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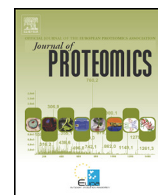

Coherent correlation networks among protein biomarkers of beef tenderness: What they reveal

Mohammed Gagaoua [a,b,c], E.M. Claudia Terlouw [a,b], Abdelghani Boudjellal [c], Brigitte Picard [a,b,*]

[a] INRA, UMR 1213 Herbivores, F-63122 Saint-Genès-Champanelle, France
[b] Clermont Université, VetAgro Sup, UMR 1213 Herbivores, BP 10448, F-63000 Clermont-Ferrand, France
[c] Equipe Maquav, INATAA, Université Frères Mentouri Constantine, Route de Ain El-Bey, 25000, Constantine, Algeria

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ABSTRACT

The development of proteomic biomarkers for meat tenderness remains an important challenge. The present study used *Longissimus thoracis* (LT) and *Semitendinosus* (ST) muscles of young bulls of three continental breeds (Aberdeen Angus, Blond d'Aquitaine and Limousin) to i) identify cellular pathways robustly related with meat tenderness, using potential protein biomarkers and ii) describe biochemical mechanisms underlying muscle to meat conversion. Correlation networks reveal robust correlations, i.e. present for at least two breeds, between potential meat tenderness biomarkers. For the two muscles of the three breeds, DJ-1 and Peroxiredoxin 6 were consistently correlated with Hsp20 and μ-calpain, respectively. For the three breeds, μ-calpain was related to Hsp70-8 in the LT muscle. Various correlations were muscle specific. For the three breeds, DJ-1 was correlated with Hsp27 for the ST, and with ENO3 and LDH-B for the LT muscle. Overall, in the LT, more correlations were found between proteins related to the glycolytic pathway and in the ST, with the small Hsps (Hsp20, 27 and αB-crystallin). Hsp70-Grp75 appeared involved in several relevant biological pathways. At the scientific level, results give insights in biological functions involved in meat tenderness. Further studies are needed to confirm the possible use of these biomarkers in the meat industry to improve assurance of good meat qualities.

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1. Introduction

In recent years, there has been a growing interest in the relationship between proteins and related genes, and meat quality traits. Functional proteomics aim to elucidate the biological function of proteins combining electrophoretic and protein sequencing technologies. They may be used to identify molecular markers, or biomarkers, that predict meat sensory qualities, including tenderness [1–9]. Such biomarkers are quantifiable indicators of biological processes and may help increasing our understanding of the biochemical processes related to various meat qualities [6,10]. Better knowledge of muscle to meat conversion would i) facilitate genetic selection, ii), help to evaluate the potential sensory quality of future meat products of existing animals, and iii) orient rearing systems and genetic choice to obtain desired meat qualities.

Today, a substantial amount of data exists on proteins that are related to various meat quality aspects. Studies show good coherence in the biological pathways involved in the development of meat quality. Generally, the proteins involved in meat quality are related to protective functions, glycolytic metabolism, mitochondrial activity and apoptosis, proteolysis, and cell structure [2]. However, the relative impacts of these biological pathways on meat quality development differ considerably between studies and even between breeds and muscles [3]. Similarly, although these studies find associations between proteins of similar or different biochemical pathways, the exact proteins that are correlated often differ. These differences may be explained by differences in the physical and physiological characteristics of the animals studied [11]. We need to get further insight in the functioning of proteins of similar and different biological pathways and ultimately, their relationship with meat quality development. One step would be to identify proteins showing robust correlations, that is, proteins that are correlated irrespectively of their physiological or physical environment.

The aim of the present study was to identify correlations between proteomic markers, existing in more than one breed or muscle and to discuss the underlying biological pathways. Therefore, we evaluated levels of Heat shock proteins (Hsps), and proteins involved in metabolism, structure, oxidative resistance and proteolysis in two muscles (*Longissimus thoracis* and *Semitendinosus*) of three continental beef breeds (Aberdeen Angus, Blond d'Aquitaine, and Limousin). These breeds differ in leanness and earliness and the muscles in metabolic and contractile properties. The proteins studied are potential biomarkers of bovine meat tenderness [3,12–14].

