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Differences in the energetics of collagen denaturation in connective tissue from two muscles

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ABSTRACT

The thermal denaturation of collagen the perimysium of intramuscular connective tissue isolated from bovine *Semitendinosus* (ST) and *Perctoralis profundus* (PP) muscles was investigated using a range of heating rates in differential scanning calorimetry (DSC) and analyzed by application of the Kissinger–Akahira–Sunose (KAS) and Lumry-Eyring models. Thermograms showed a broadening of endotherms and a shift towards higher temperatures as the thermal scanning rate increased. These features are consistent with the two-step process of a reversible transition between native and unfolded collagen molecules followed by an irreversible transition between unfolded and denatured states. There were small differences between muscles in the onset temperatures of the thermal transitions at heating rates of 0.5 K min^{-1}, while both the KAS and Lumry-Eyring models yielded similar values for the effective activation energy of the whole two-step process, the Lumry-Eyring model allowed a greater insight into differences in the reversible and irreversible steps between the perimysium both muscles. Reversible unwinding of the triple-helical collagen molecules in the perimysium from ST muscle required more energy than in PP muscle. It is speculated that the presence of large amounts of elastin in the perimysium of ST muscles may influence this due to a protein crowding mechanism, or by affecting the covalent cross-linking of the collagen.

1. Introduction

The thermal denaturation characteristics of collagen are important in many fields, including soft tissue injury (burns), ultrasonic physiotherapy treatments, tissue engineering and not least in food science. Collagen is the mayor fibrous protein present in the intramuscular connective tissues (IMCT). Denaturation of collagen in IMCT has long been known to be one of the contributory factors to changes in the texture of meat on cooking, and the acceptability of different cuts of meat has a relation to their collagen content [1] and the degree of covalent crosslinking between collagen molecules. IMCT has been shown to have a remarkably high resistance to solubilisation on heating compared to other connective tissues. Mohr and Bendall [2] demonstrated that the amount of collagen solubilised on heating from tendons attached to the bovine *Sternomandibularis* muscle was 5–6 times greater than the amount solubilised from the IMCT of the same muscle, and that this difference in thermal stability is due to a much high density of heat-stable crosslinks in the IMCT collagen.

The thermal stability of IMCT has been studied previously using DSC at a constant heating rate of 10 K min^{-1} [3,4] or 5 K min^{-1} [5,6]. However it is known that both the apparent enthalpy of denaturation (ΔH) and the peak temperature of the endotherm (T_{max}) of collagen denaturation vary with scanning rate [7]. Within muscle, collagen is found in two distinct connective tissue structures; the endomysium, separating individual muscle fibres, and the thicker perimysium, separating fascicles. The perimysium is the IMCT component that has greatest influence on the toughness of cooked meat [8,9]. The purpose of this paper is to provide a sound description of the thermal denaturation behaviour of specifically the perimysial IMCT from two different bovine muscles.

1.1. Background theory and previous analyses of collagen denaturation

Miles [10] suggested that the denaturation of collagen is not a process that can be described by equilibrium thermodynamics, where the temperature simply affects the effective equilibrium constant K^{Eff} between two interchangeable states. He showed that collagen denaturation behaviour varies strongly with the rate of heating and is largely irreversible (i.e. a second thermal scan at a constant heating rate does not show a strong endotherm). A temperature-dependent K^{Eff} and irreversible denaturation are not characteristics of a system based on equilibrium between native and denatured states. Miles [10] proposed an alternative model, based on an irreversible transfer of collagen

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molecules from the native to the denatured state, governed by a rate process that is highly temperature-dependent. The author assumed that a system containing N molecules of native collagen will denature at any constant temperature T following first-order kinetics, thus:

$$\frac{dN}{dt} = -k(T)N \tag{1}$$

And, at a constant rate of heating, β, this yields

$$\frac{1}{N}\frac{dN}{dt} = -\frac{kT}{\beta} \tag{2}$$

According to this theory, if N is the initial number of collagen molecules at the initial temperature T_i,

$$\frac{1}{N}\frac{dN}{dt} = \frac{-k(T)}{\beta}\ exp\left(\frac{-1}{r}\int_{T_i}^{T} k(T)dt\right) = \frac{\partial C_p}{\Delta H} \tag{3}$$

where ∂C_p is the apparent perturbation in the specific heat capacity due to denaturation and $\Delta H$is the enthalpy of denaturation.

In order to model the function k(T), Miles [10] used three theoretical approaches: an approach based on the Arrhenius equation (which, with some simplifying assumptions, yields a model identical to that developed in study of bacterial thermolysin [11] (this model also coincides with the Ozawa [12,13] model); an approach based on absolute rate theory, and an approach based on the mathematically-convenient "D and z" formulation used to describe thermal death of microorganisms, where D is the time to reduce the number of native molecules 10-fold, and z is the increase in temperature required to reduce D 10-fold. Miles [10] applied all three models to data from isothermal experiments and experiments at constant scan rates to denaturation data on the collagen of the lens capsules from bovine and porcine eyes. He found that, within experimental limits, the three theoretical approaches were indistinguishable in comparison with the experimental variability in the data. Miles [10] also showed that the endothermic denaturation peak is asymmetric, as predicted by theory, and that the width of the peak at half-height increases with scanning rate.

