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NMR and DSC studies during thermal denaturation of collagen

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Abstract

Epimysial and intramuscular connective tissues from calf and cow muscle were studied by NMR and DSC. Water proton NMR transverse relaxation times (T_2) were measured at 10°C for both native and thermally-denatured at 90°C for 30–360 min. DSC measurements were used to determine the temperature and the variation enthalpy of sol→gel transition. According to the heating time, significant differences were observed between tissues. NMR discriminated the type of collagen whereas DSC distinguished the age of tissue. Differences were related to the degree of protein hydration, emphasising the complementary information from these two analytical tools.

Keywords: Collagen; Thermal Denaturation; NMR; DSC

1. Introduction

According to Ricard-Blum and Ville (1989) there are 13 genetic variants of collagen in mammalian tissues. In epimysium tissue, the most abundant type of collagen is type I collagen which is composed of two α1(I) chains and one α1(I) chain (Bailey, 1987; Pearson & Young, 1989). Aggregation of type I collagen results in the formation of thick fibrils with a not very high thermal stability (Light & Champion, 1984; Rochdi, 1984). A high proportion of intramuscular collagen type III collagen composed of three identical α1(III) chains. Type III collagen forms fine fibres. Intramolecular cross-links are formed from lysine and hydroxylysine which stabilise the collagen molecules and give tensile strength to the connective tissue which becomes increasingly thermostable with ageing. Thermal denaturation of collagen induces unfolding of the triple helix. The molecule of collagen can be dissociated to form the α, β and γ components which are monomers, dimers and trimers of α chains, respectively (Veis, 1964).

Nuclear magnetic resonance (NMR) is a non-destructive technique which is used to study food or protein hydration (Hills, 1992a,b; Koenig & Brown, 1993; Otting & Liepinsh, 1995) because the ^{1}H NMR relaxation times provide an insight into the dynamics of water macromolecules and their local environment. In this study, transverse relaxation times (T_2) were measured for different native connective tissues and after denaturation at 90°C for different times.

2. Material and methods

2.1. Preparation of tissues

Epimysium and intramuscular connective tissue was extracted from *postrigor* (1 day after slaughter) *Longissimus dorsi* and *Pectoralis profundus,* respectively, from both a 3-month old calf and a 6-year old cow. The extracted tissues were washed 4 times in acetone to remove all fat and then dried using P_2O_5.

The extracted tissue was rehydrated by adding deionised distilled water to dried samples and left for 2 days at 4°C. The moisture content ($\frac{\text{water weight}}{\text{dry matter}}$) was 4. The rehydrated samples were placed in NMR tubes and sealed immediately.

Thermal solubility was determined by the method of Kopp and Bonnet (1982). Dry matter contents were measured after drying at 106°C for 24 h.

2.2. Thermal denaturation

Samples were heated at 90°C for different times from 30 up to 360 min. They were then cooled at 4°C.

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2.3. NMR measurement

All NMR measurements were performed on a Bruker PC20 spectrometer. The probe was thermostatted at 10°C. The samples were left at 10°C for 10 min before NMR measurements. Transverse relaxation measurements were performed using the CPMG (Carr–Purcell–Meiboom-Gill) pulse sequence [90°-(τ-(180°−2τ)$_{10}$−180°-τ-acquisition)$_{160}$]. The echo time (2τ) was 1 ms. The relaxation delay was 5 s and 4 accumulations were performed. Decay curves were fitted by using a non-linear regression program based on residual absolute minimisation of sum-squares. Experimental data were always best fitted with two exponentials. The following general model was therefore chosen to describe the relaxation behaviour:

$$y(t) = A\exp\left(-\frac{t}{T_{2s}}\right) + B\exp\left(-\frac{t}{T_{2l}}\right)$$

The first relaxation time T_{2s} and the corresponding population $P_{2s}[P_{2s} = 100 \times \frac{A}{A+B}]$ characterised the water with the shortest relaxation time.

2.4. Differential scanning calorimetry

Calorimetric measurements were performed with a Setaram DSC-111 differential scanning calorimeter. A standard experimental cycle was applied as previously described (Kopp, Bonnet & Renou, 1989). The analysis of the thermogram allows the determination of the beginning of the melting temperature (T_{bm}) and variation enthalpy ΔH associated with sol→gel transition

Calorimetric measurements and thermal solubility were performed after 2 days at +4°C with water: collagen ratio 4 (w/w).

3. Results

The variations of T_{2s} and T_{2l} are shown in Figs. 1 and 2, respectively. It is noteworthy that T_{2s} values rose steeply for all tissues in the first 30 min and then increased slowly with time. The behaviour of T_{2l} varied according to the tissue. For epimysium from cow or calf the T_{2l} values decreased in the first 30 min while, for intramuscular tissue, T_{2l} went through a maximum after 30 min of heating and then decreased steadily. The populations P_{2s} decreased (Fig. 3) and, for young animals, the decrease in amplitude was less marked.