* Corresponding author.
E-mail address: brigitte.picard@clermont.inra.fr (B. Picard).

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2. Material and methods

2.1. Animals and sample collection

The study was part of the larger European ProSafeBeef project (FOOD-CT-2006-36241) and organised in two replicates (2 consecutive years, during the spring/summer seasons) in a balanced experimental design. It used 71 young bulls: Aberdeen Angus (AA; n = 21), Blond d'Aquitaine (BA; n = 25) and Limousin (Li; n = 25). At 12 months of age, they were subjected to a 105 day finishing period until slaughter. They were housed in 6 m × 6 m straw-bedded pens with 4 animals of a same breed to a pen. Diets consisted of concentrate (75%) and straw (25%). Before slaughter, all animals were food deprived for 24 h to limit the risk of carcass contamination by microbes in the digestive tract during evisceration, but had free access to water. At a live weight around 665 kg, the animals were slaughtered at the experimental abattoir of the INRA Research centre in compliance with the current ethical guidelines for animal welfare. Bulls were directly transported (4.5 ± 0.1 min) in a lorry (3 × 2 m livestock compartment) from the experimental farm to the experimental abattoir situated at 2 km from the rearing building, with 2 bulls of the same home pen per transport to avoid social isolation stress. After unloading, they were slaughtered within 3 min using standard industry procedures and respecting EU regulations. Slaughter procedures from the loading at the farm to bleeding took 10.6 ± 0.1 min. Slaughter took place between 08.00 h and 10.00 h am. Bulls were stunned by penetrative captive bolt prior to exsanguination [15]. The carcasses were not electrically stimulated and they were chilled and stored at 4 °C from 1 h until 24 h *post-mortem*. *Longissimus thoracis* (LT, mixed fast oxido-glycolytic) and *Semitendinosus* (ST, mixed fast glycolytic) muscle samples were excised 30 min *post-mortem* and frozen in liquid nitrogen before storage at −80 °C until protein extractions for Dot-Blot analysis or Myosin Heavy Chain(MyHC) isoform quantification.

2.2. Extraction of proteins for Dot Blot

Total protein extractions were performed to use subsequently the soluble fractions for Dot-Blot analysis according to Bouley et al. [16]. Briefly, 80 mg of muscle was homogenized in a denaturation/extraction buffer containing 8.3 M urea, 2 M thiourea, 1% DTT and 2% CHAPS. After 30 min of centrifugation at 10,000 *g* at 8 °C, the supernatant was stored at −20 °C until use. The protein concentrations of the extracts were determined according to the Bradford method [17] using the Bio-Rad Protein Assay. Bovine serum albumin (BSA) at a concentration of 1 mg/mL was used as standard.

2.3. Immunological protein quantification

The abundances of the 18 biomarkers (including intact proteins, their fragments and complexes) listed in Table 1 were quantified by the Dot-Blot technique according to the protocol described by Guillemin et al. [18] using specific antibodies previously validated by western-blot. Briefly, Western blots were used in order to check the specificity of all the antibodies. An antibody was considered specific against the studied protein when only one band at the expected molecular weight was detected by Western blot. Western blots with the 18 primary antibodies show that all the antibodies bound specifically to the bovine protein with the expected theoretical molecular weight.

Proteins evaluated belong to five different biological pathways (Table 1): muscle fibre structure (Actin, MyBP-H, CapZ-β and MyLC-1F); metabolism (ENO3, LDH-B and MDH1); proteolysis (μ-calpain); oxidative resistance (DJ-1, Prdx6 and SOD1); and Heat shock proteins (αB-crystallin, Hsp20, 27, 40, Hsp70-1A/B, 70-8 and 70-Grp75) were determined according to [11,12]. After quantification, a ratio corresponding to small Hsp per Hsp70s was calculated using the following equation: s/70 = (Hsp20 + Hsp27 + αB-crystallin) / (Hsp70-1A/B + Hsp70-8 + Hsp70-Grp75).

