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Proteome Analysis of the Sarcoplasmic Fraction of Pig *Semimembranosus* Muscle: Implications on Meat Color Development

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Two-dimensional electrophoresis was used to investigate sarcoplasmic protein expression in pig *Semimembranosus* muscles sampled 20 min after slaughter. Two groups (light and dark) of 12 animals were selected from 1000 pigs, based on meat *L** values measured 36 h postmortem. Twenty-two proteins or fragments ($p < 0.05$) were differentially expressed. Muscles leading to darker meat had a more oxidative metabolism, indicated by more abundant mitochondrial enzymes of the respiratory chain, hemoglobin, and chaperone or regulator proteins (HSP27, αB-crystallin, and glucose-regulated protein 58kDa). Conversely, enzymes of glycolysis were overexpressed in the lighter group. Such samples were also characterized by higher levels of glutathione S-transferase ω, which can activate the RyR calcium channels, and higher levels of cyclophilin D. This protein pattern is likely to have severe implications on postmortem metabolism, namely, acceleration of ATP depletion and pH fall and subsequent enhanced protein denaturation, well-known to induce discoloration.

KEYWORDS: Proteomics; pig; *Semimembranosus*; *L value; GSTO; cyclophilin D**

INTRODUCTION

Despite considerable achievements in the field of genetic improvement of pigs, for example by controlling HALn and RN- mutations, the meat-processing industry still suffers from a large variability in the quality of raw pork. One obvious reason is that meat quality results not only from the animal's genome but also from the response of genes to external factors such as fattening and slaughter conditions, meat processing, etc. Thus, the traditional genetic approach, based on the detection of polymorphic genes, or the traditional physiological approach, based on monitoring the expression of a limited number of genes, does not completely take into account the complexity and multiplicity of interwoven biochemical mechanisms. Functional 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. (*1*), it is considered appropriate to study specifically sarcoplasmic proteins, as they contain the majority of enzymes and regulators of protein expression.

Proteomics has been successfully applied to describe postmortem modifications of pig muscle proteins (*2*, *3*) or to finely characterize PSE (pale soft exsudative) zones in *Semimembranosus* muscle (*4*). Other studies have investigated the correlation between proteins and fragments abundance with meat quality attributes such as texture (*5*) or *L** value and drip loss (*6*). Monitoring the *L** value is of particular interest since it is a relatively simple measure indicative of PSE meat (*7*).

The overall objective of the present work was to identify biochemical mechanisms responsible for meat color variability. For that purpose, a differential proteome analysis was performed on the sarcoplasmic proteins of *Semimembranosus* muscles, characterized by high or low *L** values measured at 36 h postmortem.

MATERIALS AND METHODS

Animals, Sampling, and *L Value Measurement.** The sampled pig population was composed of 1000 pigs that originated from F2 crossing between Pietrain and a synthetic line (Large White × Duroc × Hampshire). Pigs were slaughtered at a live weight of 110 kg. Twenty minutes after slaughter, a 5 g sample was taken from the dorsosuperficial region of *Semimembranosus*, frozen in liquid nitrogen, and stored at −80°C until used. The *L** value was measured with a Minolta CR 300 chromameter at deboning 36 h postmortem, on the internal surface of the muscle near the emergence of collateral of the femoral vein and artery. This region is the most frequently affected by PSE defect.

