compared with the fibrils directly isolated from tendons. In the same work by Yang et al. (2012), cross-linking agents were found to increase the tensile strength and strain at failure of the reconstituted fibrils.

In this sample preparation, a mouse tail was stored at 4°C and thawed at room temperature. Many previous studies reported no effect of frozen storage on the mechanical properties of tendons and ligaments (for example, Woo et al., 1986, Moon et al., 2006). The tail sample was obtained from one mouse. One tail was composed of many tendon fascicles, and many fibrils were able to be isolated enough to carry out this experiment. It was confirmed that there were no significant differences in the tensile properties of fibrils between the present study and our previous study using another mouse (Yamamoto and Sugiura, 2009). The fibrils were isolated from the fascicles by stirring in distilled water. In our previous study, AFM imaging of the isolated fibrils was performed. It was found that the isolation procedure did not induce any obvious damages on the surface of the fibrils (Yamamoto and Toda, 2013).
In this tensile test method, the both ends of the fibril were wound onto the tips of the microneedles. This technique worked very well to grip the fibril. The adhesion between the tips and the fibril was strong enough to carry out tensile test. There was no slippage between the tips and the fibril, and the fibril was broken between the tips. Rappaport (1967) achieved to determine the maximum tension exerted by cleavage furrow of echinoderm eggs using a similar method by two microneedles.

In the present experiment, the diameter of dried fibrils was measured with SEM imaging after tensile testing. The cross-sectional area of the dried fibrils was calculated from the diameter. And also, in the previous studies, the cross-sectional area of dried fibrils was determined using AFM or SEM imaging (Svensson et al., 2013, Yang et al., 2012). Yang et al. (2012) reported that the swelling of ~50% in fibril diameter was found upon hydration in physiological saline solution using AFM imaging. Shen et al. (2010) reported that the diameter of fibrils increased by ~120% upon hydration using AFM imaging in liquid and SEM imaging in vacuum. Spitzner et al. (2015) reported that the diameter of fibrils increased by ~15% between the dry and hydrated states using AFM imaging. The amount of increase in the diameter of fibrils was different in each study. Further studies are needed to determine the effect of hydration on the diameter of fibrils.

5. Conclusion

The single collagen fibrils directly isolated from mouse tail tendons were stretched to failure in physiological saline solution using our original tensile test method. Their tensile strength and tangent modulus decreased significantly with increasing their diameter. On the other hand, their strain at failure increased significantly with increasing their diameter.

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