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ARTICLE

Proteome Changes in Bovine Longissimus Thoracis Muscle during the First 48 h Postmortem: Shifts in Energy Status and Myofibrillar Stability

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Changes in the insoluble protein fraction of bovine longissimus thoracis muscle from eight Norwegian Red (NRF) dual-purpose young bulls during the first 48 h postmortem were investigated by two-dimensional gel electrophoresis (2DE) and matrix-assisted laser desorption ionization−time of flight tandem mass spectrometry (MALDI−TOF MS/MS). Significant changes were observed in a total of 35 proteins, and of those, 26 were identified and divided into three different groups: metabolic enzymes, cellular defense/stress proteins, and structural proteins, according to their predicted function. The majority of the metabolic enzymes identified are involved in the energy metabolism of the cell, while the cellular defense/stress proteins can be related to regulation and stabilization of the myofibrillar proteins. Both easily soluble proteins as well as structural proteins were identified in the insoluble protein fraction. We have studied the changes in solubility during postmortem storage by comparing the postmortem changes in protein composition between the soluble and insoluble protein fractions. We have identified two metabolic enzymes (2,3-bisphosphoglycerat mutase and NADH dehydrogenase) and one protein involved in the stress responses/apoptosis of the cell (Hsp70) that have not been identified previously in the insoluble protein fraction. The occurrence of these easily soluble proteins in the insoluble protein fraction could be due to precipitation or aggregation, thereby going from a soluble to an insoluble state.

KEYWORDS: MALDI−TOF MS; muscle proteins; postmortem; proteomics

1. INTRODUCTION

The conversion of muscle into meat is a sequence of changes and events, with many poorly characterized pathways. In recent years, the application of proteomics in meat science has developed with the aim to achieve a better understanding of mechanisms behind meat product quality (*1*, *2*). The involvement of postmortem degradation of myofibrillar proteins in meat tenderness has been described in a number of studies (*3*−*5*). Particularly, the degradation patterns of structural proteins have been described in detail (*6*, *7*). However, the techniques applied in these studies are more suitable for looking at single proteins. Proteome analysis, however, enables a characterization of complex mixtures of cellular proteins.

Previous proteome analyses of porcine (*8*−*11*) and beef muscles (*12*, *13*) during postmortem storage have shown that many metabolic enzymes and cellular defense/stress proteins, as well as structural proteins, change in abundance during postmortem storage. However, many of these studies have focused on either the soluble protein fraction or the total protein fraction. The aim of this study was to identify and compare proteome changes of the insoluble and soluble protein fractions in bovine longissimus thoracis muscle. We have in the present study analyzed the insoluble protein fraction in bovine longissimus thoracis muscle during postmortem storage. Proteome changes of easily soluble proteins have previously been analyzed for the same samples (*14*).

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2. MATERIALS AND METHODS

2.1. Animals and Sampling. The experiment included eight Norwegian Red (NRF) dual-purpose young bulls (approximately 13 months of age, 450 kg live weight) from a performance test station (GENO-Breeding and AI Association) in 2003. The bulls were transported (1 h) to a commercial slaughterhouse (Nortura, Rudshøgda, Norway) and slaughtered shortly after arrival. The carcasses were electrically stimulated (ES) (90 V) approximately 30 min postmortem. The hot boned M. longissimus thoracis muscles were packed and kept at 12 °C for the first 10 h postmortem and at 4 °C for the rest of the storage period. A piece of muscle tissue was taken at 1 and 48 h postmortem, snap frozen in liquid nitrogen, and stored at −80 °C until further analysis. Thus, the experiment included two sampling times after slaughter (1 and 48 h postmortem) on eight animals (biological replicates).

