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AGE RELATED CHANGES IN THE THERMAL TRANSITION OF TURKEY LEG FLEXOR TENDON COLLAGEN

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A differential scanning calorimetric, thermogravimetric and electron microscopic investigation has been carried out on the uncalcified areas of turkey leg flexor tendon as a function of age. Rehydrated samples exhibit an increase of thermal stability with age. The ΔH_D values drop from about 11 $cal \cdot g^{-1}$ in the first weeks of life down to 7 $cal \cdot g^{-1}$ after the 11th week.

At about 11 weeks, the collagen fibril diameter distribution passes from unimodal to multimodal. The DSC curves as well as the TG-DTG curves recorded from dried samples do not show any appreciable difference with ageing. The variations in thermal behaviour of rehydrated samples and fibril diameter distribution could be related to modifications in water binding with ageing.

Keywords: ageing, differential scanning calorimetry, dynamic thermogravimetry, electron microscopy, tendon collagen

Introduction

Numerous progressive changes in the physical and chemical properties of collagen are related to the ageing process. Mechanical behaviour [1–3], crimping [4] and molecular packing parameters [5–7], solubility [8], denaturation temperature [9] and diameter [10] of the collagen fibrils undergo strong variations with ageing. Most of these variations have been related to the decrease in the ratio between reducible and stable intermolecular crosslinks with ageing [9, 11]. A close relationship has been found between the amount of thermostable crosslinks and age related changes in the thermal stability of rat tail tendon and rat skin collagen [12, 13]. A high density of stable covalent crosslinks occurring laterally between molecules and as-

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sumed to be present in fibrils with large diameters has been invoked to explain the high tensile strength of connective tissues containing fibrils with a large diameter [10]. Furthermore, the variation in the physical and chemical properties of collagen with ageing can also be related to the gradual change in proteoglycan composition. Collagen fibril formation and growth, as well as their assembly, are strongly influenced by the interaction of collagen with the highly hydrated proteoglycans [14]. Furthermore, proteoglycans, which have been suggested to alter the charge distribution and water binding in collagen [14], stabilize the triple helical structure of collagen, as shown by the increase of the melting point of collagen caused by their presence [15].

In order to characterize the morphological and physical modifications of turkey leg flexor tendon with ageing we have carried out differential scanning calorimetric, thermogravimetric and electron microscopic investigations of the uncalcified area of tendons from animals of different ages.

Unlike most tendons, turkey leg tendon undergoes calcification with ageing. The calcification of collagen fibres begins when the animal is about 11–13 weeks old in the middle part of the tendon and increases up to maturity. The area nearer to the distal tendon sheath remains uncalcified. X-ray and electron microscopic investigations [16, 17] indicate an evident relationship between collagen molecular packing and inorganic deposits in the calcified areas of the tendons.

The results of this study suggest a relationship between the morphological and thermal parameters of flexor tendon collagen, which undergo the sharpest variations at about 11–13 weeks at the beginning of the calcification process.

Experimental

Materials

Flexor leg tendons were obtained from 4–37 week old Nicholas female turkeys (kindly provided by SIPA-Verona, Italy). The tendons were removed by dissection immediately after sacrifice, freed from external collagenous sheet, washed in distilled water and stored at −18°C until used. The tendons dissected from animals younger than 11 weeks do not show any radiographic evidence of calcification along their length. The calcifying areas of tendons obtained from 11–37 week old turkeys display a variable degree of calcification along their length. The samples were, however, al-

ways dissected in the uncalcified region of the tendons, below the onset of calcification.

In order to examine specimens at the same moisture content [18] all the samples were stored in distilled water for 1 h and subsequently dried at room temperature at a constant relative humidity of 50% for 24 h (dried samples). Some of these samples were stored in distilled water for 22 h before being examined under wet conditions (rehydrated samples).

