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Proteome Analysis Applied to Meat Science: Characterizing *Post Mortem* Changes in Porcine Muscle

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The aim of this work was to test the application of two-dimensional electrophoresis (2DE)-based proteome analysis in studying muscle tissues and meat of pork, and to use this technology to characterize as many of the changes that occur in pig muscle proteins during *post mortem* storage of the carcass as possible. For this purpose, 2DE proved to be a powerful tool, as it is far more sensitive and shows a higher resolving power than conventional SDS−PAGE, allowing for the precise and semiquantitative recognition of approximately 1000 individual muscle proteins in every 2DE display. In this study, we have chosen to analyze the subset of muscle proteins that have molecular masses of 5−200 kDa, and can be reproducibly separated in the pH span of 4−9. By comparing 2DE patterns of muscle samples taken immediately after slaughter (time 0), as well as those taken 4, 8, 24, and 48 h *post mortem*, we have estimated the relative changes of individual muscle proteins during the *post mortem* storage of the carcass. In this paper, of these changes we report the 15 most notable.

Keywords: *Proteome analysis; porcine muscle proteins; 2DE; meat quality; post mortem metabolism; molecular markers for meat quality*

INTRODUCTION

The postgenomic era has seen the rapid development of new methods for studying the complex patterns of protein and gene expression in tissues and cells. This novel field, commonly termed functional genomics, is focused on dissecting the molecular networks that form the basis for cellular functions and physiologic processes. For recent reviews, see refs *1* and *2*. Proteome analysis, defined as the analysis of the protein content expressed by a genome (*3*), aims to characterize the complex patterns of expressions and turnovers of cellular proteins. Two-dimensional electrophoresis (2DE) is a cornerstone technique of proteome analysis, and is a powerful tool for studying correlated up- and down-regulation of cellular proteins. Basically, the 2DE analysis aims to display the complement of cellular proteins by separating the individual proteins according to their isoelectric point along the *X*-axis, and then according to their molecular mass along the *Y*-axis. Hence, the 2DE pattern displays the relative expression of a complement of the proteins in a cell or a tissue type. For a review of 2DE technology, see ref *4*.

Meat quality is a complex trait influenced by genetic components, by the handling of animals during production, transportation, and slaughter, and by the handling of the meat during the slaughter process.

Immediately after slaughter, there is in the muscle tissues as in all other organs a shift of biochemical processes in response to the halt of the respiratory system and the circulation of blood. In muscle tissues, the morphological and ultrastructural changes as well as the changes in the energy metabolism that occur in the *post mortem* period have been well described (*5*), and it is now known that the rate and extent to which the *post mortem* metabolic processes run greatly influence important traits such as tenderness, water-holding capacity, and sensoric properties of meat (*5*). However, most of the individual proteins and genes that are involved in the *post mortem* metabolism of pig muscle remain to be described.

With regard to the tenderness of meat, it is well-known that the *post mortem* storage of meat greatly influences tenderness, and it is believed that degradation and denaturation of proteins during *post mortem* aging is responsible for the tenderization of meat. For a review, see ref *6*. The activities of calpains and their inhibitors, calpastatins (*7*, *8*), have been especially associated with the tenderization process. By using the methods of one-dimensional SDS−PAGE analysis, several sarcoplasmic proteins, including titin, (*9*) nebulin, troponin T, desmin, filamin, vinculin, and dystrophin, have been observed to be degraded (*10*−*13*). Nevertheless, the questions of whether and how the degradation of these proteins relates to meat tenderness are still not settled, and a more detailed characterization of the individual molecular changes that take place during *post mortem* storage is needed.

The aim of this work was to optimize the technologies of proteome analysis for separation of porcine muscle proteins, and to employ these methods to study the changes that occur in the protein profiles of pig muscle during the first 48 h of *post mortem* storage. We wanted to test whether proteome analysis is useful for observing and characterizing some of the molecular changes that occur in muscle tissues and pork during *post mortem* storage of the carcass. Such molecular changes, when

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fully characterized, will yield information about *post mortem* metabolic processes, and about proteolysis and other modifications of muscle proteins during the *post mortem* storage of pig meat.

