In situ thermal denaturation of myofibre sub-type proteins studied by immunohistofluorescence and synchrotron radiation FT-IR microspectroscopy

Thierry Astruc, Frédéric Peyrin, Annie Vénien, Roland Labas, Magali Abrantes, Paul Dumais, Frédéric Jamme

Abstract

The thermal denaturation of proteins in skeletal muscle was studied and characterised for the first time taking into account the in situ metabolic and contractile fibre types. From serial histological sections, collagen, elastin, various type I, Ila and IIX fibres and type I–Ila and Ila–IIX hybrids were identified by immunohistofluorescence. Histological sections were incubated in buffer solutions at increasing temperatures (40, 50, 60, 70 and 80°C). Protein secondary structure was investigated by synchrotron radiation FT-IR microspectroscopy on connective tissue and in muscle fibres rigorously identified for sub-type. Whatever the target protein components, increasing temperature resulted in a decrease in α-helix secondary structure and an increase in β-sheet structure. This phenomenon was more pronounced for intracellular proteins than for connective tissue. Although hybrid fibres were generally somewhat less sensitive to unfolding than the pure types, the amplitude of the thermal denaturation of intracellular proteins was practically independent of fibre type.

1. Introduction

Different technological treatments are applied to meet before consumption, cooking being the most common. Cooking meat improves palatability, preservation and protection against microorganisms. Numerous studies have characterised the heating-induced changes in meat structure and their relationships with organoleptic qualities (for review, see Hamm (1977) and Tornberg (2005)). Heating causes tissue shrinkage, increasing shear forces thus releasing juice. At molecular level, heating breaks the weak bonds between aminocoids, leading to a loss of three-dimensional protein conformation. Thermal denaturation of macromolecules first exposes hydrophobic sequences at their surface (Morita & Yasui, 1991; Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008) and secondly forms bonds between hydrophobic and oxidised residues of proteins, causing aggregates to form (Hamm, 1977; Morita & Yasui, 1991; Santé-Lhoutellier et al., 2008). However, the behaviour of meat during heating processes depends on the structure and composition of the skeletal muscles in the live source animal. Muscles are composed of connective tissue, fat and muscle fibres of different types based on twitch speed (slow or fast) and metabolism (oxidative or glycolytic; for review, see Xiong (1994) and Lefèvre, Culioli, Joandel-Monier, and Ouali (1999)). Muscle connective tissue is structured into organisational layers called epimysium, perimysium and endomysium that ensheath the muscle, bundles of muscle fibres, and each muscle fibre, respectively. Connective tissue is approximately 2% dry matter (Bailey & Light, 1989). It is composed mainly of collagen and to a lesser extent elastin (on average, about 5% of total connective tissue). Semitendinosus muscle is atypical, as it has a significantly higher proportion of elastin, at about 40% of total connective tissue. Connective tissue is largely responsible for the toughness of the meat, but the texture and water-holding capacity of the product is modulated by the heat treatments applied during cooking. Elastin is very heat-stable whereas the collagen denatures from 53 to 63°C, shrinks at between 60 and 70°C, and turns into gelatin depending on the thermally-stable crosslinks of the molecule and the temperature–cooking conditions (Stabursvik & Martens, 1980; Tornberg, 2005; Voutila, Ruusunen, & Puolanne, 2008).

The vast majority of muscle composition is muscle cells (about 90% dry matter) of four major fibre types, i.e. type I (slow-oxidative), IIa (fast-oxidative), IIb and/or IIX (fast-glycolytic), in variable proportions. Muscle with a high proportion of myoglobin-rich oxidative metabolism fibres is generally called red muscle, while muscle mainly composed of myoglobin-poor glycolytic metabolism fibres is known as white muscle. The underlying molecular basis of this typology resides in the polymorphism of myosin heavy
chains (MyHC). These characteristics make it possible to use monoclonal antibodies (mAbs) against myosin heavy chain (MyHC) isoforms to precisely identify the type I, IIA, IIX and IIB fibres in striated muscles (Schiaffino et al., 1989; Labas, Meunier, Picard, & Astruc, 2009; Picard & Cassar-Malek, 2009). One of the major values of using mAbs is to delineate hybrid fibres (I–IIa, IIa–IIX and IIX–IIB) which simultaneously express different isoforms of MyHC inside the same fibre (for review, see Pette and Staron (2000)).