It is known that variations exist between the thermal behaviour of collagen molecules in different chemical environments and from different tissues, with different co-components and different degrees of collagen crosslinking. Privalov [7] reported a 7 °C change in T_p for rat skin collagen when the pH was changed from 6 to 2.2. Blackwell and Gelman [14] showed that a range of glycosaminoglycans (GAG) present in connective tissues could stabilise collagen in solution, raising T_{max} by up to 8 °C. Judge and Aberle [3] report an increase in T_{max} of IMCT with increasing NaCl concentration, as well as effects of chronological age of the animal on the T_{max} of intramuscular collagen, which is assumed to be due to increasing concentrations of mature covalent crosslinks between the collagen molecules. Aktas [15] studied the salt concentration and pH effects on the thermal denaturation of IMCT from the *Longissimus dorsi* muscle by DSC, and found an increased T_{onset} and T_{max} with increasing pH and salt concentration, but did not observe the same in the thermal (energetic) stability. Aktas [15] assumed that the thermal stability of collagen molecules was more affected by hydrogen bonds and hydrophobic interactions than by electrostatic interactions.

Vyazovkin, Vincent and Sbirrazzuoli [16], showed that collagen denaturation involved two consecutive reactions, one reversible and a second irreversible process. They proposed an analysis of collagen denaturation using the Lumry and Eyring [17] model:

$$N \leftrightarrow^{K} U \rightarrow^{k} D \tag{4}$$

where N = native, U = unfolded and D = denatured molecules, K is the equilibrium constant of the reversible native- unfolded step and k is the rate constant for the irreversible unfolded-denatured step. Vyazovkin et al. [16] used an isoconversional method to calculate the apparent activation energy to denature collagen as a function of the extent of conversion (α) between the N and D states ($0 < \alpha < 1$), with an effective rate constant k^{ef} given as

$$k^{ef} = \frac{Kk}{1+k} \tag{5}$$

Vyazovkin et al. [16] stated that this approach avoids the need to make assumptions about the form of the function k(T), which was necessary in the Miles [10] paper. In their experiments, Vyazovkin et al. [16] used commercially-available powdered bovine Achilles tendon collagen which is not stabilised by collagen-GAG interactions. By representing the temperature dependence of K by the van't Hoff equation, Vyazovkin et al. [16] inferred that the apparent activation energy $E_{ef} \approx E + \Delta H$ at $\alpha \to 0$ and $E_{ef} \approx E$ at $\alpha \to 1$, where E is the activation energy of the irreversible U-D step and ΔH the enthalpy of the reversible N-U step.

Koga [18] reviewed the development of non-isothermal kinetics analysis based on Kujira and Akahira [19] who studied the traces of mass-loss during thermal degradation and adjusted the Arrhenius-type equation to correct for mass-loss. The equation obtained was;

$$\log t = \left(\frac{E_a}{2.303RT}\right) - \log\left(\frac{A}{g(\alpha)}\right), \tag{6}$$

which showed the relation of α to E_α (apparent activation Energy). Using calculations of E_α at different α, Kuijira and Akahira [19] obtained a plot similar to that of Vyazvkin et al. [16]

Using the assumption that the irreversible thermal transitions follow the relationship $d\alpha/dt = f(\alpha)k(T)$ and that the k(T) function follows an Arrhenius-type relation (i.e. $k(T) = k_o \exp.-(E_\alpha/RT)$), there are a number of isoconversional approaches that have been applied to thermal transitions generally, and these are reviewed and compared by Starink [20]. For DSC experiments at a constant scanning rate (β), the reaction rate (in our case, the collagen denaturation rate) is governed by both f (α) and k(T), and Starink's evaluation of the accuracy of a variety of approaches for measuring the activation energy stresses the requirement for highly dependable measures of both these functions for accurate prediction of E_α. The isoconversional methods generally assume that α is equivalent at all heating rates at T_{max}, and, based on the following relationship:

$$\int_0^{\alpha} \frac{d\alpha}{f(\alpha)} = \frac{k_0}{\beta}\int_0^{T_{max}} exp\left(\frac{-E_a}{RT}\right) \tag{7}$$

Starink [20] concludes that there are a number of well-known methods and methods with a potential for high accuracy in prediction of E_a, all based on the method that E_a is determined from the linear gradient of plots of: lnT^{y}_{max} *vs* $\frac{1}{RT_{max}}$.

The Ozawa plot [12,13] is a method where y = 1(y = E/RT). Ozawa plots use t versus α and dα/dt versus α to describe the denaturation process. By assuming that, at the peak of the DSC endotherm in a constant scanning rate experiment, the degree of denaturation reaction is a constant independent of heating rate, the Ozawa method predicts the following relationship:

$$\ln\beta = a - b\left(\frac{E_a}{RT}\right) \tag{8}$$

The ASTM standard method E698 (Standard Test Method for Kinetic Parameters for Thermally Unstable Materials) is based upon the Flynn-Wall corrected form of the Ozawa (FWO) method of analysis.