MATERIALS AND METHODS

Animals and Tissue Samples. The animals were treated and slaughtered according to the Danish government regulations on treatment of livestock.

The animals used in this study were seven littermates originating from a cross between a Danish Landrace/Yorkshire sow and a Hampshire boar. The pigs were raised on a commercial farm and slaughtered at an average live weight of 100 kg at the experimental slaughter plant at the Danish Institute of Agricultural Sciences (DIAS). After 1 h *post mortem*, the carcasses were stored at 4 °C. Both females and males, as well as both RN- and wild-type animals, were included in the study.

Muscle samples were taken from the carcasses immediately after exsanguination (time 0) and after 4, 8, 24, and 48 h *post mortem*. The samples were taken from the longissimus dorsi muscle, at the position of the last rib. Biopsies were removed as described in ref *14* and were frozen immediately in liquid N_2. Muscle samples were kept at −80 °C until the time of protein extraction.

Extraction of Muscle Proteins. Pieces of 100 mg frozen muscle tissues were cut and weighed at −20 °C to minimize artifactual protein degradation. Frozen muscle tissue (100 mg) was homogenized in 1 mL of 8 M urea, 2 M thiourea, 50 mM DTT, 1% CHAPS-O, and 1% carrier ampholytes (Ampholyte 3-10, Pharmacia, Uppsala, Sweden), in a hand-held glass homogenizer. Crude extracts were transferred to an Eppendorf tube and vigorously shaken for 2 h at room temperature, followed by a 30 min centrifugation step at 10000*g*, to remove unextracted cellular components, high-molecular mass protein complexes, and unsoluble proteins, as only soluble proteins can be separated by 2DE analysis. Extractions with SDS were carried out as described in ref *15*. Extractions including removal of nucleic acids were performed as described in ref *16*. The protein concentration in muscle extracts was analyzed using a Coomassie-based protein assay (Bio-Rad, Hercules, CA), and was consistently found to be in the range of 3−4 mg/mL.

Two-Dimensional Electrophoresis (2DE). The preparation and running of 2DE analysis were based on the methods described in ref *17*. The first dimension of protein separation was made in immobilized pH gradient strips (IPG strips). The IPG gradients spanning the pH regions of 4−9, 4−7, and 3−10 have been tested for separating pork muscle proteins. For the *post mortem* study, we have chosen 3 mm × 180 mm strips that span the pH range of 4−9. These strips were cast in our laboratory according to the methods described in ref *17*. Fifty micrograms of protein was loaded onto every IPG strip, by including the protein extraxt when rehydrating the IPG strips. Rehydration was performed for 20 h at room temperature. Isoelectric focusing of IPG strips was performed using a Pharmacia Multiphor instrument equipped with a Heto temperature controler run at 19 °C. Several voltage gradients have been tested (data not reported). In our hands, the ideal conditions for focusing were used with an initial 3 h step at 50 V, whereafter the voltage was gradually increased to 3500 V, and kept at this voltage until reaching steady state, or a maximum of 700 000 volt hours. Current and power were limited to 0.05 mA and 0.2 W per IPG strip, respectively. IPG strips were frozen at −80 °C immediately after focusing, and kept frozen until they were separated in the second dimension.

SDS−PAGE was used for the second-dimension separations, running continuous 10% acrylamide gels according to the methods described in ref *18*. We have used ESA, Investigator (Genomic Solutions, Chelmsford, MA), which allows the simultanous casting and running of 10 SDS−PAGE plates (25 cm × 30 cm). The focused IPG strips were equilibrated to the SDS−PAGE buffer systems by being washed for 15 min in a reducing equilibration buffer [1% DTT, 6 M urea, 2% SDS, and 30% glycerol, in 50 mM Tris-HCl (pH 8.8)], followed by a 15 min washing in an alkylating buffer [5% iodoacetic acid, 6 M urea, 2% SDS, and 30% glycerol, in 50 mM Tris-HCl (pH 8.8)]. Equilibrated strips were drained for excess fluid, mounted onto the SDS−PAGE apparatus, and sealed in place with boiling 1% agarose in Laemmli running buffer.