Myosin represents about 43% of myofibrillar proteins in mammals and is extensively involved in gelation events during heating (Fretheim, Samejima, & Egelandsdal, 1986; Liu, Foegeding, Wang, Smith, & Davidian, 1996; Morita & Yasui, 1991, for review, see Xiong (1994) and Sun and Holley (2011)).

Several studies report that myosin unfolding increases during heating and that myosin from white muscle denatures and aggregates at a lower temperature than myosin from red muscle, in rabbit (Boyer, Johandel, Roussilhe, Culioli, & Ouali, 1996), chicken (Liu et al., 1996), cattle (Egelandsdal, Martinsen, Fretheim, Pettersen, & Harbitz, 1994; Fretheim et al., 1986) and salmon (Lefèvre et al., 1999; Stabursvik & Harbit (Boyer, Johandel, Roussilhe, Culioli, & Ouali, 1996)). The thermal denaturation of myosin varies more between red and white muscles than between muscles from different animal species (Lefèvre et al., 1999; Stabursvik & Martens, 1980).

On the other hand, Xiong and Brekke (1990a, 1990b) did not find differences in the unfolding of myofibrillar proteins of leg (red) and breast (white) chicken muscles but instead found differences in protein–protein association and gelation. The time-course evolution of the molecular organisation of myosin during heating is pH-dependent (Lefèvre et al., 2007; Penny, 1967; Stabursvik & Martens, 1980; Vega-Warner & Smith, 2001; Xiong, 1994), with white fibres being more sensitive to the physicochemical environment than red fibres. Red muscles generally have higher ultimate pH values than white muscles (Lefèvre et al., 1999; Vega-Warner & Smith, 2001), which may partly explain the variation in thermal stability observed between myosin extracted from muscles with different fibre type proportions.

However, none of these studies have considered the numerous molecular interactions found in biological muscle tissues that have a high degree of structural organisation. This important parameter can only be integrated by implementing techniques that do not destroy sample structure during sample preparation. Various studies have tracked the thermal denaturation of muscle proteins during heating using infrared microspectroscopy, where the infrared spectra revealed an increase in β-sheet and a decrease in α-helical structures of proteins (Bocker et al., 2007; Kirschner, Ofstad, Skarp, Host, & Kohler, 2004; Kohler, Kirschner, Oust, & Martens, 2005; Wu et al., 2007). This structural evolution was much stronger in myofibres than connective tissue (Kirschner et al., 2004). However, to our knowledge, no study has been conducted to characterise the effects of fibre type criteria on the in situ evolution in molecular structure of muscle proteins.

An advantage of FT-IR microspectroscopy is that it can be easily combined with histological investigations. In addition, the elongated morphology of muscle cells makes it possible to realise serial cross-sections where the same cells are present on several consecutive sections. Each section can then be used in parallel to characterise individual cells using histological staining and then to perform microspectroscopy studies. This approach enables spectral measurements on tissue components and rigorously-identified cell types. However, FT-IR microspectroscopy is hampered by the fact that laboratory sources limit spatial resolution to 20 square microns. However, a confocal geometry microscope used with a bright-source synchrotron radiation makes possible the in situ spectral acquisition of biological tissues at subcellular scale.

The aim of this study was to investigate the effect of increasing temperature on unfolding evolution of proteins located in different compartments of muscle tissue. Myosin isoforms and extracellular matrix proteins were identified by immunohistochemistry. The in situ evolution of protein denaturation was followed on strictly-identified biological compounds and myofibre sub-types using FT-IR microspectroscopy coupled with the synchrotron beamline to enable the acquisition of high-quality spectra of size-limited perymysium excluding the surrounding muscle cells, thus improving measurement accuracy.