Differential scanning calorimetry

Curves were recorded for dried or rehydrated samples using a Perkin Elmer DSC-1B calorimeter. The measurements were carried out in the temperature range of 30–150°C at a scan rate of 4 deg·min^{-1}. The curves recorded using this scan rate shifted to higher temperatures with respect to those recorded with a scan rate of 1 deg·min^{-1} while the ΔH_D values did not show any appreciable variation. The use of 4 deg·min^{-1} was found to be preferable in terms of noise level and baseline. Sealed aluminium pans, recommended for volatile compounds, were used. The area of the transition peak was measured by graphical integration. The denaturation temperature was measured at the mid point of the transition peak. In order to express the ΔH_D values with respect to the dry collagen content the water content of the samples was determined by dynamic thermogravimetry.

Dynamic Thermogravimetry

Thermogravimetric investigations were carried out on dried samples using a Perkin Elmer TG-7. Heating was performed in airflow (20 cm^3·min^{-1}) using a platinum crucible at a rate of 1–5 deg·min^{-1} up to 950°C. The weight of the dried samples was around 3 mg.

Transmission electron microscopic investigations

Wet samples were fixed for 2 h in 2% glutaraldehyde solution in 0.1 M phosphate buffer (*pH* 7.3). After dehydration in graded alcohols, the specimens were stained with uranyl acetate and lead citrate. Thin sections were observed in a Siemens Elmiskop 101 electron microscope.

The fibril diameters were measured on micrographs using a Leitz ASM image analyser (Leitz, Wetzlar).

Results

Differential scanning calorimetry

The DSC curves obtained from rehydrated samples show one broad endothermic peak which occurs at increasing values of temperature with ageing. A typical curve obtained for a sample dissected from an 11-week-old turkey is shown in Fig. 1.

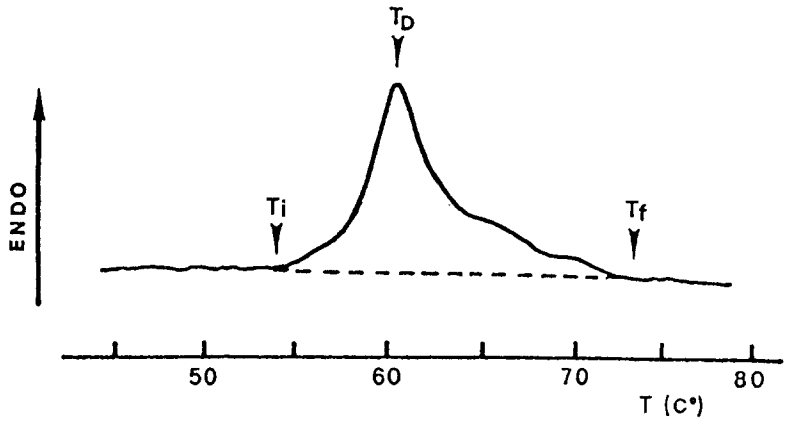

Fig. 1 DSC curve of a rehydrated sample dissected from a 11-week-old turkey

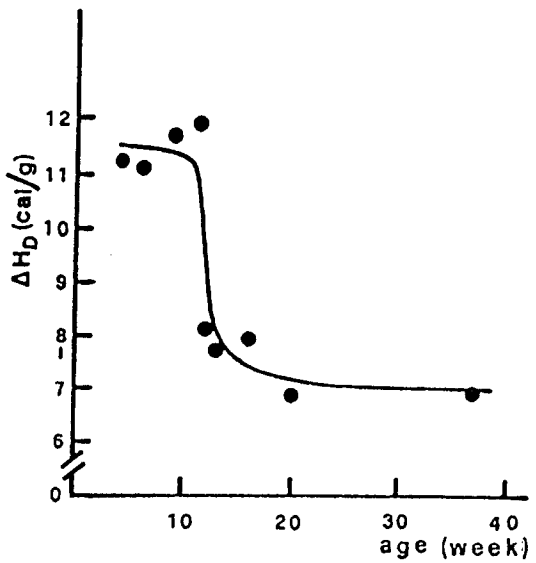

Fig. 2 ΔH_D values of rehydrated samples plotted as a function of age

T_i is the temperature where the endothermic peak starts to develop, T_D is the denaturation temperature and T_f is the temperature at which the curve returns to the baseline. The enthalpy value of collagen denaturation (ΔH_D) undergoes a strong variation around 11 weeks as can be seen in Fig. 2. T_D and T_i values increase with ageing while T_f does not change appreciably and exhibits a mean value of 72°C as shown in Table 1.

