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Original paper

Structural alteration of collagen fibres – spectroscopic and mechanical studies

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Fourier Transform Near Infrared Raman Spectroscopy has been used to monitor the molecular changes of collagen in a tendon subjected to strain. In the Raman spectrum of the unstrained tendon, some protein bands, mainly assigned to collagen, can be observed: amide I (1666 cm^{-1}) and III (1266 and 1248 cm^{-1}) vibrational modes and skeletal (C–C) stretching vibrations (816 and 940 cm^{-1}). The position of these bands is changing with the increasing strain values. It is concluded that elastin and non-helical domains of collagen are initially involved in the load transfer and triple helices of collagen are gradually joining this process.

Key words: *collagen, tendon, material properties, Raman spectroscopy*

1. Introduction

Collagen is a main component of extracellular matrix and the most abundant protein in human body (30% of the total protein mass). So far, about 27 distinctly different types of the collagen have been identified and described. About 90% of collagens are fibril-forming types I, II, III, V and XI. Type I collagen predominates in bones, tendons, ligaments, blood vessel walls and skin. Type I is usually accompanied by types III and V. Type II is the major collagen in cartilage and usually occurs simultaneously with collagen type XI [1], [2].

The structural unit of fibril-forming collagens is tropocollagen. It is a protein approximately 300 nm long and 1.5 nm in diameter. Tropocollagen is made up of three polypeptide chains, each consisting of 1050 amino acids and forming a characteristic left-handed helix, twisted together into a right-handed triple helix. A characteristic feature of collagen molecule is the presence of repeating triplet of glycine and two other amino acids, one of them being usually proline or hydroxyproline. Hydroxyproline is involved in hydrogen bonding between polypeptide chains. The helix-forming Gly-Pro-Hyp tripeptide sequence is known to be the most stable in collagen. Non-triple helical structures often occur in the end of the collagen molecule and are involved in the covalent intermolecular cross-linking [2]–[6].

Tendon is a typical collagen-rich structure of a hierarchical organization. It is composed of collagen molecules, fibrils, fibre bundles, fascicles and tendon units that run parallel to the tendon's long axis. Type I collagen constitutes about 60–70% of the dry mass of tendon. Other collagen types (III and V) also are pre-

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sented. Tendon also contains small amounts of elastin (~2%) [2], [7], [8].

Collagen is responsible for mechanical durability of biological load-bearing structures like tendons. Types I and III collagen are mainly characterized by very high tensile strength equal to about 120 MPa [9]; however, they undergo failure at low values of strain, i.e. 3÷4% [10]. Young's modulus of collagen ranges from 120.0±10.0 MPa [11] to 130.6±63.7 MPa [12]. GACKO [13] proved that an average value of elasticity modulus for collagen was around 100 MPa.

Structural changes can be studied by FT-IR-Raman spectroscopy. This technique, introduced in 1986 by Hirschfeld and Chase, is a powerful tool for the in situ investigation of biological materials, including soft tissues, because of many advantages: a high sensitivity and specificity, non-invasiveness, and a fast spectrum collecting [14]–[16]. Spectroscopic methods provide information about the morphologic composition of tissues and allows us to determine small biochemical changes in their components, caused by diseases or other pathological processes [15], [17], [18]. Recent studies have shown that FT-Raman spectroscopy can be used for the examination of the molecular deformation of natural materials under strain [19], [20].

The knowledge of the strain-induced changes in the collagen structure is important in the understanding of the biomechanics of tissues rich in collagen. Hence, the aim of this study was to evaluate structural alteration of tendon during uniaxial tensile tests in order to assess collagen strain-induced remodelling.

2. Materials and methods

Tendon samples were obtained by gentle scrapping domestic pigs' tails with scalpel. Immediately after dissection the material was placed in saline solution (0.9% NaCl) and stored at temperature of 4 °C. The specimens were measured after sampling without any kind of pre-treatment.