2. Materials and methods

2.1. Animal and samples

The experiment was carried out on bovine M. Semitendinosus (ST) from a 10-year-old Charolais cow slaughtered in a commercial slaughterhouse. The entire Semitendinosus muscle was excised 24 h postmortem and brought to the lab for the experiment. pH measured directly in six points of the muscle using a puncture pH electrode (WTW model TFK 150/E connected to a pH meter (WTW model pH315i, Weilheim, Germany) was 5.55 ± 0.02.

Five muscle samples of about 1 cm³ were excised taking into account fibre direction, and frozen in isopentane cooled to −160 °C with liquid nitrogen (−196 °C). Serial cross-sections (10 μm thick) performed using a cryostat (Microm, HM 560) were collected on glass slides and stained with Hematoxylin–Eosin–Safran to visualise general structure. One of the 5 samples was then chosen for subsequent experimentation due to its easily identifiable morphology and representative composition in terms of muscle fibre, adipocyte and connective tissue content.

For this selected sample, serial sections cut transverse to fibre direction were collected and mounted on glass slides for histology (sections of 10 μm thick) and on infrared transparent BaF2 windows (6 μm-thick sections) for infrared microspectroscopy. The number of sections were voluntarily reduced to limit erosion of the muscle block so as to identify the compounds and fibres from the first to the last studied section. Eight sections needed for the FT-IR measurements were surrounded by 14 other histology sections to enable reliable compound and morphology identifications.

2.2. Histology

2.2.1. Histochemistry

Sections were stained with Hematoxylin–Eosin–Safran to contrast the tissue and Picrosirius red to reveal the connective tissue.

2.2.2. Immunohistochemistry

Elastin was identified using a mouse anti-elastin primary polyclonal antibody (E4013 Sigma) and Alexa Fluor 488-labelled goat anti-mouse IgG secondary antibody (A 11001, Invitrogen). The collagen fibres were identified using a rabbit anti-bovine collagen I primary polyclonal antibody (MD 20121, Miobiproducts, Zurich, Switzerland) and revealed by a Cyanine Cy3- labelled goat anti-rabbit IgG secondary antibody (111–165–008, Jackson). Cell outlines were stained using a rabbit anti-laminin primary polyclonal antibody (I9393 Sigma) with a goat anti-rabbit IgG Cyanine Cy3-labelled secondary antibody (111–165–008, Jackson). Fibre typing was performed according to Labas et al. (2009).

Briefly, slow and fast myosin heavy chain (MyHC) isoforms were identified using mouse monoclonal antibodies specific to MyHC isoforms BA-D5 (MyHC-I), SC-71 (MyHC-II) (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and 55 8H2 simultaneously revealing MyHC-I and MyHC-IIx (AGRO-BIO, La Ferté Saint Aubin, France). The different primary MyHC antibodies were revealed by an Alexa Fluor 488-labelled goat anti-mouse IgG secondary antibody (A 11001, Invitrogen).
To reveal antigen expression in muscle tissue, the cross-sections were incubated with both primary antibodies against MyHC and laminin overnight in a humidified box at 4 °C. After washing, primary antibody binding was revealed by incubation for 1 h in the dark at room temperature with both labelled secondary antibodies (Alexa 488 anti-mouse IgG and CY3 anti-rabbit IgG). Controls were performed without the first and without the second antibody to validate the results.

Myofibre subtypes I, IIA, IIX and hybrid I–IIA and IIA–X were deduced according to their responses to the different antibodies.

2.2.3. Observations and images acquisition

Observations and image acquisitions were performed with a photonic microscope (Olympus BX 61) coupled to a high-resolution digital camera (Olympus DP 71) using Cell F software.

HES and picrosirius red-stained sections were observed and images were acquired in bright-field mode while immunohistochemistry images were acquired in fluorescence mode (Cyanine 3: 550/570 nm; Alexa Fluor 488: 495/519 nm).