Table 1 Typical temperatures and enthalpy changes measured from DSC curves of rehydrated samples of turkey leg flexor tendon of different age

Age / week	T_i / °C	T_D / °C	T_f / °C	ΔH_D / cal·g^{-1}
4	51.2±0.5	85.0±0.4	64.3±0.3	11.2±0.5
6	54.0±0.6	59.9±0.4	72.0±0.7	11.1±0.6
9	54.7±0.5	60.1±0.6	72.3±0.5	11.7±0.5
11	54.5±0.4	60.5±0.5	74.2±0.4	11.9±0.5
12	56.2±0.7	61.2±0.7	72.5±0.3	8.1±0.7
13	58.0±0.6	61.0±0.6	72.7±0.7	7.7±0.6
16	57.8±0.5	62.0±0.7	73.1±0.5	7.9±0.7
20	59.1±0.7	63.9±0.8	72.8±0.9	6.8±0.7
37	59.2±0.9	64.2±0.7	73.2±0.7	6.9±0.8

Each value is the mean ±standard deviation for 20 determinations

The DSC curves recorded for dried samples show one endothermic transition with a large initial speed. A typical curve obtained for a sample of 13-week-old turkey is shown in Fig. 3. With increasing age of the animal ΔH_D values do not change appreciably and assume a mean value of 9.1±0.7 cal·g^{-1}.

The values of T_i, T_D and T_f fluctuate around the mean values of 95, 115 and 119°C respectively.

Dynamic thermogravimetry

The TG-DTG curves obtained for a dried sample dissected from a 13-week-old turkey are shown in Fig. 4. The DTG curve shows three well resolved peaks; the first one at 68±1°C corresponds to a weight loss of 13.5±0.4% due to water loss, the second one at 332±1°C to a weight loss of 47.8±0.4% due to collagen depolymerization and decomposition and the third one at 600±1°C to a weight loss of 38.5±0.4% due to combustion of compounds produced during the decomposition process. The temperatures

of maximum rate of weight loss and the weight losses associated with the three peaks do not change appreciably with the age of the animal.