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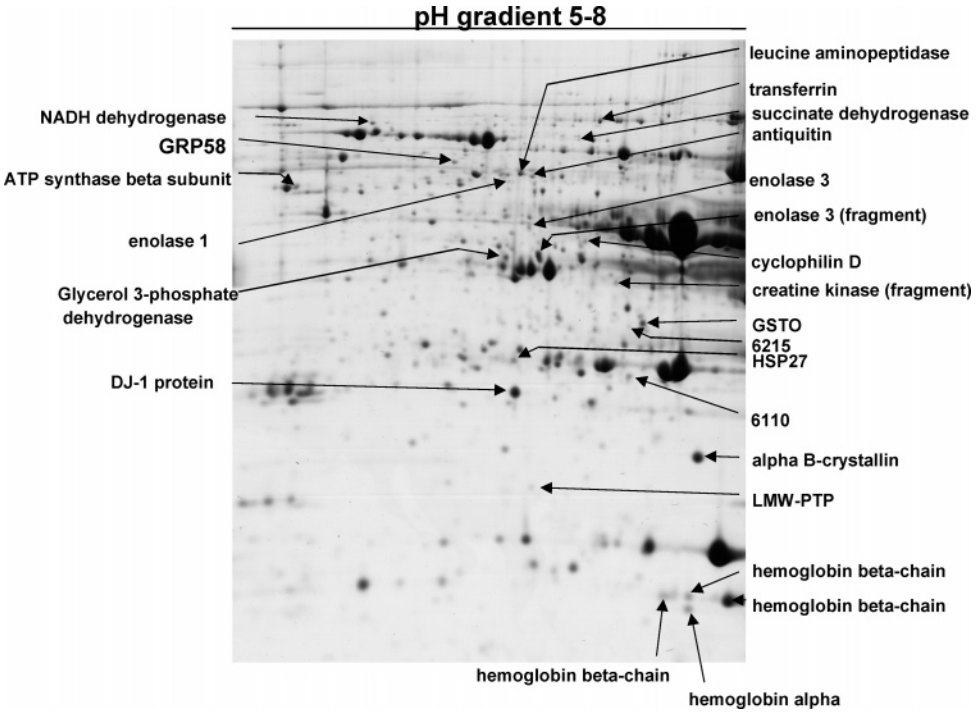

Figure 1. 2DE gel of *Semimembranosus* muscle sarcoplasmic proteins. Arrows indicate the identified proteins that were differentially expressed between dark and light groups. The numbers correspond to the spot number.

Among 1000 animals, two groups of 12 animals (chosen by pair within 12 half sibs families) were selected on the basis of extreme L^* values of meat.

Sarcoplasmic Protein Extraction and Electrophoresis. Frozen muscle was homogenized, using a glass bead agitator MM2 (Retsch, Haan, Germany), in 40 mM Tris (pH 7), 2 mM EDTA, and a protease inhibitors cocktail (mixture of AEBSF, aprotinin, leupeptin, bestatin, pepstatin A, and E-64, Sigma) at 4 °C at the ratio of 1:4 (w/v). The homogenate was centrifuged at 4° C for 10 min at 10000*g*. The supernatant, referred to as the sarcoplasmic extract, was stored at −80 °C. The protein concentration was determined by the Bradford assay (Bio-Rad). Nine hundred micrograms of sarcoplamic proteins was included in a buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.4% (v/v) carrier ampholyte, and bromophenol blue. Samples were loaded onto immobilized pH gradient strips (pH 5−8, 17 cm, Bio-Rad), and isoelectric focusing was performed using a Protean IEF cell system (Bio-Rad). Gels were passively rehydrated for 16 h. Rapid voltage ramping was subsequently applied to reach a total of 85 kV h. In the second dimension, proteins were resolved on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) gels using Protean II XL system (Bio-Rad). Gels were Coomassie Blue (colloidal blue) stained. Three gels were produced per sample, resulting in the production of 72 gels.

Image Analysis and Statistical Treatment of Data. Gels were visualized and analyzed using the two-dimensional electrophoresis (2DE) image analysis software PDQuest (Bio-Rad). Detected and matched spots were normalized by expressing the relative quantity of each spot as the ratio of individual spot quantity on the total quantity of valid spots. Relative quantities were expressed in ppm. For one sample and one spot, the mean of the three values (corresponding to the gels in triplicate) was calculated. The resulting set of averaged spot quantities was submitted to a one-way analysis of variance (ANOVA) using Statistical Analysis System (SAS) software. A spot was considered significant when it was associated to $p < 0.05$ in ANOVA. Clustering of significant spots by hierarchical ascendant classification (method of Ward based on Euclidian distances) 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 a Montage In-Gel Digest96 ZP kit (Millipore). Peptides were analyzed using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer Voyager DE-Pro model (Perseptive BioSystems). Proteins were identified from their peptide mass fingerprinting, searching the NCBI (National Center for Biotechnology Information) protein sequence database 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