Visualization of Proteins. Coomassie staining, as well as silver staining, was used to visualize protein patterns in the 2DE matrix. The silver staining methods from refs *19* and *20* were tested. Optimal staining of porcine muscle proteins was achieved by using a slight modification of the method of ref *20* as follows. Gels were fixed overnight in 40% ethanol and 10% acetic acid. Sensitizing was carried out with 0.25% glutaraldehyde in 0.5 M sodium acetate. Staining was carried out in 0.018 M NaOH, 0.29% NH_3, and 8.8 g of $AgNO_3$/L, and the gels were developed in 0.044% formaldehyde and 0.16 g/L citric acid. Staining was stopped with 7% acetic acid, 20% ethanol, and 1% glycerol.

Image Analysis. The 2DE gels were scanned using a U-Max office scanner, and analyzed using the Bioimage 2D analyzer software (Genomic Solutions). First, all spot positions were recognized, and relative integrated spot intensities in the individual gels were estimated. Then, the 2DE images were matched by comparing the relative positions and integrated intensities of the individual spots on each gel. For comparative image analysis, the images were grouped, after which the relative levels of expression of individual spots were analyzed and compared within and between the image groups. The matches suggested by automated image analysis were finally individually inspected and confirmed.

Protein Identification. Major muscle proteins were recognized by comparing the 2DE patterns of the porcine muscle to those of the mouse gastrocnemius muscle 2DE database published on the ExPASy website.

RESULTS

The focus of this work was to adapt the methods of proteome analysis to analyze muscle tissues from pigs, and to test whether this technology could be used to describe some of the protein changes that occur in pig muscles during onset of *rigor mortis* and in the subsequent tenderization process. This was done by establishing and comparing 2DE analyses of muscle samples that were taken from pig carcasses during 48 h *post mortem*. The experimental design is reviewed in Figure 1. Our data show adaptation and integration of methods of 2DE analysis that allows high resolution of major porcine muscle proteins. The 2DE protein patterns we achieved are sufficently reproducible to allow comparative proteome analysis of pork muscle, and 2DE is a powerful tool for characterizing some of the major changes that occur in the proteome profiles during *post mortem* storage of pork meat.

To achieve an optimal and highly reproducible display of porcine muscle proteins, three different methods of sample preparation were tested. To keep intersample variations to a minimum, an ideal sample preparation protocol should have few individual preparative steps, and allow solubilization of as many different proteins as possible. According to the method of ref *17*, tissue samples were thawed and homogenized directly in a strongly reducing and denaturing buffer, to minimize proteolytic activity in the disrupted tissues. This Chaps-based extraction method yielded a reproducible extraction of proteins, as seen in Table 1A. Extraction of muscle tissues by the SDS boiling method described in ref *15* gave a better extraction of high-molecular mass proteins, but resulted in less reproducible IPG focusing (data not shown). Extraction methods including the