Raman spectra were recorded by an FT-Raman spectrometer Brüker RFS 100. A diode-pumped Nd:YAG laser at 1064 nm with an output of 450 mW was used as the excitation source. The Raman signal was detected by a germanium detector and 128 scans were collected. The spectral resolution was 4 cm^{-1}. The spectral range of the acquired spectra varied from 0 to 4000 cm^{-1}, with the reliability and precision of the wave number measurements approaching 1cm^{-1}. The specimens were placed in a self-made tool designed specifically for the Raman scattering studies [21]. The tool was placed directly inside the spectrophotometer. The initial specimen length was always 28 mm. Spectra were recorded for the successive phases of stretching the specimen (by 1 mm) to the sample rupture.

Mechanical tests for equivalent specimens were performed using a MTS Synergie 100 testing machine on the day of harvesting. Measurements' protocol of mechanical properties was designed to reproduce test conditions current in FT-Raman spectrometer during spectra recording in order to measure the induced load values corresponding to strains. All test parameters (test speed, relaxation time, strain level) were determined for the Raman spectra measurements and duplicated for mechanical tests. Equivalent specimens of tendons were stretched at a constant test speed υ = 0.75 mm/min to the multiplicity of strain level ε = 3.6% until to specimens failure. Between serial steps throughout the relaxations time t = 316 s one strain level was maintained (figure 1).

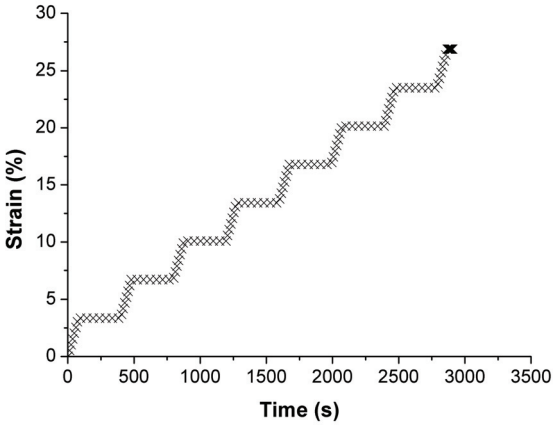

Fig. 1. Strain–time relationship for pig tail tendon

3. Results

A representative Raman spectrum of the unstrained tendon sample is shown in figure 2.

Pig tail tendon spectrum is typical of type I collagen-rich tissues. The major peaks in tendon spectra are attributed to proteins: $\nu(CH_2)$ (~2942 cm^{-1}), $\delta(CH_2,CH_3)$ (~1450 cm^{-1}), $\nu(CC)$ (~940 cm^{-1}) and amide bands with maxima of 1666 cm^{-1} (amide I) and 1249 cm^{-1} (amide III). The amide I vibration is dominated by peptide carbonyl stretching vibration with some contribution of C–N stretching and N–H in-plane bending. Amide III vibration results from C–N stretching and N–H in-plane bending. The position of