Starink [20] also recommends the widely-used generalized Kissinger model, especially in cases where precision of determination of transformation rates is limited, or the KAS method, where y = 2. The KAS method makes assumptions that are reasonable for the transitions in a

broad range of solids where (E_a)/RT is in the range of 15–60. However, cross-linked fibrous collagen with intact telopeptides have a Tmax of around 65 °C and Eα in the range 160–1300 KJ Mol-1, so that the value of (Eα)/RT lies outside that range. It is important to distinguish between cross-linked fibrous collagen ad non-crosslinked collagen, where a denaturation temperature of about 36–38 °C has been reported by Leikina et al. [21]. Starink [20] makes a case for y = 1. 92 to be a generally more accurate model, but his considerations are again based on the range of values for (Ea)/RT found in the thermal transitions of a broad range of solids.

The question of the applicability of any of the models discussed above can be discussed in the light of the assumptions and simplifications in each model. As noted previously by Miles [10], several of the models for the form of f(α) yield indistinguishable results in relation to the experimental errors in practical investigations. Unlike the approach used by Miles and colleagues [10,22–23], rate-isoconversional models do not require assumptions about the form of f(α).

The Ozawa and KAS approaches have the advantage of a very simple analysis. Provided that we critically assess the limitations of the model, these isoconversional plot models seem a reasonable and practical procedure to empirically describe the denaturation of collagen-containing matrices. It is clear from the foregoing that the thermal denaturation process depends on the molecular composition of collagen, glycosaminoglycan content, ionic environment, cross-linking and water content of the connective tissue. It is reasonable to assume that kinetic parameters may vary between the intramuscular connective tissues of different muscles on this basis, and that the endomysial intramuscular connective tissue may vary from the perimysial intramuscular connective tissue. Previous DSC studies of IMCT [3–6] do not distinguish between endomysium and perimysium.

The aim of the present study is to compare the generalized Kissinger method (KAS) described by Mittemeijer et al. [24] to the advanced isoconvertional method developed by Vyazovkin [25] in the analysis of the collagen thermal denaturation behaviour of collagen in the perimysium of two bovine muscles that are known to vary in their reaction to heat in terms of meat texture. The objective is to see which approach gives a good understanding of the thermal behavior of the highly cross-linked collagen in the perimysial IMCT and gives energetic values of practical use for scientific investigation and the meat industry.

2. Materials and methods

Commercially- available Aberdeen Angus steers, produced in a pastoral system and finished on grain for their last 30 days, were slaughtered (at live weight ~400 kg) in a local commercial slaughterhouse. The *M. Semitendinosus* (ST) and *M. pectoralis profundus* (PP) muscles were removed from the right-hand side of chilled carcasses at 5 days post-mortem.

2.1. IMCT- perimysium extraction and collagen content

Small strips of perimysial connective tissue were dissected from subsamples of each muscle as described by Latorre, et al. [26]. Each strip of perimysial connective tissue was then hydrolyzed in 5 ml HCl (6 M) at 110 °C for 16 h. After hydrolysis, samples were neutralized and the hydroxyproline concentration was determined by the spectrophotometric determination of hydroxyproline by the colorimetric method of Bergman and Loxley [27].

2.2. Differential scan calorimetry (DSC)

The denaturation kinetics of the perimysial samples were analyzed using a Rheometics Scientific SP DSC, fitted with an Intracooler-I1 unit. Samples (10-15 mg strips) of hydrated perimysial connective tissue were encapsulated in a small sample pan (part n° L7168). Non-isothermal DSC curves were obtained with different heating rates β = 0.5, 1, 2, 5, 10, 15 and 20 K min^{-1}, in the range between 298 and 363 K. Argon was used as sweeping gas and high purity sapphire as a reference. DSC scans were performed in triplicate and the temperature reproducibility of reported thermograms was ±0.5 K. Enthalpies of denaturation (peak areas) and mean denaturation temperatures were recorded.

2.3. Statistical analysis

Data are expressed as mean, standard deviation and standard errors (SE) of three repeats. Comparison between values from the two muscles were calculated by Student's *t*-test and considered significantly different at $p < 0.05$.

3. Results

Fig. 1 shows DSC endotherms for the collagen in perimysium from bovine PP muscle (Fig. 1a) and perimysium form bovine ST muscle (Fig. 1b) at heating rates between 1 and 20 K min^{-1}. As heating rate increases, the endothermal peaks become broader and the position of the peak shifts to higher temperatures. This pattern of changes with heating rate is consistent with the theoretical predictions of the Lumry-Eyring model [28]. At the lowest heating rate, two peaks in the endotherms for perimysium from both muscles were observed.

