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PROTEOME ANALYSIS OF THE SARCOPLASMIC FRACTION OF PIG SEMIMEMBRANOSUS MUSCLE: CORRELATION WITH MEAT L* VALUE

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Introduction

Despite considerable achievements in the field of genetic improvement of pigs, the meat processing industry still suffers from a large variability in the quality of raw material. One obvious reason is that meat quality results not only from the animal genetic equipment but to the response of genes to external factors such as breeding and slaughter conditions, post-slaughter treatments etc. Thus, the traditional genetic approach, based on monitoring the expression of a limited number of candidate genes, does not completely take into account the complexity and multiplicity of interwoven biochemical mechanisms. Post-genomics tools (transcriptomics, proteomics) appear suitable to describe relationships between various metabolic pathways, thereby assisting in proposing mechanisms of meat quality development. For that particular purpose, as reported by Hamelin et al. (2004), it is considered appropriate to study specifically sarcoplasmic proteins, as they contain the majority of enzymes and regulators.

Proteomics has been successfully applied to describe *post mortem* modifications of pig muscle proteins (Lametsch et al., 2002; Morzel et al., 2004) or to finely characterize PSE zones in *semimembranosus* muscle (Laville et al., 2005). Other studies have investigated the correlation between proteins and fragments abundance with meat quality attributes such as texture (Lametsch et al., 2003) or L* value and drip loss (Hwang, 2004). Monitoring L* value is of particular interest for meat destined to ham production since it is a relatively simple measure, indicative for example of defective PSE meat (Mueller and Domel, 2000). However, measure can be performed only after carcass preparation. Earlier predictors, probably of a molecular nature, would constitute an improvement to the phenotypic measurement.

Objectives

The overall objective of the present work is of a double nature: contributing to the identification of biochemical mechanisms responsible for meat color characteristics and searching for early predictors of technological quality of pig meat destined to ham production. For that purpose, a differential proteome analysis is performed on the

sarcoplasmic proteins of *semimembranosus* muscles, characterized by high or low L* values measured at 36 hours *post mortem.*

Methodology

Animals, sampling and L value measurement*

In order to generate important genetic variability in meat quality traits, the studied population was composed of 1200 pigs originated from F2 crossing between Pietrain and a synthetic line (Large White x Duroc x Hampshire). Pigs were slaughtered at a liveweight of 110 kg. 30 min after slaughter, a 5g sample was taken from the dorso-superficial region of *semimembranosus*, thereby avoiding the inner part of the muscle that may be affected by PSE zones (Laville et al., 2005). L* value was measured with a Minolta CR 300 chromameter 36h *post mortem*, on the internal surface of muscle near the bone.

Sarcoplasmic protein extraction and electrophoresis

Muscle was homogenized in 40mM Tris (pH 7), 2mM EDTA and a protease inhibitors cocktail (SIGMA) at 4°C, at the ratio of 1:4 (w/v). The homogenate was centrifuged at 4° C for 10mn at 10000g. Supernatant, referred to as the sarcoplasmic extract, was stored at -80°C. Protein concentration was determined by the Bradford assay (Bio-Rad). 900µg of sarcoplamic proteins were included in a buffer containing 7M Urea, 2M Thiourea, 2% (w/v) CHAPS 0.4% (v/v) carrier ampholyte and bromophenol blue. Samples were loaded onto immobilised pH-gradient strips (pH 5-8, 17cm Bio-Rad) and isoelectric focusing was performed using a Protean IEF cell system (Bio-Rad). Gels were passively rehydrated for 16h. Rapid voltage ramping was subsequently applied to reach a total of 85 kVh. In the second dimension, proteins were resolved on 12% SDS-PAGE gels using Protean II XL system (Bio-Rad). Gels were Coomassie Blue stained. Three gels were produced per sample.

Image analysis and statistical treatment of data

Gels were visualised and analysed using the 2DE image analysis software PDQuest (Bio-Rad). Detected and matched spots were statistically analysed using SAS software. A spot was considered significant when it was associated to $p<0.05$ in ANOVA and/or pair-wise Student test. Clustering of significant spots was also performed using xlstat software.