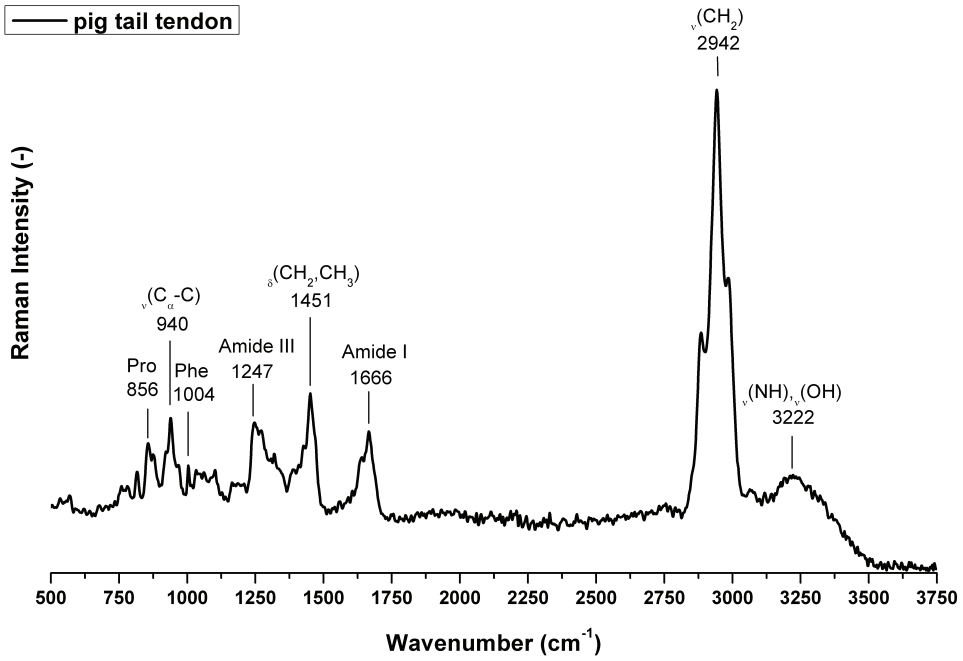

Fig. 2. Raman spectrum of the unstrained pig tail tendon

Table1. Major bands identified in tendon spectra

Peak position (cm^{-1})	Assignments		References
816	ν(CC), δ(CO_2)	protein backbone, amino acids	[7], [8], [22]
856	ν(CC), δ(CCH)	proline	[7], [22]–[24]
875	ν(CC), δ(CCH)	hydroxyproline	[7], [15], [23], [24]
922	ν(CC)	proline	[7], [24]
940	ν(CC)	α helix	[16], [23], [25], [26]
1004	ν(CC)	phenylalanine	[7], [15], [22]–[24], [26]
1248	ν(CN), δ(NH)	amide III, polar triple helix of collagen, elastin	[7], [15], [23], [24], [26]
1266	ν(CN), δ(NH)	amide III, non-polar triple helix of collagen	[7], [15], [23], [24], [26]
1451	ν(CH_2, CH_3)	amino acids side chains	[22]–[26]
1666	ν(C=O)	amine I, collagen, elastin	[7], [15], [23]–[26]
2940	ν(CH_2, CH_3)	amino acids side chains	[23], [25], [26]
~3225	ν(NH), ν(OH)	amide A, B, water	[16], [23]

Legend: ν – stretching mode, δ – bending mode.

amide bands is sensitive to the secondary structure of protein. Amide I band in the unstrained tendon spectrum is strongly asymmetric and its deconvolution (figure 3) with the Lorentz function yields few components in 1600–1700 cm^{-1} region which can be mainly assigned to: collagen (1631 and 1666 cm^{-1}), water (1641 cm^{-1}), elastin (1653, 1675 and 1683 cm^{-1}), and amino acids (1606, 1617 and 1698 cm^{-1}). In amide III region, two strong peaks are observed: band assigned to unordered (1248 cm^{-1}) and triple helical (1267 cm^{-1}) structures of collagen. Weak shoulders of amide III band (1239, 1256 and 1274 cm^{-1}) probably origin from elastin. The bands near 875, 856 and 920 cm^{-1} can be assigned to the C–C stretching vibrations of amino acids characteristic of collagen: hydroxyproline and proline. The band near 1004 cm^{-1} is assigned to the phenyl ring breathing mode of amino acid phenylalanine. Table 1 lists the wave numbers of the bands observed and their assignment.

For tendon specimens subjected to increased strain some substantial changes in the shape of the spectral line were observed (figure 4).