2.3. Infrared microspectroscopy

2.3.1. Sections preparation for FT-IR measurements

Six serial cross-sections (6 μm) set down on BaF2 windows were incubated for 3 min in 0.06 M phosphate saline buffer, pH 5.6 (i.e. close to the mean muscle pH of 5.55) maintained at 20, 40, 50, 60, 70 and 80 °C, respectively. The sections were rinsed for 1 min in ultrapure water to eliminate any phosphate salt liable to crystalize on the section during drying. A non-incubated control kept at 20 °C at room temperature (20 °C NIC) was also performed. All sections were dried at room temperature and stored 2 days under vacuum with CuSO4 crystals until FT-IR spectra acquisition.

2.3.2. Spectra acquisitions

The experiment was performed at the SOLEIL synchrotron facility (Gif-sur-Yvette, France) on the SMIS infrared beamline. Average current in the storage ring was 400 mA. Infrared spectra of thin histological muscle sections were recorded using a NicPlan IR microscope (Thermo Scientific) coupled to a Magna-IR 560 FTIR spectrometer. The microscope used Schwartzschild type objectives for IR radiation plus a 10x Nikon objective for visible light. Data were collected in transmission geometry with a 32x upper IR objective with a numerical aperture (NA) of 0.65, and a lower objective operating at constant 10x magnification. The upper aperture was set to 10 × 10 μm².

IR spectra were collected point-by-point in a 4000–650 cm⁻¹ range and recorded at a spectral resolution of 4 cm⁻¹. For each myofibre, about 20 IR spectra were collected by accumulating 64 scans, while for collagen and elastin, 15 spectra were collected along a line in a selected area by accumulating 128 scans. For each section, a reference spectrum based on 128 myofibre scans and 256 collagen and elastin scans was recorded from the region of interest in a sample free area in order to correct irrelevant gas contributions from water vapour and CO₂ in the microscope light path. Data acquisition was performed using Omnic software (v7.3, Thermo Scientific, USA).

2.4. Spectra treatments

IR measurements are affected by scattering, which biases spectra baselines. In order to study chemical variations of interest, all spectra were preprocessed prior to multivariate analysis, using the Unscrambler software (v9.8, Camo Software AS, Norway).

At first, extended multiplicative signal correction (EMSC) was used as a flexible method for correcting these spectra artifacts (Kohler et al., 2005) by removing physical light scattering effects from chemical light absorbance effects. The representative reference spectrum for the dataset was calculated as the average of all spectra. In order to improve the spectral resolution, a second derivative, based on a 9 Savitzky-Golay smoothing points and using a third polynomial degree, was applied. The second derivative was multiplied by (−1) for clarity.

2.5. Statistical analysis

Principal component analysis (PCA) was run using the Unscrambler software (v9.8, Camo Software AS, Norway). PCA is applied to the infrared spectra for handling large data sets without preliminary assumption (also named unsupervised approach). PCA is a powerful chemometric method to reveal variances or combination of variables among multivariate data. Here, the computation of PCA is based on Non-linear Iterative Projections by Alternating Least-Squares (NIPALS) algorithm. Score plots were used to show similarity maps making it possible to compare spectra regardless of sample categories. Correlation loading plots derived from first and second principal component X-loading plots were used to reveal and identify characteristic vibrational absorption bands. In this study, the spectral domain was focused on the amide I band (i.e. 1700–1600 cm⁻¹ range).

To highlight the evolutions in the secondary structure of muscle fibres according to cell type, the peaks of the negative second derivatives for the 1655 and 1628 cm⁻¹ wave numbers were measured and expressed as means ± standard error of the mean. Data were analysed using the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA) by one-way analysis of variance (ANOVA) using the general linear model procedure and an unpaired Student’s t-test to determine levels of statistical significance between heating groups. Correlation analysis was performed using Pearson and Spearman tests. Results of Pearson correlations were kept after validation by Spearman tests.

3. Results and discussion

3.1. Components localisation and fibre type identification

The results demonstrated the power of histochemistry for component localisation. HES staining identified muscle fibres, adipocytes and connective tissue and made it possible to select an area of the section based on its representativity in these various muscle components. Furthermore, we exploited the presence of an easily-identifiable blood vessel to identify the region of interest on the different serial sections (Fig. 1A). Laminin, elastin and collagen were localised by immunohistofluorescence (Fig. 1B–D). Different fibre types were identified as described previously (Labas et al., 2009). Fig. 1B illustrates an example of the identification of type-II muscle fibres using the SC-71 MyHC-II antibody.

