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

Thierry Astruc [a,*], Frédéric Peyrin [a], Annie Vénien [a], Roland Labas [a], Magali Abrantes [a], Paul Dumas [b], Frédéric Jamme [b,c]

[a] INRA de Clermont-Ferrand-Theix, UR370 QuaPA, F-63122 St Genès Champanelle, France
[b] Synchrotron SOLEIL, BP48, L'Orme des Merisiers, F-9112 Gif-sur-Yvette, France
[c] INRA, U1008 Caractérisation et Elaboration des Produits Issus de l'Agriculture, F-44316 Nantes, France

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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, IIa and IIx fibres and type I–IIa and IIa–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 meat 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 aminoacids, 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 denaturates 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 time–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

* Corresponding author. Tel.: +33 4 73 62 41 56; fax: +33 4 73 62 42 68.
E-mail address: Thierry.Astruc@clermont.inra.fr (T. Astruc).

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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, Roussihle, Culioli, & Ouali, 1996), chicken (Liu et al., 1996), cattle (Egelandsdal, Martinsen, Fretheim, Petttersen, & Harbitz, 1994; Fretheim et al., 1986) and salmon (Lefèvre, Fauconneau, Thompson, & Gill, 2007). 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, Skarpeid, 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 cm^3 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, Mdbioproducts, 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 (L9393 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 S5 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).