Protein identification by mass spectrometry

Spots of interest were excised and the proteins were subjected to in-gel tryptic digestion and peptide extraction using Montage In-Gel Digest96 ZP kit (Millipore). Peptides were analysed using a MALDI-TOF mass spectrometer Voyager DE-Pro model (Perseptive BioSystems). Proteins were identified from their peptide mass fingerprinting using the MASCOT software (http://www.matrixscience.com). The initial search

parameters allowed a single trypsin missed cleavage, carbamidomethylation of cystein, partial oxidation of methionine and a m/z error of 25 ppm.

Results & Discussion

Among 1200 animals, 2 groups of 12 animals (chosen by pair within 12 families) were selected based on extreme L* value of meat. Average L* values were 61.3 and 43.2, in the groups later referred to as 'light" and "dark" respectively.

Figure 1 shows a example of a 2-D gel of sarcoplasmic proteins. Proteins of interest, discussed below, are circled. Out of 290 matched spots, 25 spots showed a significant differential abundance between the two groups. Among those, 17 are discussed in the present manuscript. Table 1 provides identification of spots, and information related to the validity of search results. At the exception of spots 4319, 6108, 6211 and 5214, which are potential fragments, position of all spots in the gel corresponded well with the provided theoretical MW, suggesting that 13 spots are entire proteins. Figure 2 is a cluster representation of the 17 identified proteins and their relative abundance in the two groups. The two main clusters naturally separate proteins over-represented (shadowed) and under-represented in dark samples.

All proteins over-represented in the light group are cytosolic proteins involved in glygogenolysis and glycolysis (enolase and enolase fragment, glycerol-3P dehydrogenase, glycogen phosphorylase fragment) or the associated cytosolic energy metabolism enzyme creatine kinase, in the form of fragments. These results corroborate previous findings that glycolytic muscle metabolism and proportion of type IIb glycolytic fibers enhance meat lightness in pig (Larzul et al., 1997). Regarding creatine kinase fragments, their higher abundance probably also indicates a higher proteolysis, that can be induced *intra vitam*, for example by exercise (Niikawa et al., 2002) and that is observed *post mortem* as we have described recently in PSE zones (Laville et al., 2005).

Proteins over-represented in the dark group can be divided into 4 groups: mitochondrial proteins involved in the respiratory chain (ATPase beta subunit, succinate dehydrogenase, NADH dehydrogenase), hemoglobin, chaperone proteins (HSP 27, alpha-crystallin) and antiquitin. Mitochondrial proteins clearly indicate a more pronounced oxidative metabolism, which is consistent with the inverse finding in the light group. Regarding hemoglobin, it is always present in muscle as remains of blood (Warriss, 1971). Since the pool of haem pigments is correlated with color of pig meat (Warriss et al., 1990), it comes as no surprise that hemoglobin quantity is higher in dark samples. Its higher abundance is most likely linked with the greater vascularity found in oxidative type muscles (Ruusunen and Puolanne, 2004) or with a higher hemoglobin content in the blood itself as observed in veal carcasses (Klont et al., 1999). Interestingly, the two chaperone proteins (HSP 27 and alpha-crystallin) were grouped in the same cluster, suggesting a co-regulation in the studied muscles. Their general function, including in skeletal muscle, is to offer a protection against protein denaturation. Kim et al. (2004) observed the higher abundance of one form of HSP 27 in white rather than red pig muscle. However, HSP 27 exist in many forms (Scheler et al., 1999), and the pool of immunologically detected HSP 27 have been reported to be over-expressed in oxidative fibers and in response to an increased demand in oxidative metabolism (Neufer and Benjamin, 1996). Since excessive protein denaturation is known to induce discoloration,

for example in fast pH fall PSE meat (Sayre and Briskey, 1963), the higher presence of chaperone proteins may contribute somehow to the darker color in this group. Finally, antiquitin is a protein implicated in cellular turgor pressure (Tang et al., 2002). Further investigation would be necessary to confirm that it plays a role in color development, but again, a reduced quantity would be consistent with fluid linkage and protein denaturation, as observed in excessively pale PSE meat (Monin, 1995).

Conclusions

The differentially expressed sarcoplasmic proteins in *semimembranosus* muscle confirm that increased meat lightness is associated with a more glycolytic / less oxidative metabolism. Concomitant changes in chaperone proteins and in antiquitin are observed. It would be interesting to determine whether such proteins can have an effect, even limited in the *post mortem* period, that could contribute to the installation of final color development. Further investigation is also necessary to determine whether any of the described proteins can be used as early predictor of technological quality of meat.