The double-labelling of myosin isoforms and laminin enhances the readability of fibre types by contrasting the muscle cell periphery, as previously shown by Labas et al. (2009). Fig. 1B shows the abundance of laminin in the blood vessel wall and the adipocyte environment. Collagen and elastin are observably co-localised in the perimysium (Fig. 1C and D).

The typing of muscle fibres showed the presence of type I, type IIA and type IIX fibres but also hybrid type I–IIA and IIA–IIX fibres corresponding to fibres whose properties are changing depending on various factors such as age, stress or physical activity, resulting in the co-expression of specific pairs of major MHC isoforms (Pette & Staron, 2000). These results are consistent with literature reports that have indicated the presence of these fibre types in cattle ST muscle (Labas et al., 2009; Picard & Cassar-Malek, 2009). IIB fibres seen only on several “Blonde d’Aquitaine” breed bovines muscles by Picard and Cassar-Malek (2009) were not detected in this “Charolais” breed animal muscle.
Collagen, elastin, and myofibre types identified by immunohistochemistry were localised on the unstained serial sections intended for FT-IR microspectroscopy measurements (Fig. 2). In this area, each myofibre characterised according to cell type was identified by a number. Three cells of each type were selected for measurements by FT-IR microspectroscopy, except for the I–IIA hybrids which were so rare that the chosen region only contained one cell (Fig. 2). Micro-areas chosen for IR measurement were selected for their closeness and the fact that they were easy to locate on unstained sections.

3.2. Effect of heating on myofibre morphology

Heating the tissue sections resulted in slight lateral contraction of muscle fibres, in agreement with previous observations (Tornberg, 2005). General morphology, however, was sufficiently preserved to allow easy identification of the myofibres and perimysium areas selected.

3.3. Changes in secondary structure of muscle proteins during heating

3.3.1. Muscle fibres

The amide I band situated around 1650 cm\(^{-1}\) is the most useful for analysis of secondary protein structure due to its sensitivity to hydrogen-bonding pattern, dipole–dipole interaction, and geometry of the polypeptide backbone. Indeed, the amide I band informs on components such as \(\alpha\)-helix, \(\beta\)-sheets, turns and irregular structures (Barth, 2007; Jackson & Mantsch, 1995). Second derivatives of spectra were calculated to enhance the spectral resolution of the infrared band. Fig. 3 presents the score plot and correlation loadings of principal component analysis on myofibre second derivative 1700–1600 cm\(^{-1}\) corrected spectra. The score plot shows 6 clusters gradually separating along the PC1 axis as incubation temperature of the sections increases. Ninety two percent of the variance is explained by the component 1 (PC1) and 4% by the second component (PC2). The correlation loadings plot shows that the first principal component axis is oriented to 1647–1658 cm\(^{-1}\) for negative values and to 1628 cm\(^{-1}\) for positive values. Evolutions in average spectra according to incubation and thermal treatments (Fig. 4) confirmed the results of principal component analysis. According to Kirschner et al. (2004), major changes in response to heat treatment occur in the amide I region, which exhibits two well defined peaks at 1628 and 1655 cm\(^{-1}\). The band at 1655 cm\(^{-1}\), which was assigned to \(\alpha\)-helical structure (Barth, 2007; Jackson & Mantsch, 1995), decreased with temperature, while the band at 1628 cm\(^{-1}\), which was assigned to aggregated \(\beta\)-sheet structure (Barth, 2007; Jackson & Mantsch, 1995), increased \((r = -0.903, p < 0.0001; \text{Fig. 4})\). Kirschner et al. (2004) using FT-IR microspectroscopy also found that increasing temperature decreased \(\alpha\)-helix structures and increased \(\beta\)-sheet structures in bovine longissimus dorsi myofibre proteins. However,
simply incubating the sections in buffer at 20 °C led to a slight decrease in α-helix structures. That could have been due to the pH of the incubation buffer (pH 5.6), since a low pH may break hydrogen bonds and consequently partially unfold protein chains.