The DSC thermograms showed only transition between 10 and 50°C. This temperature range corresponds to the transition sol→gel. The collagen is denaturated after heating at 90°C. The DSC results (Table 1) show a strong decrease in T_{bm} after 30 min of heating for all 4 tissues and thereafter showed little change in calf whereas, for tissues from cow, T_{bm} decreased progressively from 20°C at 30 min to 13°C at 360 min. The variation enthalpy, ΔH, associated to sol→gel transition decreases in cow tissue while, in calf, no variation was observed. These results were similar at those observed for solubility (Fig. 4). After 120 min of heating, the solubility of both tissues from calf reached a plateau whereas it increased with continued heating for cow tissue (Fig. 4). Linear relationships were found between solubility and heating time ($R^2 = 0.992$ and 0.990) for epimysium and intramuscular tissue from cow.

4. Discussion

The CPMG decay data obtained for hydrated collagen samples were multi-component as expected in such inhomogeneous or compartmentalised systems (Belton & Ratcliffe, 1985). In native collagen tissues, the

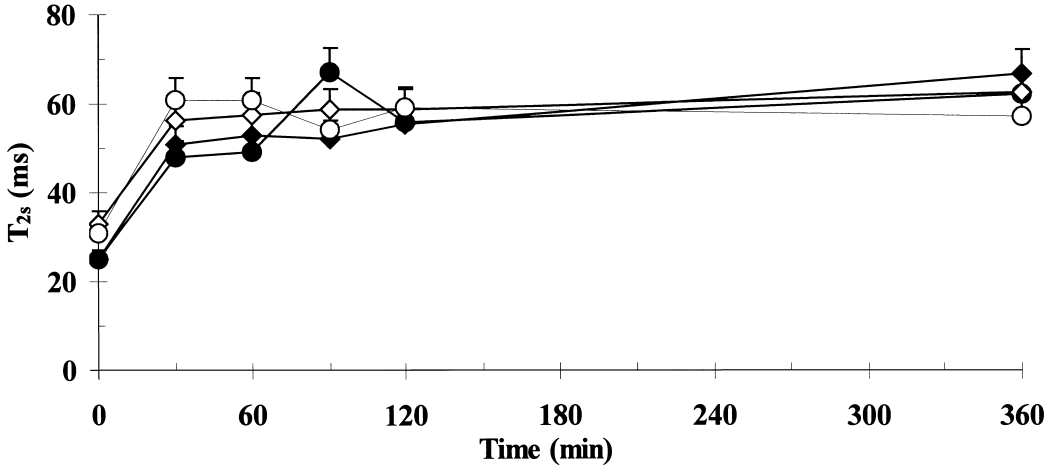

Fig. 1. Variation of T_{2s} as a function of heating time for 4 tissues: ◇: calf intramuscular, ◆: cow intramuscular, ○: calf epimysium, ●: cow epimysium.

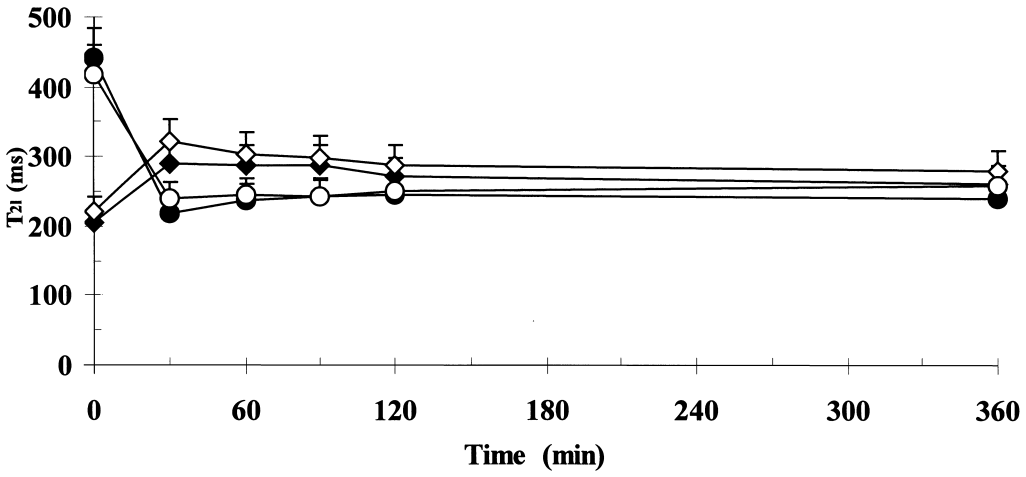

Fig. 2. Variation of T_{2l} as a function of heating time for 4 tissues: ◇: calf intramuscular, ◆: cow intramuscular, ○: calf epimysium, ●: cow epimysium.