2.2. Extraction of Muscle Proteins. Frozen muscle tissue (approximately 200 mg) was homogenized in 1 mL of TES buffer [10 mM Tris at pH 7.6, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.25 M sucrose] using Precellys 24 (Bertin Technologies, France) at 5500 rpm for 2 × 20 s. Subsequently, the homogenate was centrifuged (30 min at 7800*g*,

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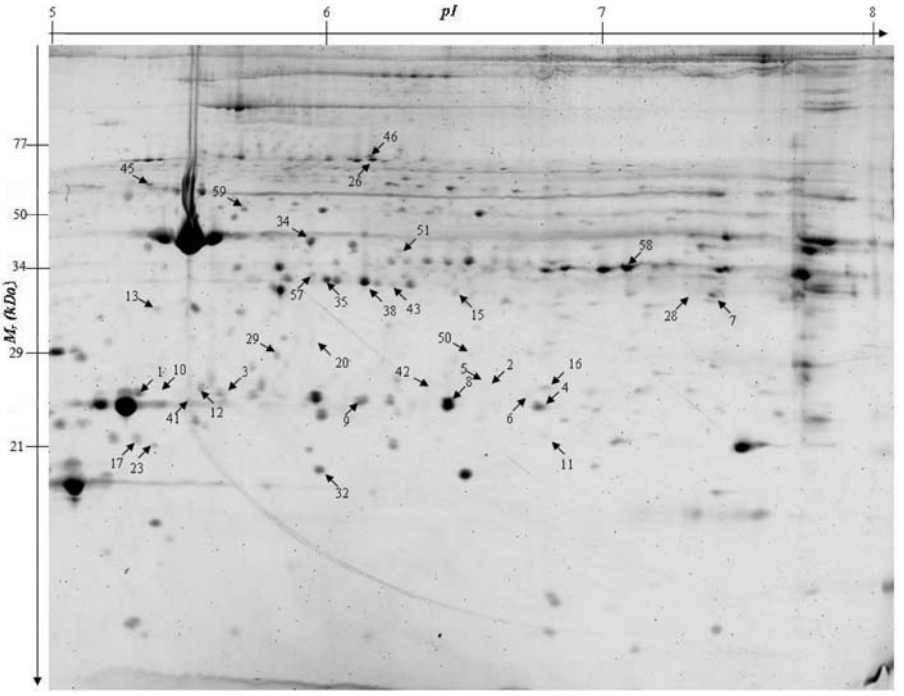

Figure 1. Representative 2DE pattern of the insoluble protein fraction extracted from bovine longissimus thoracis muscle. The protein (150 μg) was separated using a pH range of 5−8 in the first dimension and SDS−PAGE (12.5% T) in the second dimension. Protein spots that changed during the first 48 h postmortem are numbered.

Heraeus, Biofuge frescus, Germany) at 4 °C, and the supernatant was discarded. This step was repeated twice to remove all TES-soluble proteins. Finally, the pellet was homogenized in urea buffer [7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT)] using Precellys 24 at 5500 rpm for 2 × 20 s. The homogenate was shaken vigorously for 1 h at room temperature followed by centrifugation (30 min at 7800*g*) to remove any insoluble components. The concentration of proteins was measured with a commercial kit at 750 nm (RC-DC Protein Assay, Bio-Rad) in a spectrophotometer, with bovine serum albumin (BSA) as a standard.

Each sample was extracted once, while the subsequent two-dimensional gel electrophoresis (2DE) analysis of the samples were conducted twice, giving a total of 32 gels (2 sampling times × 8 biological replicates (animals) × 2 technical replicates). Each gel batch consisted of one replicate of both time points taken from one animal.