Among 1000 animals, two groups of 12 animals (chosen by pair within 12 half sibs families) were selected on the basis of extreme L^* values of meat. All animals selected were genotyped for HALn and RN- mutations (*8*, *9*) and found bearing homozygous wild-type genotypes for these two loci, (N/N; rn+/rn+), i.e., noncarriers of HALn and RN- alleles.

Average L^* values were 61.3 ± 2.5 and 43.2 ± 4.6 in the groups later referred to as "light" and "dark", respectively. On 2DE gels, out of 290 matched spots, 24 spots showed a significant differential abundance between the two groups. Twenty-two spots were successfully identified using peptide mass fingerprinting. **Figure 1** shows a representative gel of the soluble muscle proteins with the position of those 22 spots. The identification and information related to the validity of search results are shown in **Table 1**. At the exception of spots 4319 and 6211, which are probably fragments, the position of all spots in the gel corresponded well with their theoretical molecular weight (MW), suggesting that 20 spots are entire proteins. **Figure 2** shows the cluster representation of the 22 proteins in the two groups. The two main clusters naturally separate proteins overrepresented in the light group and overrepresented in the dark group.

Proteins Overrepresented in the Dark Group. They can be grouped according to their biological function. First, the ATPase β-subunit, succinate dehydrogenase, and NADH dehydrogenase are mitochondrial proteins involved in the respiratory chain and oxidative metabolism. Hemoglobin, the main

Table 1. Proteins Differentially Expressed Between Dark and Light Groups

spot no.	protein name [a]	accession no.[a]	mowse score[b]	% cov[c]	MP[d]	theorical MW/pI[e]	observed MW/pI[e]	spot ratio[f]	spot *p* value[g]
			metabolic proteins						
405	ATP synthase β-subunit	gi|104	115	38	9	38.6/4.9	57.1/5.2	–1.9	<0.001
1712	NADH dehydrogenase	gi|57110953	206	34	23	79.6/5.8	80.1/5.6	–2.1	<0.05
5614	succinate dehydrogenase	gi|284648	114	24	13	72.8/7.2	73.3/6.3	–2.3	<0.05
4323	enolase 3	gi|16878083	69	16	7	46.9/7.5	41.7/6.2	2.2	<0.001
4404	enolase 1	gi|62896593	91	19	8	47.1/7.0	57.6/6.1	1.9	<0.05
4304	glycerol-3-phosphate dehydrogenase	gi|33695088	80	29	11	37.5/5.8	37.0/6.1	1.6	<0.05
			iron binding proteins						
7005	hemoglobin β-chain	gi|3041678	130	55	9	16.1/7.1	15.8/6.6	–3.0	<0.05
7008	hemoglobin α-chain	gi|229626	85	58	6	15.0/8.7	15.5/6.7	–4.4	<0.05
7009	hemoglobin β-chain	gi|11382865	144	66	9	16.0/7.2	15.8/6.7	–3.7	<0.05
8003	hemoglobin β-chain	gi|3041678	117	55	9	16.1/7.1	15.8/6.8	–1.9	<0.001
5723	transferrin	gi|833800	353	67	45	76.9/6.7	80.1/6.3	1.4	<0.05
			indicators of proteolysis						
4509	leucine aminopeptidase	gi|1127257	126	29	13	52.5/5.8	60.5/6.1	1.3	<0.05
4319	enolase 3 (fragment)	gi|109215	163	40	18	46.8/8.1	37.4/6.2	1.6	<0.05
6211	creatine kinase (fragment)	gi|54111517	74	19	7	43.0/6.6	34.7/6.4	1.9	<0.05
			chaperone and regulator proteins						
4106	HSP27	gi|55926209	75	35	7	22.9/6.2	27.0/6.1	–1.8	<0.001
7010	αB-crystallin	gi|7441290	147	56	15	20.1/6.7	20.1/6.7	–2.7	<0.05
3505	GRP58	gi|57108151	123	31	18	56.7/5.8	63.5/5.9	–1.4	<0.05
5317	cyclophilin D	gi|27806463	145	37	14	40.5/6.1	39.0/6.3	1.7	<0.02
6217	GSTO	gi|47522916	77	45	9	27.4/6.8	30.8/6.5	5.4	<0.0001
			miscellaneous						
4513	antiquitin	gi|25108887	111	25	13	55.3/6.2	60.0 /6.2	–3.9	<0.01
4111	DJ-1 protein	gi|59858513	166	69	13	20.0/6.8	24.3/6.1	1.3	<0.001
4009	LMW- PTP	gi|14285667	69	51	6	18.0/6.8	19.0/6.2	1.7	<0.001
6215	unidentified						30.1/6.5	2.1	<0.05
6110	unidentified						26.0/6.4	0.7	<0.05