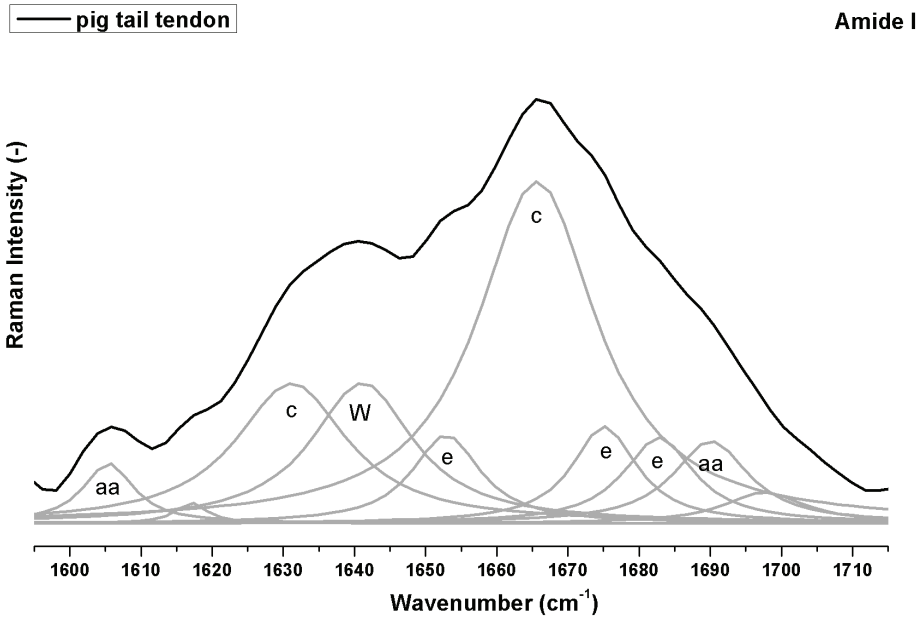

Fig. 3. Decomposition of amide I band of pig tail tendon.
aa – amino acids, c – collagen, e – elastin, W – water (hydrogen bonded and free water)

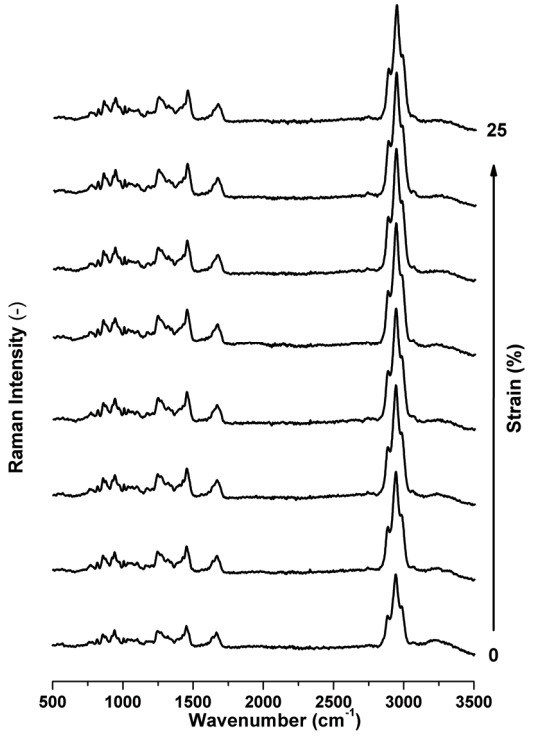

Fig. 4. Raman spectra of pig tail tendon versus strain. Spectra are shown
as the function of strain increased in 3.6% steps from bottom (0 strain) to top (25% strain)

The stress applied to chemical bonds leads to changes in interatomic distances and consequently, due to the inharmonicity of the vibrational energy, it shifts positions of the bands. Vibrational modes of peptide bonds are sensitive to the protein conformation changes [8], [21], [27]. Considerable alterations of amide bands are noted (figure 5). Negative shifts of ν(CC) bands of protein backbone are observed. Bands assigned to amino acid proline (856 cm^{-1}) and hydroxyproline (875 cm^{-1}) are shifted to lower wave