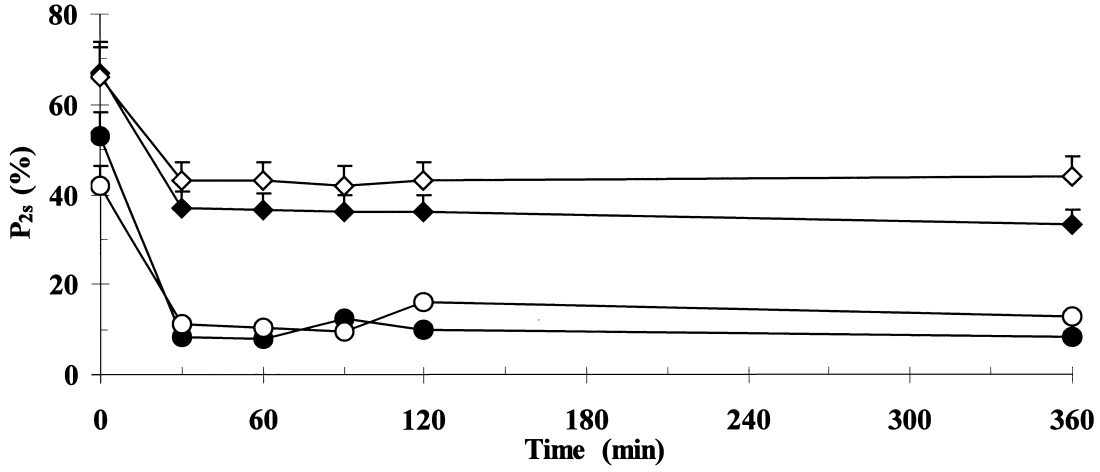

Fig. 3. Variation of P_{2s} as a function of heating time for 4 tissues: ◇: calf intramuscular, ◆ cow intramuscular, ○: calf epimysium, ●: cow epimysium.

Table 1
Effect of heating time at 90°C on DSC parameters for different tissues

	T_{bm} (°C)				ΔH (J/g DM)			
	Epimysium		Intramuscular		Epimysium		Intramuscular	
Time	Calf	Cow	Calf	Cow	Calf	Cow	Calf	Cow
0	58.6 ± 0.8	61.5 ± 0.2	61.6 ± 0.2	65.7 ± 0.2	90.6 ± 1.9	78.1 ± 1.2	58.6 ± 0.6	45.6 ± 0.2
30	14.4 ± 0.6	19.8 ± 0.2	14.5 ± 0.1	19.1 ± 0.1	18.3 ± 0.2	8.0 ± 0.6	12.0 ± 1.1	3.8 ± 1.2
60	13.7 ± 1.8	15.7 ± 1.7	14.4 ± 0.1	16.9 ± 1.6	18.8 ± 1.7	9.4 ± 0.8	11.9 ± 0.9	5.7 ± 0.7
90	13.3 ± 1.6	13.5 ± 0.8	13.7 ± 1.4	15.7 ± 0.5	18.1 ± 1.3	11.8 ± 0.6	11.6 ± 1.8	7.0 ± 0.8
120	14.6 ± 0.6	13.0 ± 0.3	14.0 ± 1.0	15.1 ± 0.7	18.3 ± 2.9	13.2 ± 1.0	11.5 ± 2.5	9.8 ± 1.5
360	14.5 ± 3.5	12.9 ± 2.6	13.6 ± 1.0	13.0 ± 0.7	18.4 ± 2.0	15.3 ± 0.6	12.3 ± 1.0	15.3 ± 1.9

T_{2s} had similar values for the different tissues but T_{2l} values in epimysium tissue were twice those in intramuscular tissues. These high values of T_{2l} could indicate that the water had a low interaction with macromolecules in the compact structure of the epimysium. Aggregation of type I collagen (epimysium) forms fibrils that are thicker than those of type III (intramuscular) which forms only thin fibrils (Bailey, 1987). Previous results (Finch, Gardner, Ledward & Menashi, 1974; Kopp et al., 1989) on native collagens showed that cleavage of hydrogen bonds (an endothermic process) lowers both both temperature (T_{bm}) and enthalpy of