From each thermogram at seven heating rates (β) from 0.5 to 20 K min^{-1}, the initial (onset) temperature of the endotherm, temperature of the peak and upper maximum temperature of the endotherm

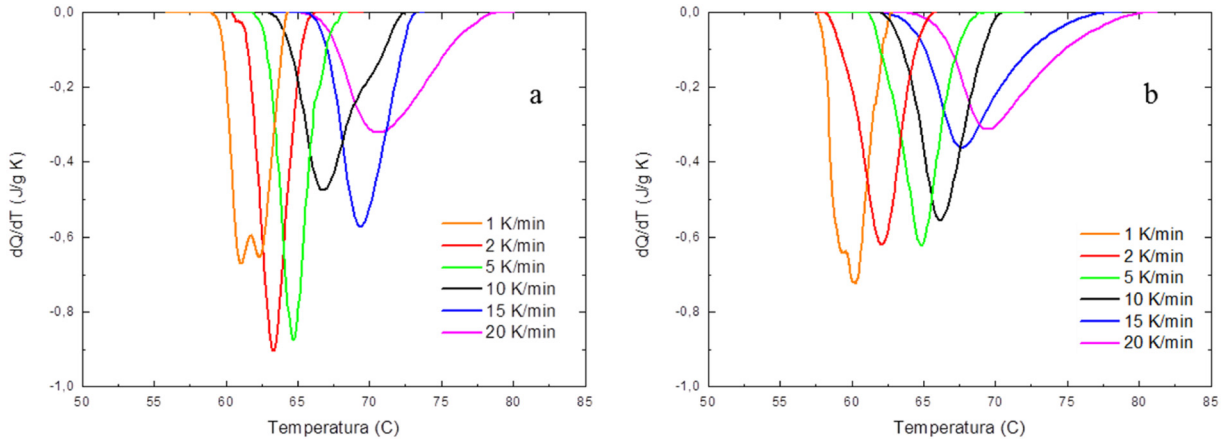


Fig. 1. Mass (weight) normalized DSC curves at different heating rates (0.5, 1, 2, 5, 10, 15 and 20 K min^{-1}) for (a) perimysium from PP muscle, and (b) perimysium from ST muscle.

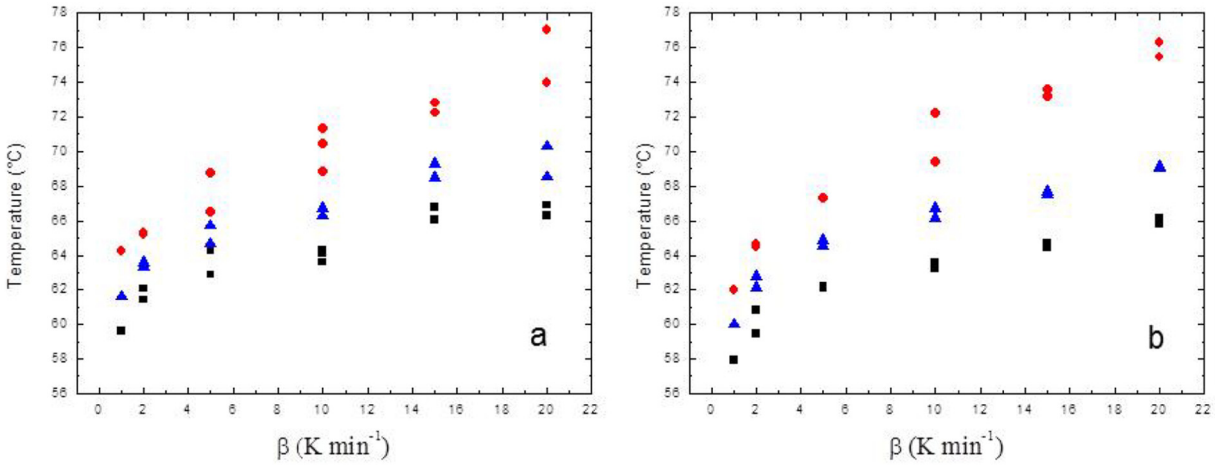


Fig. 2. Mean values of onset (Ti; squares), peak (Tp; triangles) and maximum (Tm; circles) temperatures of endotherms measured at different heating rates for (a) perimysium from bovine PP muscle and (b) perimysium from bovine ST muscle.

(Ti, Tp and Tm; respectively) were evaluated. These temperatures for each heating rate are shown in Fig. 2 for perimysium from bovine PP muscle (Fig. 2a) and perimysium form bovine ST muscle (Fig. 2b). All three temperatures showed a linear relationship with heating rate, in both tissues. Table 1 shows the regression equation for Tp (sometimes referred to as T_{max}, and usually described as the denaturation temperature) versus β for each tissue. At $\beta = 0$, Tp and Ti (the onset temperature) both demonstrated differences between perimysial IMCT from PP versus ST muscles. The collagen present in PP samples showed a higher Tp at $\beta = 0$ than in ST samples. Similar differences have previously been observed by Latorre et al. [29] in the onset temperatures for hydrothermal isometric tension and Tp for perimysium from PP and ST.

The data for IMCT from both muscles shown in Fig. 2 were analyzed by applying either the KAS or Lumry-Eyring model. Using the Mittmeijer et al. [24] modification for KAS, the activation energy (considering the total process as one step) was obtained from the linear regression slope and assuming R as the gas constant. The KAS linear regressions for IMCT from PP and ST are shown in Fig. 3 and the E values so obtained were 338(±29) and 322(±14) kJ mol^{-1}, for the perimysium from PP and ST muscles, respectively. The E value did not show differences between tissues, and both showed good regression fitting ($r^2 = 0.9233$ and 0.9827 for PP and ST, respectively).