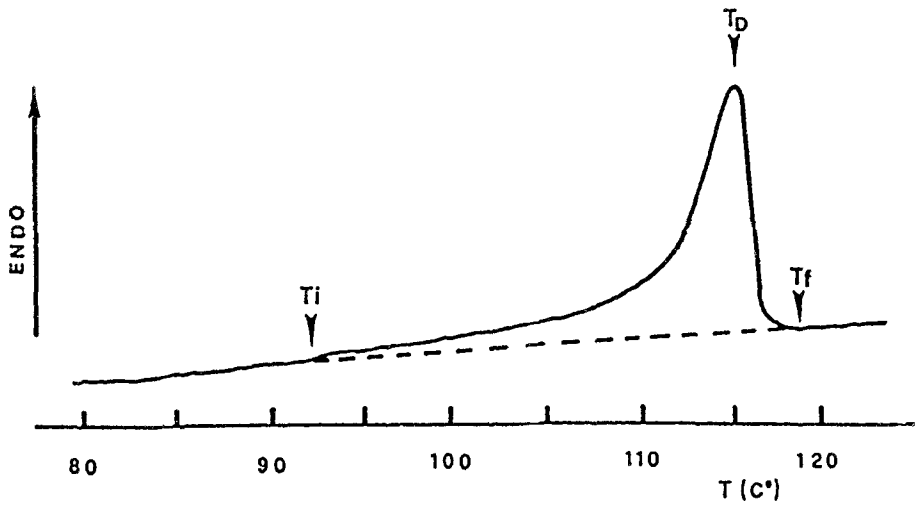

Fig. 3 DSC curve of a dried samples dissected from a 13-week-old turkey

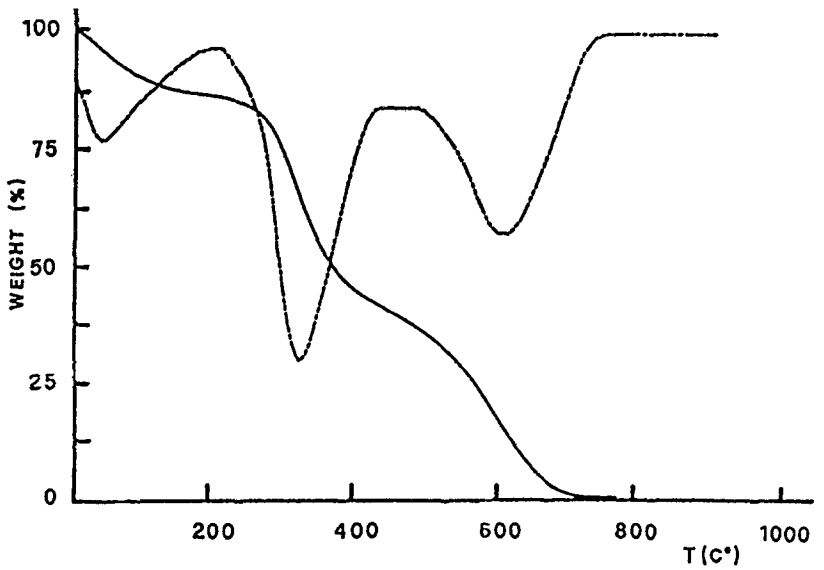

Fig. 4 TG-DTG curves obtained from a dried samples dissected from a 13-week-old turkey

Electron microscopic investigations

The collagen fibril diameter distributions determined from electron microscopy images of thin sections of tendon samples dissected from 4, 6, 13 and 20-week-old turkeys are presented in Fig. 5 a-d. At 11 weeks the collagen fibril diameter increases by a factor of about 2 (Table 2) whereas the collagen fibril diameter distribution which is fairly sharp and unimodal for younger animals, becomes broad and multimodal.

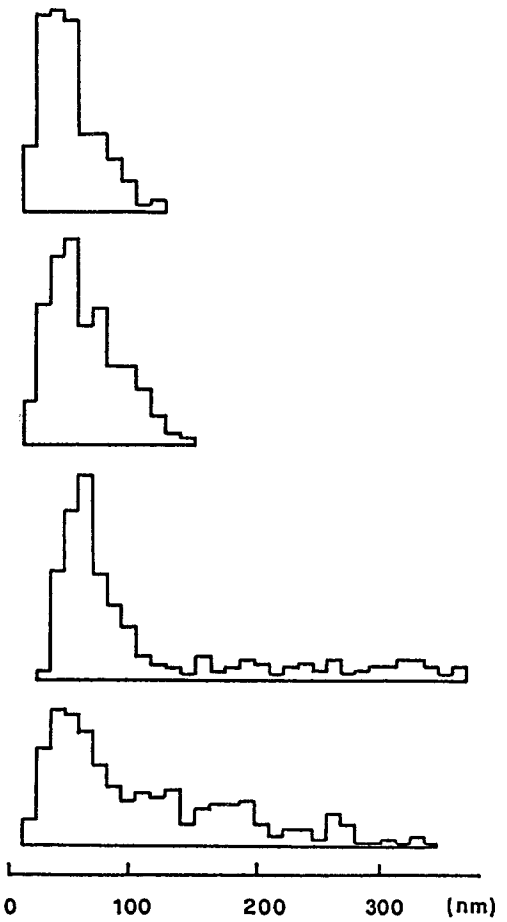

Fig. 5 Histograms illustrating the distribution of the collagen fibril diameters measured in samples of 4 (a), 6 (b), 13 (c) and 20 (d) weeks