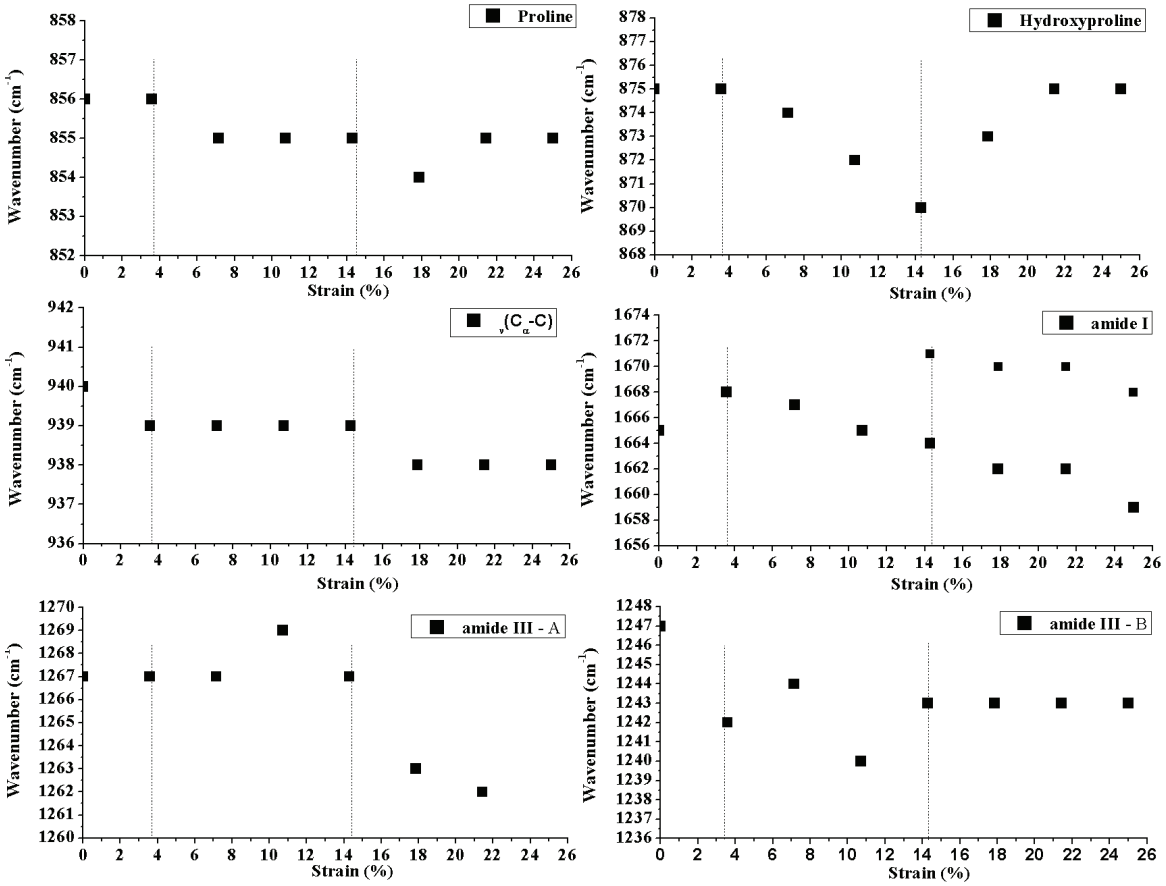

Fig. 5. Strain–band position dependencies of Raman bands in pig tail tendon spectra. A) non-polar triple helix of collagen, B) polar triple helix of collagen