2.3. 2DE. Protein separation in the first dimension was performed on immobilized pH gradient (IPG) strips (Bio-Rad), 24 cm, spanning the pH region 5−8. For analytical 2DE, 150 μg of protein was loaded onto each IPG strip by in-gel rehydration overnight at room temperature. For preparative 2DE, 500−1000 μg of protein was loaded onto the IPG strips. The isoelectric focusing was performed using the Ettan IPGPhor II unit (GE Healthcare BioSciences, Uppsala, Sweden). In the initial step, a low voltage (100 V) was applied followed by a stepwise increase to 8000 V, reaching a total of 70 000 V h. In the second dimension, proteins were separated on 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using the Ettan DALT *twelve* large format vertical system (GE Healthcare Bio-Sciences). Analytical gels were stained with Krypton staining. Each gel was immersed in a fixing solution (40% methanol and 10% acetic acid) and gently shaken for 2 × 30 min, followed by rinsing in ultrapure water for 5 min. The gels were immersed in 1× Krypton Protein Stain (Thermo Fisher Scientific, Inc., Rockford, IL), covered with aluminum foil to minimize light exposure, and shaken gently overnight at room temperature. After removal of the staining solution, the gels were immersed in destaining solution (5% acetic acid) for 5 min, followed by rinsing in ultrapure water for 2 × 15 min. Ettan DIGE Imager (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used for scanning, using excitation and emission filters of 540 and 595 nm, respectively, with an exposure value of 0.4 s. Preparative gels were stained by silver staining according to Shevchenko et al. (*15*). For repeated identification of the proteins, several preparative gels were made.

2.4. Image and Data Analyses. Analytical 2DE images were imported into Progenesis SameSpot, version 3.1 (Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, U.K.). A reference gel was selected to match all of the other gels, and artifacts and mismatched spots were removed by manual editing. The protein spots were matched across all 32 gels (2 sampling times × 8 animals × 2 technical replicates), and the spot volumes were normalized by dividing the raw volume of each spot in a gel by the total volume of valid spots in that gel. The data in the resulting table were log-transformed and imported into Unscrambler version 9.6 (CAMO A/S, Norway) for validation of the data by principle component analysis (data not shown). Significance testing was performed in the 50-50 MANOVA program (http://www.langsrud.com/stat/ffmanova.htm), where the rotation test was used to calculate adjusted p values (*16*).

2.5. Mass Spectrometry (MS) Analysis. Protein spots of interest were excised from silver-stained gels with pipet tips and extracted from the gels. Samples were prepared for MS analysis according to methods described by Jia and co-workers (*12*), with minor modifications. After the addition of 30 μL of trypsin digestion buffer, the gel pieces were incubated on ice for 45 min before incubation at 37 °C overnight. For purification of digested proteins, a column was prepared by packing a small plug of C18 material (3 M Empore C18 extraction disk, Varian, St. Paul, MN) into a Gelloader tip (20 μL, Eppendorf, Hamburg, Germany). The sample was directly eluted from the column with 0.8 μL matrix solution [15 mg/mL α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Leipzig, Germany) in 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA)] and spotted directly on the matrix-assisted laser desorption ionization (MALDI) plate (MTP 384 target plate ground steel TF, Bruker Daltonics, Bremen, Germany).

An Ultraflex MALDI−tandem time of flight (TOF/TOF) mass spectrometer with the LIFT module (Bruker Daltonics) was used for protein identification. Peptide calibration standard I (Bruker Daltonics) was used for external calibration. A peak list was generated using the program package FlexAnalysis 2.4 (version 1.1.3, Bruker Daltonics) with median baseline subtraction of 0.8 in flatness and smoothing by the Savitzky−Golay filter of m/z 0.2 in width. For interpretation of MS and MS/MS spectra, BioTools 3.0 (version 1.0, Bruker Daltonics) was used. Proteins were identified by peptide mass fingerprinting (PMF) using the database

Table 1. Identified Proteins from Bovine Longissimus Thoracis Muscle Changing in Abundance during the First 48 h Postmortem[a]