[a] Protein names and accession numbers were derived from the NCBI database. [b] The MASCOT baseline significant score is 68. [c] Percentage of coverage of the entire amino acid sequence. [d] Number of matched peptides. [e] MW and pI, theoretical (recorded in NCBI database) and observed (calculated from the spot position on the gel). [f] Spot ratio: average quantity in light group/average quantity in dark group. A negative sign was added when the protein was overexpressed in dark group. [g] *p* value from the ANOVA analysis (effect of *L** group).

iron-binding protein in serum, is represented by four spots in the same cluster. Interestingly, the two chaperone proteins [heat shock protein (HSP)27 and αB-crystallin] were grouped in the same cluster, suggesting a coregulation in the studied muscles. Two other proteins are aldehyde dehydrogenase 1 (antiquitin 1), a protein implicated in cellular turgor pressure, and the glucose-regulated protein 58 kDa (also named GRP58, Erp57, and ER-60 protease), a molecular chaperone thiol-dependent oxidoreductase.

Proteins Overrepresented in the Light Group. Enolase 1, enolase 3, and glycerol 3-phosphate are involved in glycolytic metabolism but are classified in different subclusters. Two protein fragments (creatine kinase and enolase 3) are associated with a leucine aminopeptidase and the glutathione transferase ω (GSTO), which catalyzes the binding of glutathione to different compounds such as ROS (reactive oxygen species) and also has an effect on opening the ryanodine receptor calcium channel (*10*). In another subcluster, DJ-1 protein, a chaperone protein involved in the cellular response to stress, is associated with two proteins: peptidylprolyl isomerase D (cyclophilin D) and the low molecular weight protein tyrosine phosphatase (LMW-PTP), which has been shown to regulate several receptors (*11*). Finally, also overrepresented in the light group is transferrin, a protein synthesized in liver, which binds free iron to deliver it to the cells through the vascular system.

DISCUSSION

Metabolic Proteins. Overexpression of mitochondrial proteins in the dark group clearly indicates a more pronounced oxidative metabolism. In contrast, the light group is characterized by overexpression of some cytosolic proteins involved in glycolysis (enolases 1 and 3 and glycerol-3P dehydrogenase). These results corroborate previous findings that glycolytic muscle metabolism and proportion of type IIb glycolytic fibers enhance meat lightness in pigs (*10*).

Iron-Binding Proteins: Hemoglobin and Transferrin. Hemoglobin was more abundant in the dark group, in accordance with Hwang (*11*). Because the pool of heme pigments is correlated with the color of pig meat (*12*), it obviously contributes to the darker color. Hemoglobin is always present in muscle as remains of blood (*13*). Its higher abundance could be linked to a higher blood flow in muscle, to a higher hemoglobin content in the blood, or/and to the degree of bleeding. Blood flow in muscle depends first on vascularity, which is more developed in oxidative muscles (*16*). In the dark group characterized by a more oxidative metabolism, higher vascularity therefore certainly contributes to a higher level of hemoglobin. Furthermore, blood flow also depends on a number of factors, particularly exercise and degree of stress (*17*). The effect on blood flow of stress and muscular activity at slaughter was not assessed in the present study, but this aspect probably deserves further attention.