Fig. 4 shows the results of the methods described by Vyazovkin et al. [16] to calculate the E_{ef} by application of the Lumry-Eyring model. The dependence of the activation energy on the extent of conversion for collagen denaturation in the perimysium form both the PP and ST muscle is in agreement with the results of Vyazovkin et al. [16] and confirms that the denaturation of collagen in perimysium is in multiple steps. According to the Lumgry-Eyring model the $E_{ef} = E_a$ at $\alpha \to 1$ corresponds to the energy required for the irreversible step in the two-step denaturation process, and the energy of reversible step ($N \leftrightarrow U$) is taken as the E_{ef} at $\alpha \to 0$, where $E_{ef} = E_a + \Delta H$. The values obtained from these assumptions for the energy involved in the reversible and irreversible processes are shown in Table 2 for perimysium from the PP and ST muscles.

Table 1
Values of Tp extrapolated to zero heating rates and fitted linear regression equations of Tp versus β for perimysial IMCT of *Pectoral profundus* (PP) and *Semitendinousus* (ST) muscles. Tp values are means ± standard errors (SE).

	IMCT-PP	IMCT-ST
Tp ($\beta = 0$)	62.73(±0.44)	61.77(±0.51)
Regression eq.	$Tp = 62.73 + 0.369\,\beta$	$Tp = 61.77 + 0.392\,\beta$
R^2	0.89531	0.88876

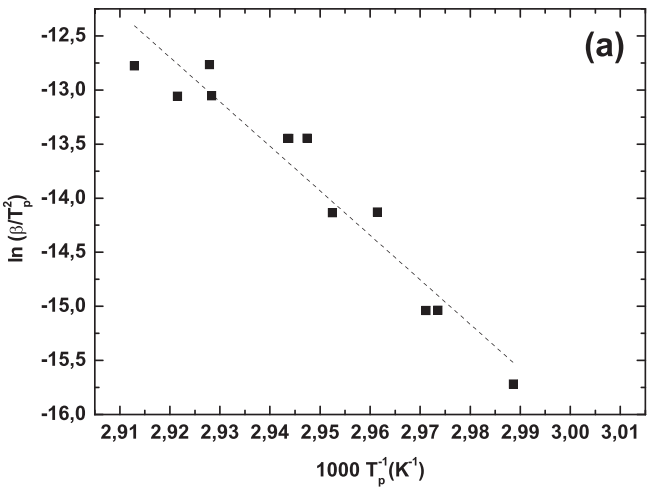
The amino acid hydroxyproline is only found in large quantities in collagen, representing approximately 14% of the total amino acids in mammalian fibrous collagens, and therefore the hydroxyproline content of a tissue is used to measure its collagen content [27]. The hydroxyproline content per gram of each tissue is also shown in Table 2, from which it can be seen that the perimysial IMCT from the ST muscle contains approximately double the collagen content of the perimysium from the PP muscle.

As a baseline for comparison of the KAS and Lumry-Eyring models, the area under each endotherm at each heating rate was taken as a measure of the total energy (Q) consumed in the collagen denaturation process. These values for the perimysium from each muscle are presented for each heating rate in Table 3 as Joules per gram of wet perimysium. Although there is no consistent pattern of variation in Q with heating rate for either tissue, there are small but significant differences between the values for each muscle. Table 4 shows the mean and standard error values of Q averaged across all values of β, which are significantly lower ($p < 0.05$) perimysium from the ST muscle than from the PP. As noted in Table 2, the IMCT for the PP muscle has lower collagen content than the IMCT from the ST muscle. The value of Q for each tissue expressed as J mg^{-1} collagen is also shown in Table 4. This calculation of the denaturation energy on the basis of collagen content yield an even larger difference between the two tissues.

4. Discussion

4.1. Comparison of denaturation energy values and denaturation temperature values with previous literature

In general, both the temperature and the energy values reported in this study show reasonable consistency with previous literature. The predicted temperature of the onset of denaturation (Ti) reported by Miles [10] for lens capsule collagen was in the range of 50–52 °C, with measured values falling in the range 50–56 °C with the thermal scanning rates used (1–20 K min^{-1}). This agrees with recent data of Sun et al. [29] who used second harmonic generation imaging to show changes is rat-tail tendon stability with a Ti of 54 °C. This also accords with the data of Lewis and Purslow [30] showing a reduction of the strength of intramuscular perimysial connective tissue isolated from bovine *Semitendinosus* muscle heated for one hour at temperatures below 60 °C. It is important to contrast these data from cross-linked fibrous collagen with the denaturation temperatures for non-cross-linked collagen. Leikina et al. [21] carried out DSC analysis on soluble, uncross-linked collagen, and reported Tp values between 38 and 41 °C for using heating rates between 0.004 and 1 °C/min.