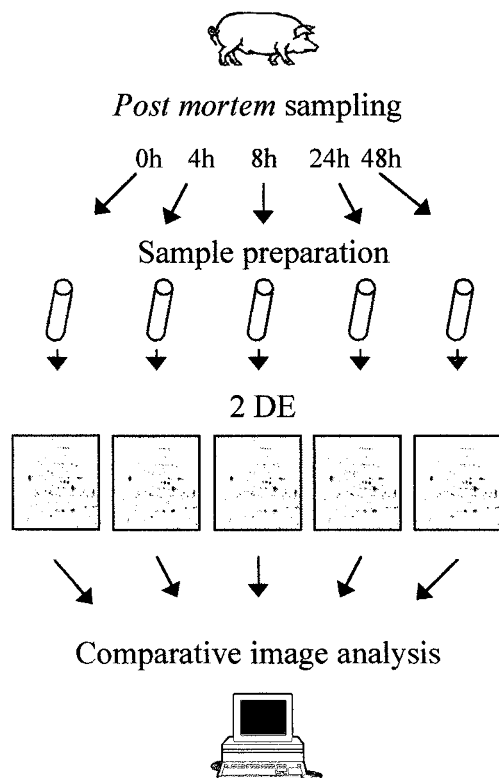

Figure 1. Experimental design of *post mortem* proteome analysis. Muscle samples were collected from seven pigs at 0, 4, 8, 24, and 48 h *post mortem*. Samples were homogenized and separated by 2DE analysis. Computer-assisted comparative image analysis allowed detection of porcine muscle proteins that change during *post mortem* storage of all the animals.

Table 1. (A) Comparison of Protein Extraction Methods and (B) Protein Concentrations in *Post Mortem* Muscle Extracts

(A) Protein Extraction Methods[a]

protein (μg/μL)	
Christensen et al. (*16*)	Görg et al. (*17*)
3.5	2.7
2.9	3.3
2.9	3.9
3.3	4.2
3.0	3.6

(B) Protein Extraction *Post Mortem*[b]

hours *post mortem*	protein (μg/μL)
0	2.7
4	3.0
8	2.9
24	3.2
48	3.3

[a] Ten 100 mg pieces were cut from a single 100 g piece of frozen porcine muscle tissue. The 100 mg pieces were homogenized in 1 mL of either nuclease-supplemented buffer (*16*) or in a reducing and denaturing lysis buffer (*17*). Nuclease-treated samples were subsequently lyophilized and redissolved in lysis buffer. The protein concentrations were determined as described in Materials and Methods. [b] Five 100 mg pieces of *post mortem* muscle tissues were homogenized in 1 mL of lysis buffer according to the method of ref *17*. The protein concentrations were determined as described in Materials and Methods.

removal of nucleic acids, as described in ref *16*, did not result in a better recovery of protein, as seen in Table 1A. Figure 2 shows a comparison of 2DE-separated muscle proteins prepared with or without nuclease

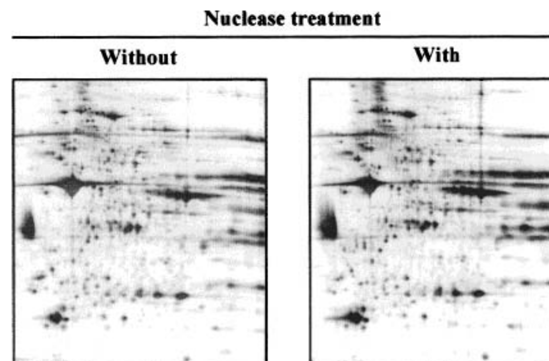

Figure 2. Extraction of porcine muscle proteins. Extraction of porcine muscle proteins with and without nuclease treatment has been compared. One hundred milligram pieces were cut from a single 100 g piece of frozen porcine muscle tissue. The 100 μg pieces were homogenized in 1 mL of either nuclease-supplemented buffer (according to ref *16*) or in a reducing and denaturing lysis buffer (according to ref *17*). Nuclease-treated samples were subsequently lyophilized and redissolved in the above-described lysis buffer. Protein aliquots (50 μg) were separated on IPG 4-9.

treatment. We observed that neither the separation nor the sample load was enhanced by removal of DNA/RNA during extraction; hence, this additional step seemed to be disadvantagous for the extraction of porcine muscle proteins, and we have chosen the Chaps-based method of ref *17* for comparative proteome analysis of *post mortem* muscle. Different focusing conditions and voltage gradients were tested to find the optimal resolution and reproducibility (data not shown). We have found that a rather prolonged initial step using low voltage is beneficial for optimal focusing of porcine muscle proteins. As for optimal focusing gradients, we have tested the separation of pig muscle proteins on pH gradients of 4−9, 4−7, and 3−10, as seen in Figure 3. While the pH gradient of 4−7 gave the highest resolving power in a rather narrow zone, the gradient of pH 4−9 allowed the maximum range of well-seprated muscle proteins. It is clearly seen in Figure 3 that the pH gradient of 3−10 did not display well-resolved proteins in the zone at pH >7. Hence, in this study, we found the pH gradient of 4−9 to be optimally suited for separating and analyzing porcine muscle proteins, and this gradient was selected for the analysis of *post mortem* conditions.