Interestingly, the second iron-binding protein transferrin showed a higher representation in the light group. Hamelin et al. (*1*) observed an overexpression of transferrin in hypertrophied lamb muscles. These muscles were also characterized by a more glycolytic metabolism and a less developed vasculature. The overexpression of transferrin was therefore interpreted as a

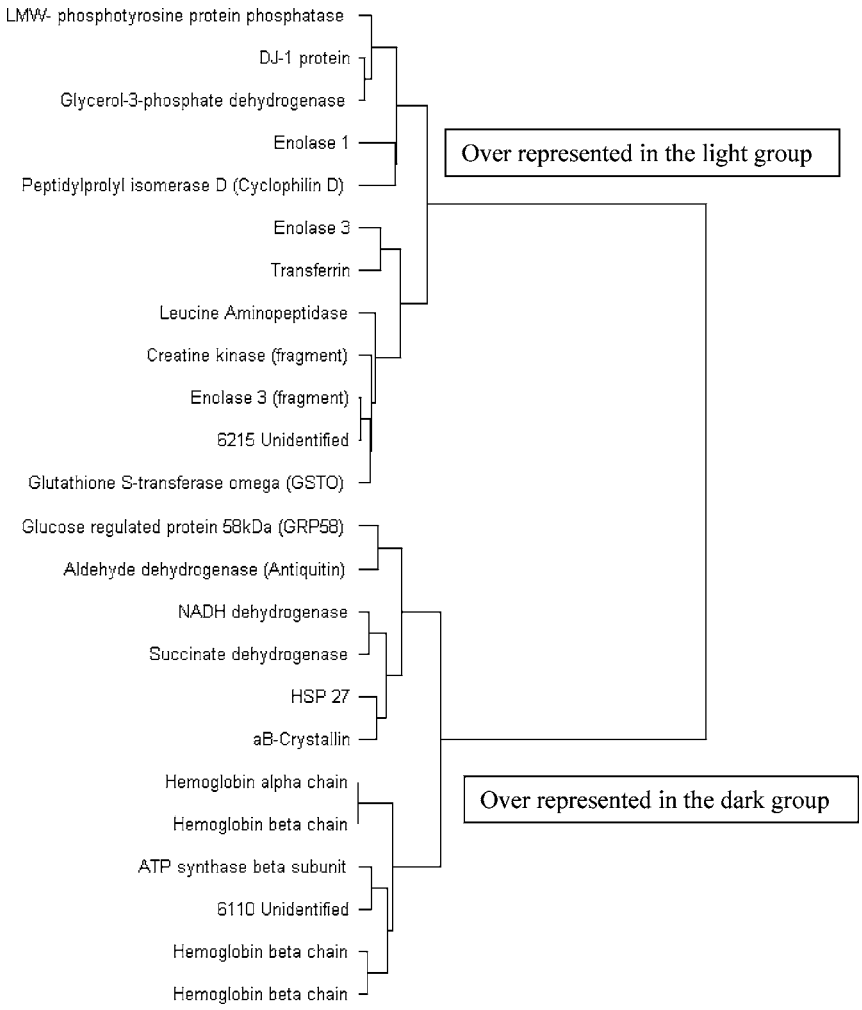

Figure 2. Clustering representation of the 24 significant spots.

mechanism to compensate iron deficit related to a lower level of blood supply. This hypothesis may apply to this study and would be in agreement with effects of chronic hypoxia described in Lopez-Barneo et al. (*18*) since hypoxia induces up-regulated expression of glucose degradation enzymes and transporters as transferrin.