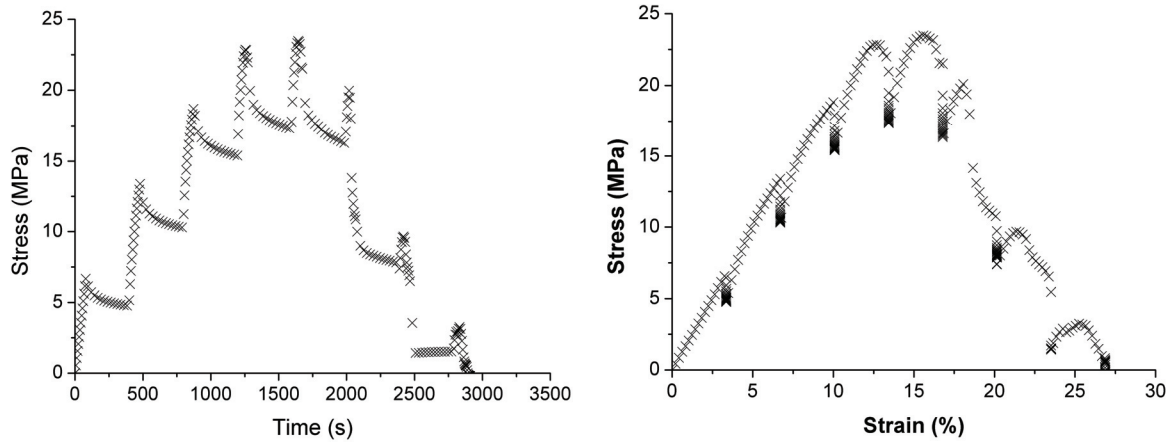

Fig. 6. Example of relationships: A) stress–time, B) stress–strain for pig tail tendon

numbers as the value of the strain increases. Bands near 1004 cm^{-1} and 1451 cm^{-1} do not change their positions.

On the basis of progressive elongation of equivalent specimens of tendons in the directions of fiber arrangement, the stress–time (figure 6A) and stress–strain (figure 6B) relationships were determined. The stress relaxations were noted in each step of testing procedure. Degree and rate of relaxations increase with an increase in strain values. For each rectilinear section in stress–strain relationships an incremental tangent modulus of elasticity (E_p) was determined (table 2).

Table 2. Incremental tangent modulus of elasticity (E_p) for individual strains' range for progressive elongation of pig tail tendon

ε(%)	E_p(MPa)
0÷8.36	154.3±66.3
3.6÷7.2	196.7±57.5
7.2÷10.8	191.2±32.5
10.8÷14.4	218±86.9
14.4÷18	243.6±78.3
>18	–

4. Discussion

A relatively homogeneous structure of pig tail tendons, i.e. the high content of type I collagen, is a perfect substitute model for human collagenous tissues in the research of collagen structural changes caused by strains. Moreover, animal tissues are easily available and can be investigated just after sampling without prolonged freezing. Proteolytic processes, which start in the tissues after death, and a long-term storage at low temperatures have a disadvantageous influence on a protein structure and change mechanical properties of tissues [28]–[30].

As the present studies prove, the application of strain shifts several bands in the Raman spectrum of tendon. Peak positions explicitly change in stages (figure 5).

The majority of vibrational modes do not exhibit alterations when the strain is in the range of 0–3.6%. Initially, the maximum of the amide I band position is shifted to higher wave numbers. COLOMBAN [27] and SIRICHAISIT [20] did not observe radical changes in the position of the amide I contour in the spectra of silkworm and spider fibres subjected to stress. However, an upshift of this mode related to the transition of α-helical to β-pleated sheet structures was perceived by CHURCH [19] for stretched wool fibres. In our opinion, the change in the amide I position is connected with collagen fibres reorganization into the force associated with the stretching and straightening of waves and twists. Those variations occur due to changes in the H-bonded network or other structural reorganizations in the collagen structure [20]. A slight decrease of amide bands' intensity (1267 and 1668 cm^{-1}) testifies to collagen fibres' reorientation into stretching [31]. These phenomena (fibres ordering and arrangement in the direction of effect loads) cause that the value of an incremental tangent modulus of elasticity E_p for this range of strains is relatively low in comparison with that in the next stages (table 2).