spots	identified proteins	NCBI accession number (source)	matched peptides/percent sequence coverage (%)	experimental ~pI/~MW (Da)	theoretical pI/MW (Da)	fold change
		Metabolic Enzymes				
2	2,3-bisphosphoglycerate mutase (fragment)	gil78369370 (bovine)	9/37	6.60/25900	6.03/30271	+3.7
6	adenylate kinase 1	gil61888850 (bovine)	8/45	6.75/23200	8.40/21764	+3.1
7	creatine kinase M chain (fragment)	gil4838363 (bovine)	11/30	7.40/33000	6.63/43172	+3.1
16	creatine kinase M chain (fragment)	gil4838363 (bovine)	8/23	6.80/23000	6.63/43172	+2.1
58	glycerol-3-phosphate dehydrogenase 1	gil78365297 (bovine)	13/36	7.10/36500	6.42/38236	−1.4
20	NADH dehydrogenase (ubiquinone) Fe−S protein 3, 30 kDa	gil27807353 (bovine)	9/31	5.90/30000	6.25/30378	−1.9
		Cellular Defense/Stress Proteins				
1	heat-shock 27 kDa protein 1	gil71037405 (bovine)	4/27	5.35/24200	5.77/22722	+4.9
3	heat-shock 27 kDa protein 1	gil71037405 (bovine)	13/53	5.60/23500	5.77/22722	+3.5
4	crystallin, αB	gil27805849 (bovine)	15/72	6.80/23200	6.76/20024	+3.5
10	heat-shock 27 kDa protein 1	gil71037405 (bovine)	4/27	5.40/24200	5.77/22722	+2.8
12	heat-shock 27 kDa protein 1	gil71037405 (bovine)	11/49	5.55/23900	5.77/22722	+2.4
32	heat-shock protein, α-crystallin-related, B6	gil119224088 (bovine)	7/60	6.00/20800	5.95/17525	+1.6
46	heat-shock 70 kDa protein 1A	gil40254806 (bovine)	27/44	6.20/72500	5.55/70492	−1.5
26	heat-shock 70 kDa protein 1A	gil73853769 (bovine)	27/46	6.20/69000	5.68/70530	−1.7
9	heat-shock 27 kDa protein 1	gil71037405 (bovine)	7/39	6.15/23400	5.77/22722	−2.9
8	heat-shock 27 kDa protein 1	gil71037405 (bovine)	18/72	6.45/23200	5.77/22722	−3.0
		Cell Structure Proteins				
5	actin, α1, skeletal muscle (fragment)	gil134024776 (bovine)	14/60	6.60/25100	5.23/42338	+3.2
13	F-actin-capping protein subunit β (CapZ β)	gil13124696 (bovine)	11/40	5.40/30800	5.36/31616	+2.3
28	troponin T fast skeletal muscle type	gil21039002 (bovine)	11/35	7.35/33000	8.70/30684	+1.7
34	actin, α1, skeletal muscle	gil27819614 (bovine)	7/19	5.95/42000	5.31/42451	+1.6
41	actin, α1, skeletal muscle (fragment)	gil27819614 (bovine)	13/29	5.50/23900	5.31/42451	+1.5
51	actin, α1, skeletal muscle	gil134024776 (bovine)	7/22	6.30/40000	5.23/42338	+1.4
59	actin, α1, skeletal muscle	gil134024776 (bovine)	9/42	5.70/55500	5.23/42338	−1.3
57	capping protein (actin filament) muscle Z-line, α1	gil134085807 (bovine)	10/54	5.85/33200	5.53/33082	−1.4
45	capping protein (actin filament) muscle Z-line, β	gil28603770 (bovine)	14/42	5.40/58100	6.02/34176	−1.5
38	troponin T1, skeletal slow	gil41386697 (bovine)	13/30	6.15/33200	5.71/31265	−1.6

[a] Of the 35 spots changed during postmortem storage of bovine muscle with a 5% significance level, we identified 26 spots.

search program MASCOT (http://www.matrixscience.com/), searching against the mammalian NCBInr 20070216 database. Search parameters within MASCOT were set with a MS tolerance of 100 ppm and a MS/MS tolerance of 0.5 Da, and the maximum of missed cleavage sites was 1 (see the Supporting Information). Carbamidomethyl (C) and oxidation (M) were used as fixed and variable modification, respectively. MS/MS analysis and repeated MASCOT-based database searches of precursor ions recognized in the PMF search were performed to confirm the PMF-based protein identification. The number of peptide matches, sequence coverage, pI, and molecular weight were used to evaluate the database search results.