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Tables and Figures

Table 1. Identification of 17 differentially represented proteins (SC: Mascot score, MP: number of matched peptides, C: percentage of sequence coverage)

Spot	Identity	Accession number	SC	MP	C	Theoretical Mr/pI
0405	beta-subunit ATP synthase	gi|104	115	9	38	38.6/4.9
1712	NADH dehydrogenase	gi|57110953	206	23	34	79.6/5.8
4106	HSP 27	gi|55926209	75	7	35	22.9/6.2
4304	glycerol-3-phosphate dehydrogenase	gi|2149959	91	10	48	39.9/5.0
4319	enolase (fragment)	gi|109215	163	18	40	46.8/8.1
4404	enolase	gi|4503571	60	8	19	47.1/7.0
4513	antiquitin	gi|25108887	111	13	25	55.3/6.2
5214	glycogen phosphorylase (fragment)	gi|55636467	76	12	13	97.1/6.7
5314	enolase	gi|57086343	132	18	33	55.4/8.5
5614	succinate dehydrogenase	gi|284648	114	13	24	72.8/7.2
6108	creatine kinase (fragment)	gi|54111517	62	8	18	43.0/6.6
6211	creatine kinase (fragment)	gi|54111517	74	7	19	43.0/6.6
7005	hemoglobin chain D	gi|5542425	198	13	91	16.0/6.7
7008	hemoglobin alpha chain	gi|70237	90	6	58	15.0/8.7
7009	hemoglobin chain D	gi|5542425	190	11	77	16.0/6.7
7010	alpha-crystallin	gi|7441290	147	15	56	20.0/6.7
8003	hemoglobin chain D	gi|5542425	175	13	91	16.0/6.7

Figure 1. 2-D electrophoretic separation of sarcoplasmic proteins of *semimembranosus* pig muscle. Identified proteins differentially represented between the two groups are circled and numbered.