The change in secondary structure with increasing temperature is the result of intracellular protein denaturation. Indeed, myosin is denatured at about 54 and 58 °C, whereas actin, actomyosin complex and titin are denatured at around 80 °C and the transition temperature of sarcoplasmic proteins is about 65–67 °C (for review, see Tornberg (2005)). Sarcoplasmic proteins, which are soluble at low ionic strength, may be less implicated in the phenomenon than myofibrillar proteins, given that they could have been extracted while the sections were being incubated in heated buffer solution.

Compared with muscle fibres, second derivative spectra of connective tissues – including elastin and collagen – showed a slight shift, as the peaks related to α-helix and β-sheets switched from 1655 to 1658 cm⁻¹ and from 1628 to 1635 cm⁻¹, respectively. According to the results of Kirschner et al. (2004), the connective tissue spectra in the amide I band during heating show less dramatic alteration than myofibre spectra. The elastin-rich area shows little change in amide I bands as a function of heating temperature, which indicates that in situ, elastin is heat-stable up to 80 °C (Fig. 4, Elastin).

Measurements made in the perimysium area where immunohistofluorescence did not detect elastin showed little heating temperature-related variability in the 1658 cm⁻¹ band (Fig. 4, Collagen). However, the 1628 cm⁻¹ band assigned to β-sheet structure showed higher variability, especially from 60 °C. Sections incubated at 70 and 80 °C show higher levels of β-sheet structure than sections incubated at lower temperatures.

The most pronounced changes in molecular structure post-60 °C are consistent with the onset of connective tissue coagulation observed at 60 °C by electron microscopy in bovine ST muscle (Jones, Carroll, & Cavanaugh, 1977; Leander, Hedrick, Brown, & White, 1980). These results were confirmed by differential scanning calorimetry (DSC) data showing that collagen begins to contract at 60 °C (Voutilai et al., 2008) up to a peak temperature at around 65–67 °C (Stabursvik & Martens, 1980; Voutilai et al., 2008). However, in relation to muscle fibres, our results show that even for temperatures above 60 °C, there is little modification in the secondary structure of collagen. This can be explained by the fact that the heating time of just 3 min is not sufficient to cause a significant change in the secondary structure of the connective tissue. In addition, the muscle came from a 10-year-old animal, and it is assumed that the level of collagen crosslinking increases with age, thus increasing its thermal stability (Hamm, 1977).

The mean spectrum obtained on the histological section incubated at 80 °C was the only spectrum that showed a shoulder at
1689 cm$^{-1}$. Kirschner et al. (2004) investigating connective tissue assigned this band to aggregated β-sheet structure. This peak could be linked to the onset of the solubilisation of collagen to gelatin, as suggested by Kirschner et al. (2004).

3.3.3. Muscle fibres sub-types

To highlight the evolution of the secondary structure of muscle fibres according to their cell type, the height of the peak of the second derivatives for the bands located at 1655 cm$^{-1}$ (α-helix) and 1628 cm$^{-1}$ (β-sheet) were used. The results are presented as histograms in Fig. 5.

The results confirm a simultaneous decrease in α-helix and increase in β-sheet structures in response to increasing temperature. Although the overall evolution in secondary structure of proteins during the thermal process remained similar regardless of fibre type, small differences are noticeable: The variability of changes in α-helical structure between fibre types increases after 60 °C. Even taking into account the fact that type I–IIa cells are under-represented in the calculations, it is clear that hybrid fibres are more heat-stable than pure-type fibres at temperatures ranging from 60 to 80 °C (Fig. 5).

Compared to other fibre types, levels of β-sheet structures were lowest in type I fibre at 40 °C, and progressively increased with increasing temperature to become highest at 70 °C.

The slight heterogeneity in the degree of evolution of the secondary protein structure may be related to muscle protein expression, which can vary depending on fibre type (for review, see Xiong (1994)). Moreover, recent microgenomics analysis carried out on fibres isolated from mouse muscle showed significantly differences in gene expression profiles between type I and type IIB fibres (Chemello et al., 2011).