We have compared protein visualization by Coomassie staining as well as two different protocols of silver staining, as seen in Figure 4. All staining methods allow semiquantitative estimation of the relative levels of expression of individual proteins in the tissue samples. The comparison seen in Figure 4 shows that all silver staining methods were far more sensitive than Coomassie staining. The method described in ref *19* gave a suboptimal staining of acidic proteins, while the method of ref *20* gave a rather sensitive staining in the entire pH span, and an acceptable background staining. This silver staining method was clearly the most sensitive and reproducible for staining porcine muscle proteins, and was used for the subsequent *post mortem* study. When optimal extraction, focusing, and staining conditions were used, 1000 well-separated individual protein spots typically appeared in each gel, as estimated by computer-assisted image analysis.

Integrating the selected methods as described above, we used 2DE analysis to study protein changes that occur in porcine muscle during *post mortem* storage of

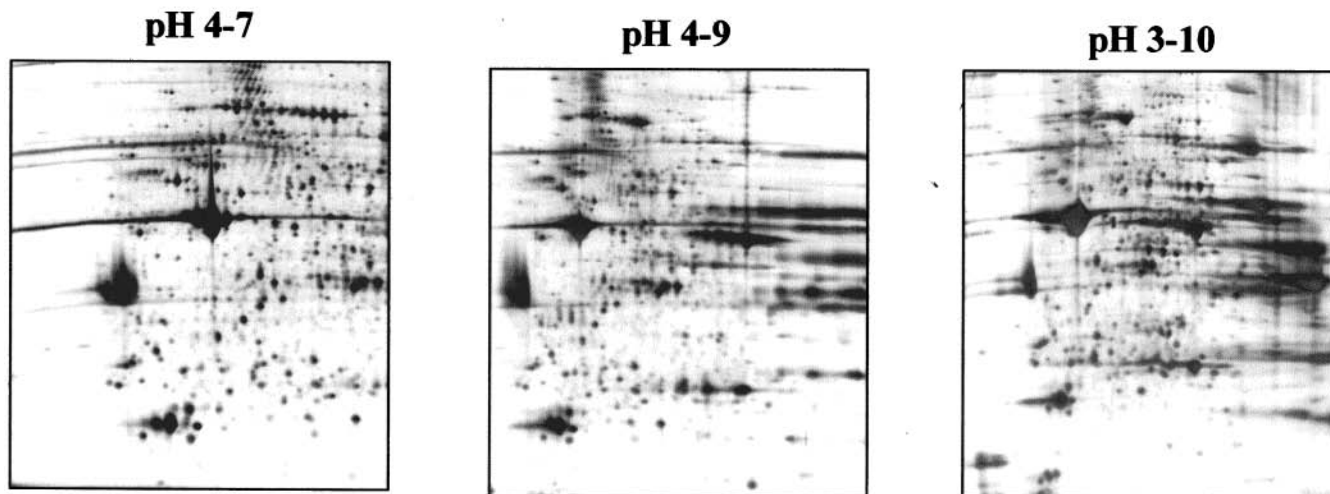

Figure 3. 2DE analysis of porcine muscle proteins. The resolving properties of three different IPG gradients were tested. Aliquots (50 μg of protein/2DE) of a representative porcine muscle extract were separated on IPG gradients spanning the pH ranges of 4–7, 4–9, and 3–10.