3. RESULTS

The experiment included two sampling times after slaughter, 1 and 48 h postmortem, from a total of eight NRF young bulls. Proteins extracted from the longissimus thoracis muscle with TES buffer were discarded, and the resulting pellet was washed twice with TES buffer and then dissolved in urea buffer. This protein fraction, which was analyzed in the present study, is referred to as the insoluble protein fraction. **Figure 1** shows a representative image of the 2DE pattern of the insoluble protein fraction. Proteins in the molecular mass region of 10−75 kDa and the pH range between 5 and 8 were included in the comparative analysis. The majority of the proteins were located within pH 5−7. A total of 300 protein spots were matched across all 32 gels, and 35 of these were found to change significantly in abundance during the first 48 h postmortem (**Figure 1**). A total of 26 spots were successfully identified, and these are listed in **Table 1**. Spots that could not be identified were either difficult to extract from the 2DE gels or present in an insufficient amount for identification by MS.

The identified proteins could be grouped according to their predicted function: metabolic enzymes, cellular defense/stress proteins and structural proteins. A total of six metabolic enzymes were identified, four of them showed higher abundance and two of them showed lower abundance after 48 h postmortem. Two spots identified as creatine kinase fragments showed higher abundance at 48 h postmortem. Adenylate kinase 1 and 2,3-bisphosphoglycerate mutase (fragment) also showed higher abundance, while NADH dehydrogenase and glycerol-3-phosphate dehydrogenase 1 showed lower abundance after 48 h postmortem.

A total of 8 of 10 cellular defense/stress proteins identified were members of the small heat-shock protein family, with nearly all of them being heat-shock 27 kDa protein (Hsp27). A total of six Hsp27 proteins were identified: four of them had higher abundance after 48 h postmortem, while the other two had lower abundance. Two α-crystallin proteins were identified, and both of them showed higher abundance after 48 h postmortem, while two Hsp70 proteins had reduced abundance after 48 h postmortem.

A total of 10 structural proteins were identified, including 3 capping proteins, 5 actins (two fragments) and 2 troponin T isoforms. One of the capping proteins showed higher abundance after 48 h postmortem, while the other two had lower abundance at 48 h postmortem. For one of the five proteins identified as actin, the abundance was lower at 48 h postmortem. One of the proteins identified as troponin T had higher abundance after 48 h postmortem, while the other had lower abundance at that time point.

4. DISCUSSION

In this study, we have investigated the proteome changes in the insoluble protein fraction of bovine longissimus thoracis muscle during the first 48 h postmortem. The goal was to obtain more knowledge about the biochemical processes taking place during this period. Furthermore, we wanted to compare our results to previously reported proteome changes in the soluble protein fraction from the same samples (*14*).

In this study, proteins in the molecular mass region of 10−75 kDa and the pH range between 5 and 8 were included in the comparative analysis. On the basis of previous experiments, the pH gradient 5−8 was found to provide useful information of the postmortem changes in the bovine muscle proteins. This limits our ability to capture some of the structural proteins because of their high molecular weight (MW) and low pI. However, previous studies have shown that we are able to analyze some of the structural proteins on the given MW and pI range.

The carcasses analyzed in this study were ES shortly after slaughter. ES results in an accelerated postmortem glycolysis, resulting in a rapid pH decline and earlier development of rigor mortis (*17*, *18*). Moreover, studies have reported that ES enhances postmortem proteolysis of myofibrillar and cytoskeletal proteins (*19*, *20*). Thus, the changes in protein solubility and abundance postmortem could be enhanced by effects of ES.

4.1. Comparison of Proteome Changes in the Soluble and Insoluble Protein Fractions. In a previous study of the soluble protein fraction (*14*), the postmortem changes in protein composition were investigated up to 24 h, while in the present study of the insoluble protein fraction, the postmortem changes were analyzed up to 48 h postmortem.

Adenylate kinase 1, glycerol-3-phosphate dehydrogenase 1, and one fragment of creatine kinase increased in abundance in both fractions during postmortem storage (*14*). This indicates a possible shift in solubility from 24 to 48 h postmortem. Two metabolic enzymes identified in the insoluble protein fraction were not found in the soluble fraction, namely, 2,3-bisphosphoglycerate mutase (fragment) and NADH dehydrogenase. Both of these enzymes have previously been identified in the total protein fraction of pig muscle (*21*, *22*). However, the appearance of these enzymes in the insoluble protein fraction of muscle has to our knowledge not previously been reported.