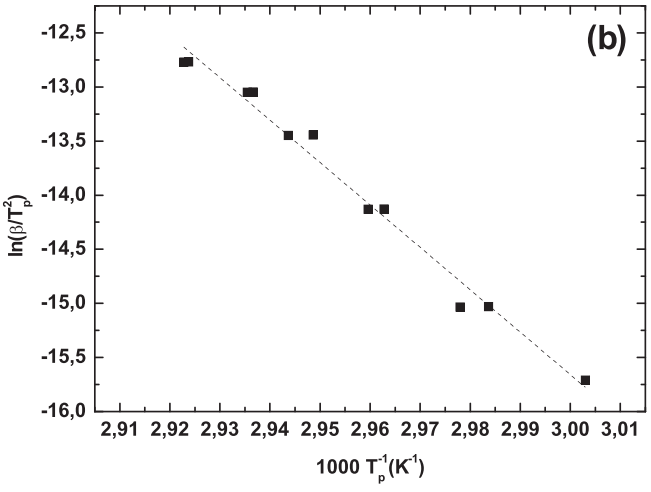


Fig. 3. Fitting the KAS model: plot of ln (βT_p^{-2}) versus (T_p^{-1}). The fitted linear slope corresponds to (E/R^{-1}) (R:gas constant 8,3144 kJ $kmol^{-1}$ K^{-1}) in the model; (a) model fit for perimysium from PP muscle and (b) model fit for perimysium from ST muscle.

Table 2
Effective activation energy by application of the KAS method and activation energy of irreversible step and standard enthalpy of the reversible step according to the Lumry-Eyring mechanism calculated by applying an isoconversional method, for collagen denaturation in perimysial IMCT from *Pectoral profundus* (PP) and *Semitendinousus* (ST) muscle. Also shown is the collagen content in perimysial IMCT from PP and ST muscle in terms of mg of collagen per gram of wet tissue.

	IMCT-PP	IMCT-ST
KAS model (one-step)(one process) $E_f \equiv E_a$ (kJ mol^{-1})	338 ± 29	322 ± 14
r^2	0.92329	0.98268
Lumry-Eyring (reversible and irreversible step $N \leftrightarrow U \rightarrow D$)		
$E_f \equiv E_a + \Delta H$ ($\alpha \rightarrow 0$) (kJ mol^{-1})	386	393
$E_f \equiv E_a$ (kJ mol^{-1}) (irreversible component $\alpha \rightarrow 1$)	263	158
ΔH (kJ mol^{-1}) (reversible component $E_f(\alpha \rightarrow 0)$-$E_a$)	123	235
Collagen content (mg/g wet tissue)	51.50 ± 0.86 (0.497)	109.99 ± 3.59 (2.54)

It is tempting to speculate on the cause of the double peak shown at the lowest heating rates in Fig. 1 for perimysium from both muscles. For the bovine ST and PP muscles, Light et al. [9] show that type III accounts for 41–42% of the total collagen in the perimysium. Type III collagen is comprised of three identical type III alpha-1 chains (circa 100 KDa each), and so has a molecular weight of approximately 300 KDa, which is roughly three-quarters of the molecular weight of type I collagen (407 kDa). Despite the fact that type III collagen is stabilised by interchain disulphide bonds not present in type I collagen [31], the enthalpy of denaturation of collagen type III is reported to be low compared to type I collagen [22]. However, without an analysis of the proportion of collagen types in the specimens used here, it is not possible to speculate that the two peaks reflect the denaturation of the two collagen types, and this is recognised as a limitation in the current study.

Brüggemann et al. [32], using second-harmonic generation imaging, reported variable T_{onset} values for collagen strands in different locations within pork muscle; some (epimysial) collagen from the outer surface of the muscle showed a T_{onset} of 57 °C, whereas other collagen fibres within the meat showed higher T_{onset} values. The fibre-forming type III collagen (not present in rat-tail tendon, but present in a wide range of other connective tissues, including intramuscular connective tissue)

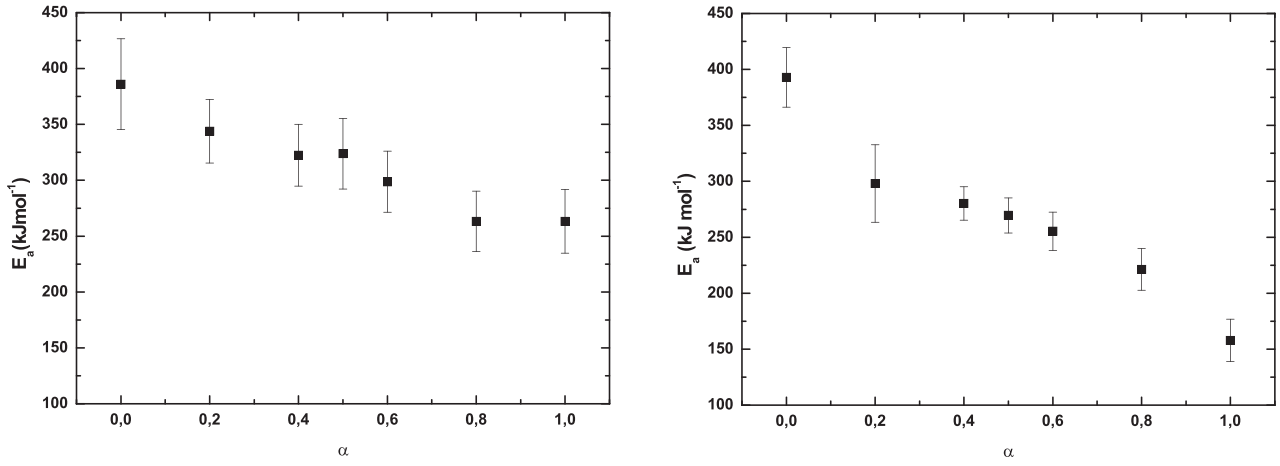


Fig. 4. Effective activation energy calculated by the Lumry-Eyring method versus the extent of conversion for (a) perimysium from the PP muscle and (b) perimysium from the ST muscle.