Indicators of Proteolysis. A leucine aminopeptidase was more abundant in light muscle. This is consistent with previous findings (*19*) where the activity of leucine aminopeptidase measured at 2 h postmortem was positively correlated with *L** value. The mechanism linking leucine release and color development remains however unclear.

In the same cluster, fragments of enolase and creatine kinase were also overrepresented in light muscles, indicating enhanced proteolysis of the parent proteins. Greater proteolysis of creatine kinase in light PSE zones of pig muscles, sampled 72 h postmortem, was previously evidenced in our laboratory (*4*). However, in the present study, the very early sampling time postmortem (20 min) suggests that proteolysis is generated by a phenomenon occurring during the life of the animals. Enolase and creatine kinase are particularly prone to oxidation, as described in pathological conditions due to oxidative stress (*20*), in aging rats (*21*), or in postmortem chicken breast meat (*22*). Although further work would be required to confirm this hypothesis, it is possible that the higher abundance of fragments, i.e., the higher susceptibility to proteolysis, may be due to increased oxidation levels. This would also be consistent with reports that creatine kinase proteolysis can be induced intra vitam by acute exercise (*23*) since strenuous exercise is a natural source of ROS and oxidative stress (*24*).

Chaperones and Regulators Involved in Oxidative Metabolism. In our view, three proteins overexpressed in dark samples, namely, GRP58, HSP27, and αB-crystallin, reflect the predominant oxidative metabolism. Present in the endoplasmic reticulum (ER), the GRP58 modulates the SERCA2b pumps, whose main function is to enhance sequestration of Ca^{2+} within the ER (*25*). GRP58 also plays a pivotal role in folding of glycoproteins in the ER (*26*). Overexpression of GRP58 reduces the frequency of Ca^{2+} oscillation (*25*), which is a characteristic of oxidative metabolism.

Similarly, the two chaperone proteins HSP27 and αB-crystallin were more abundant in dark samples. This is consistent with Neufer and Benjamin (*27*) who reported that the pool of immunologically detected HSP27 was larger in oxidative fibers, in muscles of type I fibers with high oxidative capacity, and in response to an increased demand in oxidative metabolism, for example, during sustained exercise.

The cell equipment in proteins present shortly after slaughter is very likely to have consequences postmortem. For example, GRP58 may delay the leakages of Ca^{2+} into the cytosol. Even if this delay is limited in intensity and time, it can undoubtedly have an effect on reducing the rate of pH fall, which is well-known to result in a darker meat color. As to the overexpression of chaperones, their implication in meat quality is less clear. To our knowledge, the only report has been made by Schwerin (*28*) who observed that polymorphism on the gene encoding

for a HSP70 affected pork meat quality. The general function of chaperone proteins is to offer a protection against protein denaturation, and it is not excluded that their effect can continue for some time postmortem. This would therefore delay protein denaturation, which is a commonly described phenomenon associated with discoloration, for example, in fast pH fall PSE meat (*29*).

Regulators Likely to Accelerate Postmortem ATP Depletion. GSTO belongs to the glutathione transferase family (GSTs). GSTs catalyze the binding of glutathione to different potentially toxic compounds, including ROS (*30*). However, Dulhunty et al. (*10*) have shown that GSTO has a weak activity of ROS neutralization and suggested that one of its main function is to activate the RyR (ryanodine receptor) calcium release channels of skeletal muscle. Its overexpression would therefore lead to a constant increased cytoplasmic [Ca^{2+}]. Such elevated cytoplasmic [Ca^{2+}] increase intramitochondrial [Ca^{2+}] and the subsequent ROS production (*31*, *32*). Postmortem, any increase in cytoplasmic [Ca^{2+}] accelerates ATP breakdown and pH decline.

Peptidylprolyl isomerase D (cyclophilin D) is a member of the cyclophilin family of chaperones. It is located in the mitochondrial matrix, where it can bind to the ANT (adenine nucleotide translocase) of the inner membrane, opening the mitochondrial permeability transition pore (MPTP) and stimulating hydrolysis of ATP produced by glycolysis (*33*). Intramitochondrial [Ca^{2+}] greatly enhances the binding of cyclophilin D to the ANT (*34*) and the subsequent MPTP opening, mitochondria swelling, and membrane disruption. Thus, overexpression of GSTO could contribute to enhanced ATP degradation also by stimulating cyclophilin binding.