A total of six proteins from the group of cellular defense/stress proteins were found in both protein fractions, with all of them belonging to the small heat-shock protein family. The abundance of these proteins decreased during the postmortem period in the soluble protein fraction (*14*), while their abundance increased in the insoluble protein fraction. This indicates that the small heat-shock proteins shift from the soluble to the insoluble protein fraction possibly as a result of aggregation onto myofibrillar proteins, thereby following them during extraction. α-Crystallin and Hsp70 were only found in the insoluble protein fraction in the present study. α-Crystallin has previously been identified in the insoluble protein fraction of bovine muscles (*23*, *24*), while Hsp70 has only been identified in the soluble protein fraction (*23*) and the total protein fraction of pig muscle (*22*).

In the group of cell structure proteins, only actin was identified in both of the protein fractions. However, only one spot was identified as actin in the soluble protein fraction, while five protein spots were identified as actin in the insoluble protein fraction. Even though actin is in theory a component of the insoluble protein fraction, some copies have been found to be extracted with soluble proteins (*13*). A total of five cell structure proteins were only identified in the insoluble protein fraction, three capping proteins and two troponin T isoforms. Capping proteins have not been found in previous studies concerning a comparison of soluble versus insoluble protein fractions of bovine muscle. However, some capping proteins have been identified in the total protein fraction of pig muscle (*22*).

4.2. Protein Solubility. Some of the proteins identified in the insoluble protein fraction are considered to be easily soluble proteins. Indeed, some of these proteins were found in the easily soluble protein fraction previously investigated in these samples (*14*). Furthermore, other studies have also identified easily soluble proteins in the insoluble protein fraction of muscle (*23*−*25*). This indicates that the protein solubility changes during postmortem storage. By comparison of the soluble and insoluble protein fractions from the same samples, we are able to assess changes in solubility of these proteins.

Previous studies have suggested that the changes in protein solubility were caused by higher temperatures and lower pH conditions present in postmortem tissue (*23*, *24*, *26*). Boles et al. (*25*) suggested that these same reasons resulted in proteins from the easily soluble protein fraction becoming denatured and insoluble and then forming aggregates that precipitate onto myofibrils. Furthermore, Laville et al. (*23*) speculated that the appearance of soluble proteins in the insoluble protein fraction during the first 5 days of muscle aging was because they had been subjected to chemical modifications, making them insoluble. Different mechanisms could be responsible for these changes, such as isoelectric precipitation because of pH decline (*25*, *27*), postmortem degradation or modification of proteins (*23*, *25*), and interaction of cell defense or stress proteins with myofibrils during stress conditions (*28*, *29*). Further investigations will be necessary to unravel the underlying mechanisms behind the changes that we observed in solubility of proteins during postmortem storage.

In a previous study (*23*) of postmortem changes in beef, it was suggested that the glycolytic enzymes creatine kinase and glyceraldehyde-3-phosphate dehydrogenase could be modified upon exposure to oxidative stress, which might explain their changes in solubility. Mammalian members of the small heat-shock protein family undergo increased phosphorylation under heat-shock conditions, which leads to changes in the oligomeric organization (*29*). This results in the formation of both small and large oligomers, which may be responsible for stabilization of the actin filaments, thus protecting against stress.

4.3. Metabolic Enzymes. After slaughter, the energy metabolism in the muscle is switched from aerobic to anaerobic metabolism. At that time, the two most important sources of ATP become degradation of glycogen to lactic acid and the transfer of phosphate from creatine phosphate to ADP to yield creatine and ATP (*30*). Three of the four metabolic proteins showing higher abundance in the insoluble protein fraction at 48 h postmortem, two creatine kinases and one adenylate kinase 1, are involved in the energy metabolism of the cell. All of these proteins are in some way involved in the reversible transfer of a phosphate group between ATP and ADP. Both of the proteins identified as creatine kinase are considered to be fragments, while adenylate kinase 1 is most likely phosphorylated because that particular post-translational modification lowers the pI value (*31*), but other modifications cannot be excluded. Adenylate kinase 1 has previously been

found to have a similar experimental pI value in the soluble protein fraction from the same samples (*14*). Moreover, fragments of creatine kinase have also been found to increase in abundance during postmortem storage of porcine meat (*10*). Given that both of these enzymes play an important role in energy metabolism under anaerobic conditions, these findings are in line with a higher demand for energy.