Table 3
Total denaturation energy (Q), calculated as total area under the endothermal peaks, for each heating rate (β) for collagen denaturation in perimysium from *Pectoralis profundis* muscle (PP) and *Semitendinosus* muscle (ST). Q is measured in Joules per gram of wet perimysium (w.b. = wet basis). Q values are means ± standard errors (SE); $n = 3$.

β (K min^{-1})	Q (J g^{-1} IMCT-perimysium w.b.)	
	PP	ST
1	2.0 (±0.4)	1.6 (±0.2)
2	1.9 (±0.4)	1.5 (±0.2)
5	2.2 (±0.2)	1.7 (±0.2)
10	1.9 (±0.2)	1.5 (±0.1)
15	2.1 (±0.1)	1.6 (±0.1)
20	2.5 (±0.3)	1.8 (±0.2)

Table 4
Average total denaturation energy (Q), as average of total area under the endothermal peaks at different heating rate (β) expressed as Joules per gram of wet perimysial tissue (w.b. = wet base), and as Joules per milligram of collagen in the perimysium of PP and ST muscles.

Total denaturation energy (Q)	IMCT-PP	IMCT-ST
J g^{-1} IMCT-perimysium w.b.	2.11 ± 0.23(0.10)^{a}	1.62 ± 0.11(0.04)^{b}
J g^{-1} collagen*	40.9 ± 4.43(1.81)^{a}	14.65 ± 0.98(0.40)^{b}

Tabulated values are mean ± standard deviation, with standard error of the mean (SE) in brackets. Different superscripted letters in the same horizontal row indicate significant differences ($P < 0.05$). * Calculated on the basis that hydroxyproline constitutes 13.5% of the mass of collagen.

contains interchain disulphide bonds at the c-terminal end of the triple helical collagen molecule and these are thought to confer some reversibility on denaturation. Miles and Bailey [22] report ΔH values of 1255 kJ mol^{-1} for type I collagen and 372 kJ mol^{-1} for type III, although it is not clear in their paper what the sources of the collagens are (no native connective tissue contains solely type III collagen), or what conditions (media) they were kept in.

Values in the literature for the apparent (total) enthalpy of denaturation of collagen in a variety of tissues are shown in Table 5. The absolute values for total enthalpies from both the KAS and Lumry-Eyring models reported in Table 2 are in broad agreement with the lower ranges of the values in Kj mol^{-1} shown in Table 5. Vyazovkin et al. [16] report apparent activation energies of denaturation between 375 kJ mol^{-1} at 0% conversion of N to D forms down to 160 kJ mol^{-1} at 90% conversion. The results in Table 2 are in accord with their value at 0% conversion. In terms of enthalpies reported as Joules per gram of collagen, the figure for collagen in perimysium form the PP muscle in Table 4 is consistent with the published values listed in Table 5, although the value for collagen in the perimysium from the ST muscle reported in Table 4 is lower. In this study the dry weight of the DSC samples was not taken, and so it is not possible to express values in terms of joules per gram of dry weight. This is recognised as a limitation of this study.

4.2. Differences in composition and terminal denaturation parameters between the perimysium of PP and ST muscles

The calculation of the amount of collagen per gram of wet tissue in the perimysium shows a surprising variation between the two muscles, with the apparent collagen content in the perimysium from the PP muscle being much lower than from the ST muscle. This result is surprising because it has been demonstrated by Bendall [33] that IMCT from ST muscle contains a high amount of elastin (37% by dry weight of the total intramuscular connective tissue). Bendall [33] did not analyse the elastin content of the PP muscle, but notes that generally the elastin content of most bovine muscles is low, with ST being the major exception. Bendall [33] reported that only 5.5% of the weight of the total connective tissue in bovine *Pectoralis superficialis* muscle is elastin. Rowe [34] showed that the bulk of the elastin fibres in the IMCT of bovine ST muscle is associated with the perimysium. As a significant fraction of the perimysium from ST muscle is occupied by elastin, it is surprising that the collagen content reported in Table 2 for ST perimysium is higher than for perimysium from the PP muscle. Light et al. [9] compared the connective tissue composition of six bovine muscles, including ST and PP. Although they crudely separated the endomysium form the perimysium, the collagen content of IMCT from the various muscles is only reported as a percentage of the dry mass of the whole muscle; there are no figures given for the collagen content of the perimysial fraction for each muscle.