Generally, when the strain increased from 3.6 to 14.4%, the bands in Raman spectra of stretched tendon shifted to the lower wave numbers with an increasing value of the strain. A negative shift of contour is assigned to amino acid hydroxyproline (875 cm^{-1}) and amide bands in this range are connected with a lateral compression of carbonyl groups [8]. The maxima of an amide III mode at 1246 cm^{-1} (polar, proline-poor domain of collagen) and a weak shoulder arise from elastin reduction, while the location of an amide III band at 1267 cm^{-1} (non-polar, proline-rich fragments of collagen) is stable in the strain range of 0–14.4%. Primarily, structural changes occur in the domains with a non-triple helical conformation in a collagen molecule. Such fragments with the accompaniment of elastin first transfer the loads in a tendon [2]. This contributes to tendon stiffening, by about 22% on average, in this range of strains in comparison with the first range. It is worth noticing that for strains between 10.8% and 14.4% the stiffening effect of tendon increases by next 10%, which is connected with changes in amide III band contour (figure 5).

For strain level of approximately of 14.4%, structural changes take place in all collagen molecules and cause that the layout stiffness definitely increases. In that stage, significant shifts of bands in tendon spectra are observed. The shape of amide I contour becomes plainly asymmetric. An analysis of the amide III band at 1267 cm^{-1} shows that it shifts up by 5 cm^{-1}. Changes of amide III mode position prove that triple helical structures of collagen participate in load-bearing process. After exceeding this value the character of stress–strain relationships (figure 6) indicates that load-bearing structures undergo permanent deformation and gradual failure. First, microfractures in tendon are observed, and then destruction on a macroscopic level occurs (on 20÷25% strain values).

Monitored shifts of bands (816 and 940 cm^{-1}) assigned to vibrational modes of a protein backbone to lower values of wave numbers determine its deformation during stretching. Whereby changes of ν(CC) band position are slight in an initial stage (1–2 cm^{-1}). WANG [8] is of the opinion that the C–C backbone structure accommodates most of the tensile stress imparted to the system.

Gradual tendon stretching with repeated test stopped at the multiplicity of 3.6% strains maintained throughout the definite relaxation time (316 s) allowed us to notice a stress relaxation phenomena typical of tendon [32], [33]. The relaxation of rat's Achilles tendon for 600 s at 5% strain approached, on average, 70% [32]. In the present study, at 3.6%

strain for half that time, percent relaxation equals only about 20%.

On the basis of experimentally obtained stress–strain relationships, an incremental tangent modulus of elasticity E_p was determined. The values of E_p parameter for pig's tail tendon are in general lower than those found in literature; however, the range of E_p variability is very wide and greatly depends on donor's age and species, tendon type and test and sample preparations conditions as well. With the best authors' knowledge, the uniaxial tensile test is most frequently used for the modulus of elasticity determinations. For typical uniaxial tensile test (without any test stopping), the value of elastic modulus ranges between about 1.36 GPa [34] and 416.3 MPa [31] for human's and rat's Achilles tendon, respectively. For one donor's species, changes in elastic modulus values occur with age; for example, an elastic modulus of rat's tail tendon increases during the first 4 months of life, from 330 MPa (in the first month) to 1.3 GPa (in the fourth month) [35]. Even the conditions of tendon preparation and storage affect the values of elastic modulus; for rabbit's patellar tendons OHNO [30] noticed an increase in elastic modulus with freezing process (from 931 MPa (for fresh) to 1.28 GPa (for frozen)).

Although a pig's tail tendon is mainly made up of collagen, the values of incremental tangent modulus of elasticity (E_p) are higher than those of collagen fibres. Numerous intermolecular crosslinks stabilize and strengthen this structure. Hence, the mechanical properties of tendon are determined by the cross-linked collagenous network [2], [36]–[38].

5. Conclusions

The Raman spectra of pig's tail tendon, as an example of collagen-rich tissues, prove that collagen undergo strain-induced remodelling. That process consists in reorganization and elongation of collagen fibres through stretching initially non-helical and next triple helical domains. The results obtained from spectroscopic and mechanical analyses are highly correlated. A modulus of elasticity increases with an increase in the amount of more rigid parts of collagen, which participates in load transfer processes.

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