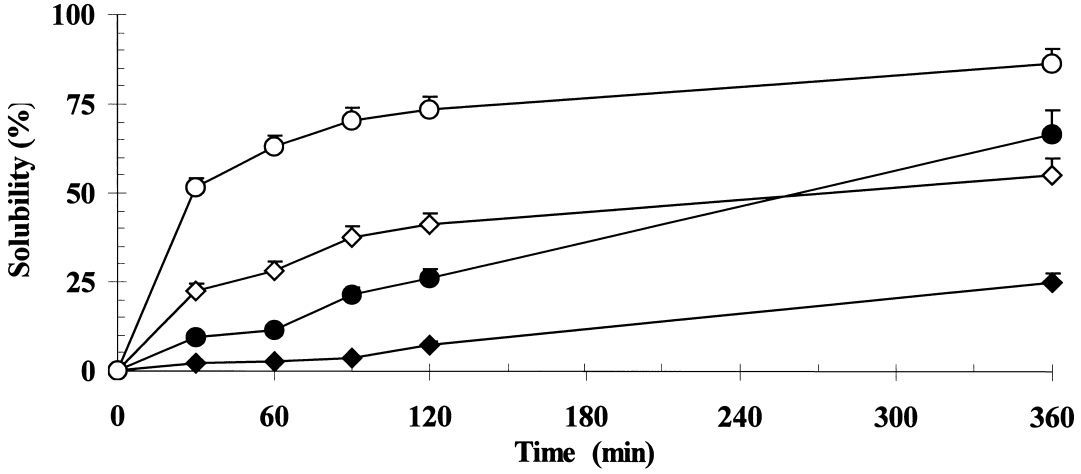

Fig. 4. Variation of the thermal solubility as a function of heating time for 4 tissues: ◇: calf intramuscular, ◆: cow intramuscular, ○: calf epimysium, ●: cow epimysium.

denaturation. Similarly, if exothermic hydrophobic bond cleavage occurs, denaturation enthalpy should increase and T_{bm} should decrease. Our DSC results from native tissues (Table 1) are consistent with a higher hydrophobicity in calf than in cow and in epimysium than in intramuscular tissues. Moreover, hydrophobic interactions are known to stabilise the cross-linked fibres (Finch & Ledward, 1972). Higher cross-linking can be displayed by the lower solubility in calf epimysium than in cow intramuscular (Fig. 4). However, the difference in denaturation enthalpy between calf and cow tissues is less than between epimysium and intramuscular tissue, while the difference in amplitude of T_{bm} is similar. These results suggest a more important contribution of hydrogen bonding in the younger (calf) tissue than in older (cow) tissue.

The thermal denaturation first causes breaking of hydrogen bonds which stabilise the native helical structure. Some intermolecular cross-links are then broken (Valin & Kopp, 1978). This difference between tissues could be the result of differences in breaking of hydrogen bonds during the first step of denaturation. The epimysium thick fibres in the native state could allow the access of water protons to specific sites after thermal denaturation. This change in water interaction induces variation in the relaxation parameters (Hills et al., 1990; Otting & Liepinsh, 1995) and a decrease in T_{2l}. After heating at 90°C for 30 min, the collagen was fully denatured but the decrease in temperature (10°C) allows the refolding of the collagen triple helix. Increasing time of heating at 90°C induces the hydrolysis of the polypeptide chains. This prevents the renauration of collagen and exposes hydrophobic area. The cleavage of these exothermic hydrophobic bonds increases ΔH and decreases T_{bm} for cow tissues while the ΔH and T_{bm} are constant for calf tissue because of the low content of cross-links. The different NMR parameters varied significantly in comparison with the native tissue. However, the different tissues had very similar T_{2l} and T_{2s} values, while significant differences between epimysium and intramuscular tissues were observed in P_{2s} values. At 10°C, which is the temperature of NMR measurements, the gelatin is in the gel state. The system is heterogeneous because of the difference in solubility between the α, β and γ components. This heterogeneity could induce different waters. The bound water associated with the various components, is expected to have the short relaxation time (T_{2s}). However, no linear relationships were found between the bound water represented by P_{2s} and thermal solubility. Woessner (1963) pointed out that, in two phase systems, the NMR observable populations and observable relaxation times are dependent on the exchange rate between the two phases and are not the values characteristic of each phase. In our system, the different fractions of water should be in exchange and the P_{2s} values should be dependent on the water-macromolecule interactions which as expected are dependent on the tissue.

5. Conclusion

The thermal denaturation is a progressive process which has been studied by using two methods: NMR and DSC. In all tissues, changes occur in the first 30 min of heating at 90°C but were more dramatic in younger tissues with fewer collagen crosslinks. Each method gives different information. The NMR results (T_{2l} and P_{2s}) differentiate epimysium and intramuscular tissues which corresponds to different types of collagen while the DSC discriminates differences due to tissue age. Intermolecular cross-links play an important role in the thermal stability. The differentiation of epimysium from intramuscular tissue observed by NMR is noteworthy. The water dynamics in the tissue depend on its

molecular structure. The availability of sites on the macromolecule induces a great variation in NMR parameters because of either an exchange of water protons with those of collagen or a formation of hydrogen bonds.

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