Thus, the two proteins GSTO and cyclophilin could contribute in an additive and/or interactive manner to faster postmortem ATP consumption and pH decline. Combined to a high temperature, this accelerates protein denaturation (*35*), which leads to paler meat.

Miscellaneous. LMW-PTP belongs to a family of enzymes that has been studied mainly with respect to their involvement in cell growth and proliferation (*36*). For example, LMW-PTP has been shown to regulate receptors to insulin or to fibroblast growth factor (*11*). LMW-PTP is inactivated by ROS or H_2O_2 (*37*), and its overrepresentation in light samples may be a compensative effect to exposure to excessive ROS. This has, however, never been reported to our knowledge.

Concerning DJ-1, it is reported to be involved in diverse cellular processes (oncogenesis, male fertility, control of protein−RNA interaction, etc.) and in the cellular response to stress (*38*) by acting as an antioxidant and/or chaperone protein. Its overrepresentation in light samples may therefore also reflect a compensation phenomenon to greater exposure to oxidative stress. Hwang (*6*) observed a negative correlation between DJ-1 abundance measured at 24 h postmortem and drip loss. Because discoloration and low water-holding capacity are linked through protein denaturation in PSE meat (*35*), DJ-1 may therefore be indicative of a PSE phenomenon.

Finally, antiquitin is a protein implicated in cellular turgor pressure (*39*). Further investigation would be necessary to confirm that it plays a role in color development.

To summarize, overexpressed proteins in dark samples all pointed to a predominant oxidative metabolism. We propose that such a cellular composition in proteins (metabolic enzymes, heminic pigments, chaperones, etc.) influences postmortem evolution, delaying pH fall and offering a protection against denaturation. In contrast, overexpressed proteins in light samples were more complex to interpret, although the first conclusion is that such muscles relied more on glycolysis as a source of energy. According to observations on glycolytic enzymes, hemoglobin, and transferrin, the hypothesis of a deficiency in muscle oxygenation at the time of slaughter, either by structural vascular deficiency or by inability of the animals to adapt their blood flow to the stressful situation, should not be excluded. Another observation is the overexpression of GSTO, which we believe is probably a key factor triggering several mechanisms. Thus, higher levels of GSTO would contribute to a constant increased cytoplasmic and intramitochondrial [Ca^{2+}] and subsequent ROS production. However, except for overrepresentation of DJ-1 and greater proteolysis of enolase and creatine kinase, we have no substantial evidence of oxidative stress, especially since the most abundant antioxidant enzymes classically reported in intra vitam or postmortem muscles (*40*, *41*) did not appear as differentially expressed. In any case, overexpression of both GSTO and cyclophilin D, together with glycolytic enzymes, is likely to have severe implications on postmortem metabolism, namely, acceleration of ATP depletion and pH fall and subsequent enhanced protein denaturation, well-known to induce lightening. The differential expression of GSTO and cyclophilin D among individual animals could explain at least a part of the variability observed in postmortem metabolism of pig muscle beyond halothane sensitivity (HALn mutation). These results bring a new view on the mechanisms relating muscle characteristics, rate of muscle postmortem metabolism, and meat quality.

ABBREVIATIONS USED

2DE, two-dimensional electrophoresis; ANT, adenine nucleotide translocase; ER, endoplasmic reticulum; GRP58, glucose-regulated protein 58 kDa; GSTO, glutathione S-transferase ω; HSP, heat shock protein; LMW-PTP, low molecular weight phosphotyrosine protein phosphatase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MPTP, mitochondrial permeability transition pore; MW, molecular weight; pI, isoelectric point; PSE, pale soft exsudative; ROS, reactive oxygen species; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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