Table 1
List of the 18 protein biomarkers of beef tenderness investigated using the Dot-Blot technique in this study.

Protein name	Gene	UniProtKB ID
Heat shock proteins		
αB-Crystallin	*CRYAB*	P02511
Hsp20	*HSPB6*	O14558
Hsp27	*HSPB1*	P04792
Hsp40	*DNAJA1*	P31689
Hsp70-1A/B	*HSPA1B*	P08107
Hsp70-8	*HSPA8*	P11142
Hsp70-Grp75	*HSPA9*	P38646
Metabolism		
ENO3 (enolase 3)	*ENO3*	P13929
LDH-B (lactate dehydrogenase chain B)	*LDHB*	P07195
MDH1 (malate dehydrogenase 1)	*MDH1*	P40925
Structure		
CapZ-β (F-actin-capping protein subunit β)	*CAPZB*	P47756
α-actin	*ACTA1*	P68133
MyLC-1F (myosin light chain 1F)	*MYL1*	P05976
MyBP-H (myosin binding protein H)	*MYBPH*	Q13203
Oxidative resistance		
DJ-1 (Parkinson disease protein 7)	*PARK7*	Q99497
Prdx6 (Cis-peroxiredoxin)	*PRDX6*	P30041
SOD1 (superoxide dismutase Cu/Zn)	*SOD1*	P00441
Proteolysis		
μ-Calpain	*CAPN1*	P07384

Compared to Western blot, Dot-Blot is a rapid technique, but with a similar coefficient of variation inter and intra assay (10%). Optimal dilution ratios of the antibodies were determined at the same time, using the conditions indicated by the supplier of the reactant and adapted to bovine muscle samples [11]. Conditions retained and suppliers for all primary antibodies are reported in Table 2. Protein extracts (15 μg) of each of the 142 muscle samples were spotted (four replications per muscle sample) on a nitrocellulose membrane with the Minifold I Dot-Blot apparatus from Schleicher & Schuell Biosciences (Germany) in a random order on the 96-spot membrane. In addition, a mixed standard sample (15 μg) was deposited for data normalization as reported by Guillemin et al. [18]. The Dot-Blot membranes were air-dried for 5 min, blocked in 10% PBS milk buffer at 37 °C for 20 min, and then incubated to be hybridized with the specific primary antibody of each protein (Table 2). Subsequently, the membranes were incubated at 37 °C for 30 min with the anti-mouse fluorochrome-conjugated LICOR-antibody IRDye 800CW (1 mg/mL).

Infrared fluorescence detection was used for quantification of the relative protein abundances. Subsequently, the membranes were scanned using the Odyssey NIR imager (LI-COR Biosciences), with an 800 nm laser, a 169 μm spatial resolution and a fixed gain of 5.

Dot-Blot images were quantified with GenePix PRO v6.0 (Axon) [18]. Each dot volume was calculated as the total dot intensity from which the median local background value multiplied by the dot area was subtracted. Because Dot-Blot offers the possibility of replicates, a data-prefiltering approach was implemented to eliminate outlier values mainly due to dust. The exclusion technique of outliers was based on the Medium Absolute Difference (MAD) and applied before repeated values were averaged. Finally, to make the data comparable between assays, the data were normalized using a regression-approach based on the used mix standard specific for each muscle. Thus, relative protein abundances were based on the normalized volume and expressed in arbitrary units.

2.4. Electrophoresis and quantification of Myosin Heavy Chain (MyHC) isoforms

The abundance of the three other proteins corresponding to MyHC isoforms was quantified by an appropriate SDS-PAGE technique [19].

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