2,3-Bisphosphoglycerate mutase serves an important function in oxygen transport, causing a conformational change in hemoglobin, which results in the release of oxygen, making it possible for local tissues to pick up free oxygen. This enzyme has previously been identified in the total protein fraction of pig longissimus muscle, where postmortem proteolysis was assessed by gel-based proteome analysis (*21*). Furthermore, Durany and Carreras (*32*) have found the enzyme in skeletal muscles of rats, rabbits, and humans. The fragment of this enzyme had higher abundance after 48 h postmortem. The need for 2,3-bisphosphoglycerate mutase diminishes after slaughter when the oxygen transfer to the muscle stops. Accumulation of fragments of this enzyme could be the result of degradation because this enzyme is no longer needed. NADH dehydrogenase is located in the inner mitochondrial membrane and catalyzes the transfer of electrons from NADH to coenzyme Q. Lower amounts of this enzyme during the postmortem time period might indicate that the need for it diminishes when the energy metabolism of the cell shifts from aerobic to anaerobic and other sources of ATP production become more important. Indeed, this enzyme decreased in abundance during postmortem storage of porcine muscle in a previous study (*22*). Glycerol-3-phosphate dehydrogenase 1 showed lower abundance after 48 h postmortem in the insoluble protein fraction. This may indicate degradation of this enzyme because energy metabolism ceases.

4.4. Cell Defense/Stress Proteins. A total of 8 of 10 proteins identified as cellular defense or stress proteins in this study belong to the family of small heat-shock proteins: Hsp27 and α-crystallin. Members of the small heat-shock protein family have a function to prevent degradation and structural damage of proteins from apoptotic processes in muscle cells (*33*). It is believed that Hsp27 and crystallin are involved in regulation and stabilization of the myofibrillar proteins. They are also involved in protection of actin filaments and other cytoskeletal proteins from fragmentation caused by stress conditions (*34*, *35*). The observation of higher abundance of these proteins during postmortem storage of bovine muscle therefore indicates a change in the stabilization of the myofibrillar proteins.

A total of six proteins were identified as Hsp27, with four of them showing higher abundance after 48 h postmortem, while the other two had lower abundance. All of these Hsp27 proteins are representing the full-length protein with some modifications, because their experimental and theoretical pI values differ. Previous analysis of porcine (*10*, *11*) and bovine (*12*−*14*) muscles have demonstrated that Hsp27 increases in abundance shortly after slaughter but decreases during storage. Both of the crystallin proteins identified increased in abundance postmortem, and the same observation was made in a previous study (*12*).

Hsp70 was identified in two spots, with both of them showing lower expression 48 h postmortem. In a previous study of proteome changes of bovine muscle, fragments of Hsp70 were found to increase in abundance in the soluble protein fraction at day 21 postmortem (*23*). Hsp70 is an ATP-dependent chaperone, which can prevent partially denatured proteins from aggregating and allows them to refold, thereby protecting cells from thermal or oxidative stress (*36*, *37*). Furthermore, Hsp70 has been found to directly inhibit apoptosis (*38*). The appearance of Hsp70 in the insoluble protein fraction indicates that the protein shifts from the soluble to the insoluble state, because it might bind to myofibrillar proteins during stress conditions shortly after slaughter and prevent their aggregation. The decreasing abundance of this protein during postmortem storage might indicate that the degradation of it starts, which may point to a change in the stability of the myofibrillar proteins.