4.3. Insights given by the Lumry-Eyring versus the KAS model on differences between the IMCT of the two muscles studied

The values in Table 2 for total apparent enthalpy of denaturation given by the KAS model do not differ greatly between the two muscles, and the effective activation energy calculated by the Lumry-Eyring method at 0% conversion ($\alpha = 0$) are in agreement with these values from the KAS model (Fig. 3 and Table 2). However, analysis using the Lumry-Eyring model exposed clear difference in the denaturation process of collagen in the two tissues as the degree of conversion increases. As α approaches 100%, the effective activation energy for collagen denaturation in the ST perimysium is much lower than in the PP perimysium. Using the normal assumptions of the Lumry-Eyring model, this implies that the energy required for the reversible native to unfolded step of the two step process (N → U) is approximately twice as great for collagen in the ST perimysium than in the PP perimysium, whereas the energy consumed in the irreversible unfolded to denatured step (U → D) is much greater in collagen of the PP perimysium versus the ST perimysium (Table 2). It is possible that the presence of elastin in the ST perimysium may partially explain these differences. Despa et al. [35] show that "crowding" of proteins can affect the transition between native and unfolded states. They compute that stearic restrictions provided by more stable proteins in a mixed system within the radius of gyration of unfolded collagen can increase the thermal stability of the collagen. While the analysis of Despa et al. [35] is primarily aimed at explaining

Table 5
Literature values of denaturation energy for collagen.

kJ mol^{-1}*	J g^{-1} (d.m)**	Tissue	Reference
860		Lens capsule	Miles [10]
1225		Collagen type I – unknown source	Miles & Bailey [22]
372		Collagen type III – unknown source	Miles & Bailey [22]
370		Sigma collagen I – extracted bovine Achilles tendon	Vyazovkin et al. [16]
518		Rat tail tendon	Miles et al. [43]
	45–60	Rat tail tendon (with varying water content)	Miles et al. [22]
	58.6	–	Miles & Ghelasvili [44]

* Energy per mol of collagen content.
** Calculated as energy per mg of dry mass (d.m.) of collagen.

the higher thermal stability of collagen in fibres and tissues versus collagen in dilute solution, their analysis may equally explain why the presence of significant amounts of elastin affects the N ↔ U transition. Hydrated elastin does not show an appreciable endotherm in DSC scans up to 90 °C [36]. As the temperature is raised above 27 °C hydrated elastin actually undergoes a well-documented "inverse temperature transition", becoming more-ordered rather than less-ordered. Therefore it is possible to speculate that the presence of thermally-stable elastin in the perimysium for ST muscles may crowd the collagen and increase the energy required to transform it into the unfolded state. An alternative explanation may be that the cross-linking of the collagen may conceivably be affected by the high elastin content in the perimysium of the ST muscle, increasing the energy required for the reversible unfolding (N → U) process. On this basis, we would have to suppose that the collagen molecules in the ST muscle are more difficult to unwind into individual helices due to the presence of elastin, but subsequently required less energy to irreversibly denature than collagen molecules in the perimysium for the PP muscle.

4.4. Practical implications

The results reported above have implications for our understanding of cooked meat quality. It is recognised that differences between the amount and cross-linking of collagen explain part of the differences in the toughness different muscles when cooked and eaten as meat [1,37–39]. The results presented here suggest that the exact nature of the complete thermal transition of perimysial collagen from native to denatured state varies between the ST and PP muscles, and these differences may be another contributor to variations in toughness between cooked muscles. In the meat science literature it is often assumed that collagen denaturation contributes to toughening of cooked meat only at cooking temperatures of 63–65 °C and above [39–41]. In this present study, the lowest or onset temperature (Ti) observed was 58 °C for perimysium of the muscles analyzed, and occurred at the lowest scanning rate. While the temperature at the maximum rate of denaturation (Tp) is often given as "the" denaturation temperature of collagen, these Ti values are important to consider in domestic and industrial cooking of meat. In conventional domestic oven-roasting of meat, it is not uncommon for the central region of the meat to go from room temperature to 70 °C or 80 °C (depending on consumers' preferences for how well-cooked they like their meat) during cooking times of 1–2 h. This implies that the heating rate in the centre of a meat roast can be as little as 0.5 °C/ min. Commercial cooking practices using lower cooking temperatures for much longer times may involve holding meat close to 60 °C for many hours [42]. Under these long-time low-temperature circumstances, the slow denaturation of intramuscular collagen at temperatures near the equilibrium values can make a significant contribution to changes in cooked meat toughness.

5. Conclusion

There are differences in the thermal denaturation of collagen in the perimysium of two bovine muscles, as evidenced by differences in onset temperatures (Ti) and effective activation energy. The variations in the endothermic DSC peaks derived at various heating rates for both perimysial tissues show features which accord with the two step N ↔ U → D process that is the basis of the Lumry-Eyring model. While the KAS model gives a reasonable estimate of the overall activation energy, the Lumry-Eyring model points to differences in the energy used in the reversible and irreversible step of the two-step denaturation process between the perimysium from the two muscles. These differences may be related to the different compositions of the two tissues, either in terms of the content of covalent collagen cross-links, or the presence of elastin in the perimysium from ST muscle.

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