Table 2 The mean diameters of collagen fibrils in the turkey leg flexor tendon as a function of age

Age / week	Mean diameter / nm
4	38
6	50
9	48
11	47
13	117
16	110
20	102
37	113

Discussion

The denaturation temperatures as well as the denaturation enthalpies of dried samples do not vary appreciably with ageing. This fact is in agreement with the results of TG data which do not show any appreciable variation in the values of temperature and weight losses associated with water release, depolymerization and combustion.

The differential scanning calorimetric measurements carried out on rehydrated samples provided completely different results.

Rehydrated samples of turkey leg tendon exhibit an increase in the thermal stability with ageing. In fact the values of T_D and T_i, determined from the broad endothermic DSC curves increase with the age of the animal.

The ΔH_D values, on the other hand, do not vary linearly with the age, dropping from about a value of 11 $cal \cdot g^{-1}$ in the first week of life, down to 7 $cal \cdot g^{-1}$ after the 11th week.

The comparison between the thermal behaviour of dried and rehydrated samples indicates an important role of the relative amount of bound and free water on collagen stability.

The abrupt variation of ΔH_D in rehydrated samples may be ascribed to differences in the state of water associated to fibrils of different diameter. In fact, at about 11–13 weeks the mean fibril diameter increases by a factor of about 2, and the collagen fibril diameter distribution varies from unimodal to multimodal, although the primary mode of fibril diameter distribution does not change appreciably (Fig. 5). These data suggest a close relationship between collagen fibril morphology and thermal behaviour with increasing age. This is confirmed also by the results obtained for samples of

4-week-old animals which exhibited the lowest values of T_i T_D and T_f and the sharpest unimodal distribution of collagen fibril diameter.

The variation in fibril diameter distribution is probably required by the tendon in order for it to fulfil its mechanical function.

It is worth to note a characteristic feature of turkey leg flexor tendon even the calcification process begins at about 11–13 weeks.

Even though our samples were always dissected from the uncalcified area of the tendon, which is well below the calcified area, the stability and the morphology of collagen fibrils in the uncalcified portion might nevertheless have some kind of relationship with the process of calcification.

It is well known that the density of stable covalent crosslinks, which are assumed to be present in collagen fibrils with large diameters, increases with age [10]. Although the thermogravimetric data do not allow to detect any variation in the stability and amount of crosslinks with age [19], a small crosslink contribution to the observed increase in the thermal stability of rehydrated samples with age cannot be excluded.

Furthermore, during development and ageing, changes have been observed in the amount and kind of proteoglycans associated to collagen fibrils [14]. Proteoglycans have been suggested also to be closely related to fibril radial growth [20] and to affect collagen water binding [14].

Therefore a variation in the amount and/or kind of proteoglycans might be invoked to explain the observed changes in the thermal behaviour and morphology of collagen fibrils with ageing.

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Zusammenfassung — Mittels DSC, TG und Elektronenmikroskop wurden unverkalkte Beugesehnenregionen von Truthahnbeinen in Abhängigkeit des Alters untersucht. Rehydratierte Proben zeigen mit zunehmendem Alter ein Ansteigen der thermischen Stabilität. Der Wert von ΔH_D sinkt von 11 cal/g in den ersten Lebenswochen auf 7 cal/g nach der 11.Woche.
Nach etwa 11 Wochen geht der Durchmesser der Kollagenfibrillen vom einer eingipfeligen in eine multigipfelige Verteilung über. Weder die DSC-, noch die TG-DTG-Kurven von getrockneten Proben zeigen irgendwelche nennenswerten Unterschiede in Abhängigkeit vom Alter. Die Änderungen des thermischen Verhaltens rehydratierter Proben und der Verteilung des Fibrillendurchmessers können den Änderungen der Bindung des Wassers in Abhängigkeit vom Alter zugeschrieben werden.