4.5. Cell Structure Proteins. There are complex interactions of biochemical processes that take place during the conversion of muscle to meat (*39*, *40*). The influence of these processes on the final texture and tenderness of the meat are still not clear, but it is well-documented that fragmentation of myofibrils takes place during postmortem storage of meat and that this is related to the tenderness of aged meat (*41*). The proteolytic degradation of structural proteins, including titin, nebulin, troponin T, desmin, filamin, and vinculin, plays a major role in the development of meat tenderness according to a number of previous studies (*3*−*5*, *9*, *41*−*43*). Of these structural proteins, only troponin T was observed in the present study. A possible explanation is that, because the other above-mentioned structural proteins have high molecular weights, they are not observed on the gels in which proteins in the 10−75 kDa range are resolved.

All of the structural proteins identified in this study, capping protein, actin, and troponin T, have previously been related to meat quality (*8*−*10*, *21*). A total of 3 of the 10 structural proteins were identified as capping proteins, with 2 of them showing lower abundance 48 h postmortem. Capping proteins play an important role in the control of actin polymerization (*44*). Hart and Cooper stated that capping proteins are a key factor in maintaining thin filament uniform length, interacting with both actin and additional components of the Z-line, and its downregulation leads to myofibrillar disarray (*45*). Given that actin and other associated structural proteins are prone to degradation postmortem, it might indicate that degradation of capping proteins also starts because the need for them ceases as a result of their sentral role in actin stabilization. Furthermore, one of the capping proteins showing lower abundance has a considerably higher molecular weight than expected, which might indicate that that particular isoform is still bound to other proteins. However, one of the identified capping proteins in the present study was found to increase in abundance. This pattern has also been observed for one isoform of capping protein in the total protein fraction in porcine muscle (*9*). The reason for this is unclear, but it might indicate different rates of degradation for diverse isoforms of the protein.

A total of five proteins were identified as actin, and only one of them showed lower abundance after 48 h postmortem. Three of the identified actins are representing the full-length protein. The degradation of actin has been demonstrated in a previous postmortem analysis of porcine muscle (*9*).

Two proteins in our study were identified as troponin T, fast and slow isoforms. The postmortem degradation of troponin T is well-known and believed to be related to tenderness (*46*). Lametsch et al. (*10*) reported that the full-length troponin T (43 kDa) decreased in abundance postmortem, while the 38 kDa troponin T fragment accumulated during postmortem storage. Both isoforms of troponin T identified in our study are considered to be representing full-length proteins. The experimental pI value for one of them was lower than expected, which might indicate phosphorylation, and it has a higher abundance 48 h postmortem. The reason why one of these isoform decreases while the other increases might be related to changes in their solubility or degradation during postmortem storage, which might differ for the fast and slow troponin isoforms.

4.6. Concluding Remarks. In this study of the insoluble protein fraction from bovine longissimus thoracis muscle during

the first 48 h postmortem, both easily soluble proteins as well as structural proteins have been identified. The advantage of investigating the insoluble protein fraction is that more structural proteins are present, which provides better opportunity to study postmortem proteolysis. By comparing the postmortem changes in protein composition between the soluble and insoluble protein fractions, we are able to look at the changes in solubility during postmortem storage. We have identified two metabolic enzymes (2,3-bisphosphoglycerat mutase and NADH dehydrogenase) and one protein involved in the stress responses/apoptosis of the cell (Hsp70) that have not previously been identified in the insoluble protein fraction. The occurrence of these easily soluble proteins in the insoluble protein fraction could be due to precipitation or aggregation, thereby going from a soluble to an insoluble state. Different mechanisms might be responsible for this change in the protein solubility, e.g., isoelectric precipitation because of pH decline and modification of proteins; however, further studies are needed to unravel the specific mechanism behind the observed changes in this study. This study indicates a connection between the stability of myofibrillar proteins and the solubility of easily soluble proteins, such as metabolic enzymes and cellular defense/stress proteins. Further studies should involve studies of the insoluble protein fraction over a longer time period postmortem, where it would be interesting to connect the rate of pH decline in the first few hours postmortem to changes in protein solubilization.

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Supporting Information Available: Details concerning each identified protein: residue number, residue size, error (ppm), number of missed cleavage sites, MS/MS score, and sequence of identified peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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