Protein extracts can be analysed to characterise the secondary structure of a specific protein or even target certain parts of the molecule. Various studies have gained knowledge on the mechanisms involved in the thermal denaturation of isolated myosin or tail sub-fragment myosin proteins (Morita & Yasui, 1991). Microspectroscopic methods cannot characterise the secondary structure of a single target protein as they focus on all the proteins under the beam, which in this study means all the myofibrillar proteins. However, microspectroscopic methods can get rid of the potentially bias-generating chemical extraction by modifying the thermal stability of the proteins studied.

These results are in disagreement with most studies on myofibrils or myosin extracted from different types of muscles, which have usually shown a greater thermal stability of myosin or myofibrils from red muscle than from white muscle (Boyer et al., 1996; Egelandsl et al., 1994; Fretheim et al., 1986; Lefèvre et al., 1999, 2007; Liu et al., 1996; Stabursvik & Martens, 1980). Protein extracts are prepared by steps that include grinding and centrifugation in buffers of high ionic strength and different pH (Lefèvre et al., 2007; Penny, 1967; Stabursvik & Martens, 1980; Vega-Warner & Smith, 2001), which can completely change their initial assembly and properties and consequently their thermal denaturation profiles. Moreover, the higher ultimate pH of red muscles compared to white muscles (Lefèvre et al., 1999; Vega-Warner & Smith, 2001; Xiong, 1994) could explain the variability in degree of protein changes depending on cell type that was observed by these authors.

However, Bertram, Kohler, Bocker, Ofstad, and Andersen (2006), who also used infrared microspectroscopy, did not find any effect of ultimate pH on secondary protein structure after cooking. They considered that the putative pH effect on protein unfolding could be masked by strong thermal denaturation. Here, our samples came from a single muscle and had suffered little treatment. Moreover, the relatively low ultimate pH of our muscle could partially...
explain the homogeneity in the behaviour of the different fibre types during heating. Indeed, Vega-Warner and Smith (2001) saw no differences in calorimetric enthalpies of myosin prepared from white and red bovine muscle at pH 5.5, which is close to the pH of our muscle and the incubation bath used (pH 5.6).

It is likely that the preservation of the structural organisation of muscle samples and consequently the maintenance of the molecular interactions promotes the thermal stability of proteins. Actomyosin complex, for example, is more heat-stable than myosin alone (Hamm, 1977; Penny, 1967). It is likely that the protein network still

![Bar chart](image_url)

**Fig. 5.** Evolution of the secondary structure of intracellular myofibre proteins with increasing temperature. The levels of alpha helices and beta sheets are measured, respectively, by the height of second derivative spectra peaks at 1655 and 1628 cm$^{-1}$. For a given temperature, different letters indicate a statistical difference significant for $p < 0.05$. 

...
in place in our study model minimises or even eliminates the differences in behaviour in response to heating depending on the type of fibre observed on extracts of isolated myosin or myofibrils.

4. Conclusion

Using FT-IR microspectroscopy made it possible to characterise the secondary structure of proteins directly on sections of muscle tissue that have not undergone any chemical treatment and have thus preserved their molecular assemblies. The secondary structure of collagen is significantly altered from 60 °C, whereas the secondary structure of elastin remains relatively stable until 80 °C. The secondary structure of myofibrillar protein clearly changes according to heating temperature, but no significant differences between fibre types were found, suggesting that proteins still bound by their molecular interactions demonstrate better thermal stability. Our study suggests that for whole beef meat products with a satisfactory ultimate pH, product composition in terms of fibre type is not determinative of the degree of heat denaturation of myofibrillar proteins during cooking.

Acknowledgements

These experiments were performed at the SOLEIL synchrotron on the SMIS beamline within the framework of Project Number 99080034. The authors thank Dr. Alain Buleon for valuable supportive discussions. The authors are grateful to the French Research National Agency (ANR-09-ALIA-008-01 Program PRONUTRIAL (2010–2013)) for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.03.012.

References