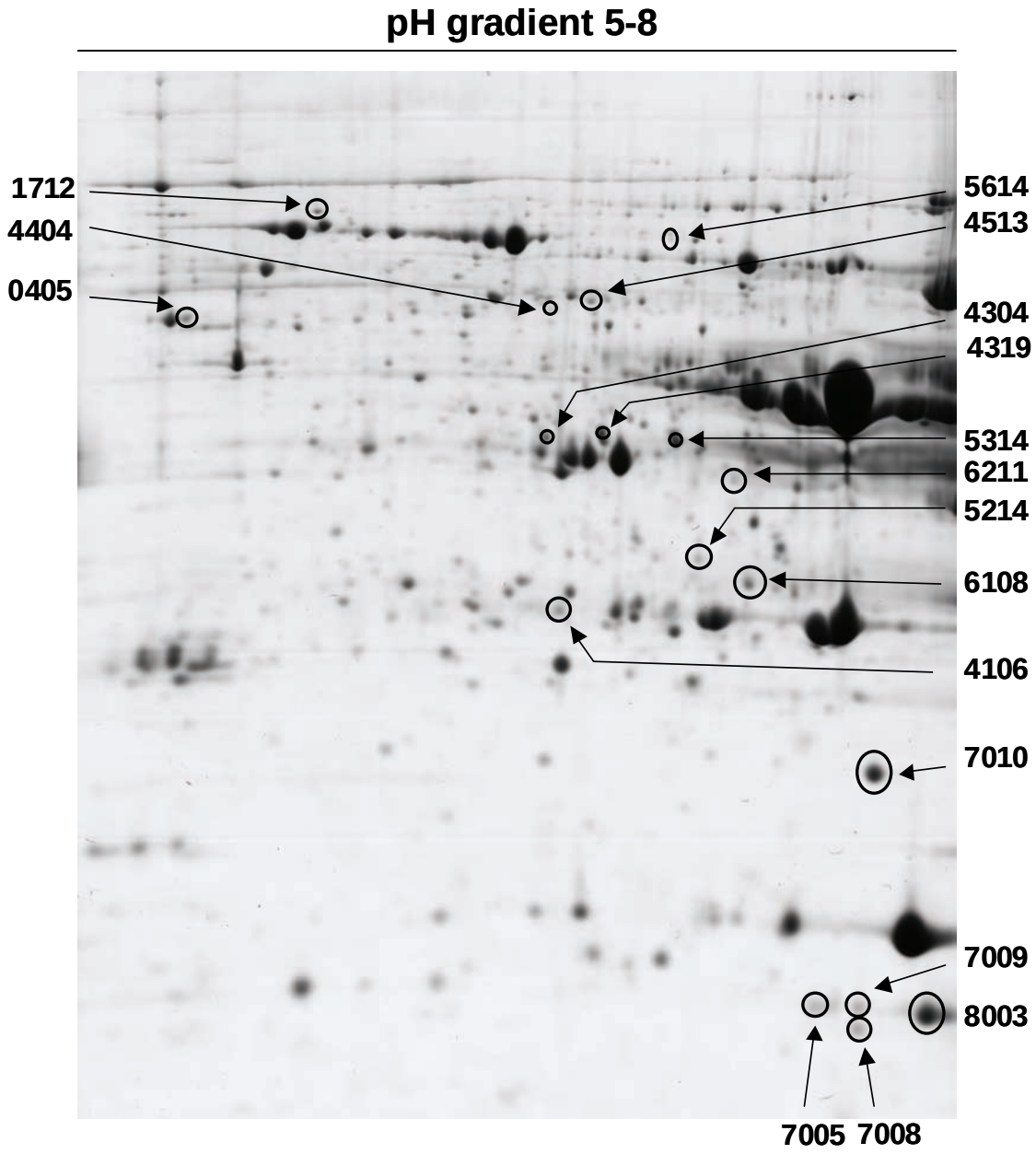
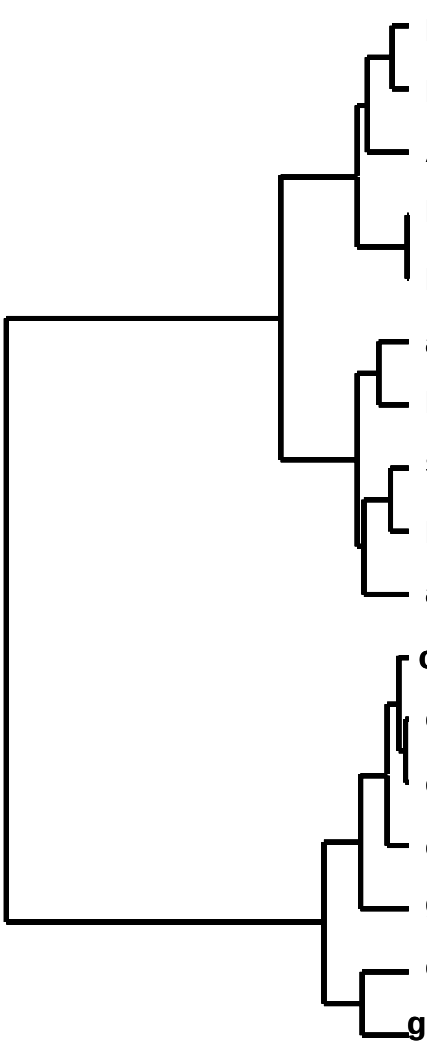


Figure 2. Cluster representation of identified proteins of interest and relative abundance (ppm) in the groups with high ("light") and low ("dark") L* value.



	Spot	Abundance (ppm) -light	Abundance (ppm) -dark
hemoglobin chain D	8003	10206	19346
hemoglobin chain D	7005	150	457
ATPase beta subunit	0405	763	1447
hemoglobin chain D	7009	96	358
hemoglobin alpha	7008	90	395
alpha-crystallin	7010	3814	10180
HSP 27	4106	920	1643
succinate dehydrogenase	5614	156	365
NADH dehydrogenase	1712	209	431
antiquitin	4513	206	822
creatine kinase (fragment)	6108	2974	1919
enolase	5314	11811	8394
enolase (fragment)	4319	11257	6904
creatine kinase (fragment)	6211	574	299
glycogen phosphorylase (fragment)	5214	926	664
enolase	4404	876	444
glycerol-3-phosphate dehydrogenase	4304	2518	1540