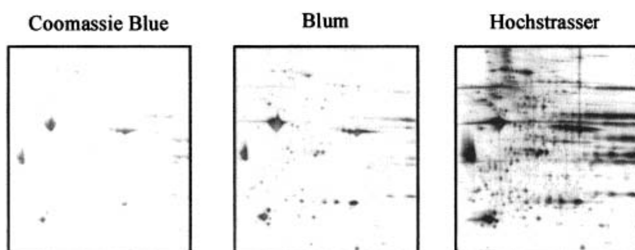

Figure 4. Detection of 2DE-separated proteins. The sensitivity of protein staining methods has been compared. Aliquots (50 μg of protein/2DE) of a porcine muscle extract were separated on IPG 4-9. Protein patterns were visualised either by Coomassie staining or by silver staining methods published by Blum et al. (*19*) and by Hochstrasser et al. (*20*).

the carcass. Comparative proteome analysis during the *post mortem* period was performed on muscle samples removed from the carcasses at 0, 4, 8, 24, and 48 h *post mortem*. Representative 2DE analyses of muscle tissues removed immediately after slaughter (time 0) and after 48 h of *post mortem* storage are shown in Figure 5. During slaughter and postslaughter handling, great care was taken to mimic the relevant *post mortem* storage conditions of pig carcasses.

For the *post mortem* study, a total of 35 silver-stained 2DE images were scanned and analyzed, and by analyzing the spot patterns of these images, we have recognized the proteins that have an altered appearance throughout the *post mortem* period.

The computer-assisted image analysis of *post mortem* samples made it possible to evaluate, match, and compare the positions and the relative intensities of the individual spots. In this study, we have focused our attention only on spots that change in similar patterns during *post mortem* development in all seven of the animals in the study. An overview of the 15 most profound and repetitive of these changes is shown in Figure 5, while the expression profiles of the 15 selected changes are illustrated in Figure 6.

Comparison of 2DE patterns allowed the following observations to be made. The overall pattern of pig muscle proteins seems to be remarkably consistent during *post mortem* aging, suggesting that proteolytic activity in the *post mortem* muscle is restricted to a few muscle proteins only; hence, the bulk of muscle proteins seems to be remarkably stable during *post mortem* processes. Also, the apparent consistency in 2DE patterns suggests that the solubility properties of most muscle proteins remain unaltered during *post mortem* storage, as the same amount of proteins can be extracted (as seen in Table 1B), and the relative aboundances of the majority of individual muscle proteins seem rather similar throughout the *post mortem* storage period. However, careful analysis of the picture has shown a number of altered proteins, some of which seem to be individual variations, while other changes can be observed in all the animals in the study. The 15 protein spots that we have chosen for further detailed analysis exhibit a similar profile in all seven animals in the study, and are therefore likely to reveal parts of the molecular basis of *post mortem* metabolism of pig muscle. In general, we observe that upcoming spots are rather more abundant than spots that disappear during *post mortem* storage, suggesting that most of the novel spots are due to fragmentation of proteins.

Figure 6 illustrates the expression profiles of the 15 most prominent *post mortem* protein changes.

DISCUSSION

In this study, we tested and integrated different methods of proteome analysis to dissect the molecular changes that occur in pig muscle during *post mortem* storage of the carcass, and to understand some of the molecular mechanisms that influence the quality of pig meat. The energy metabolism of *post mortem* muscle has been intensively studied, explaining some of the variation of meat quality (*5*). To improve our understanding of this variation, a more detailed characterization of the proteins and genes that influence meat quality is needed.

In porcine muscle, the best characterized of these proteins and genes include the ryanodine receptor (*21*), the product of the halothane gene which has a major impact on the water-holding capacity, color, and texture of pork meat (*22*), and the recently identified AMPK protein (AMP-activated protein kinase), the product of the porcine RN gene (*23*) that influences the glycogen content of muscle cells, which have an impact on the cooking yield and water-holding capacity of pork meat (*24–26*). In sheep, a mutation in the ovine Callipyge

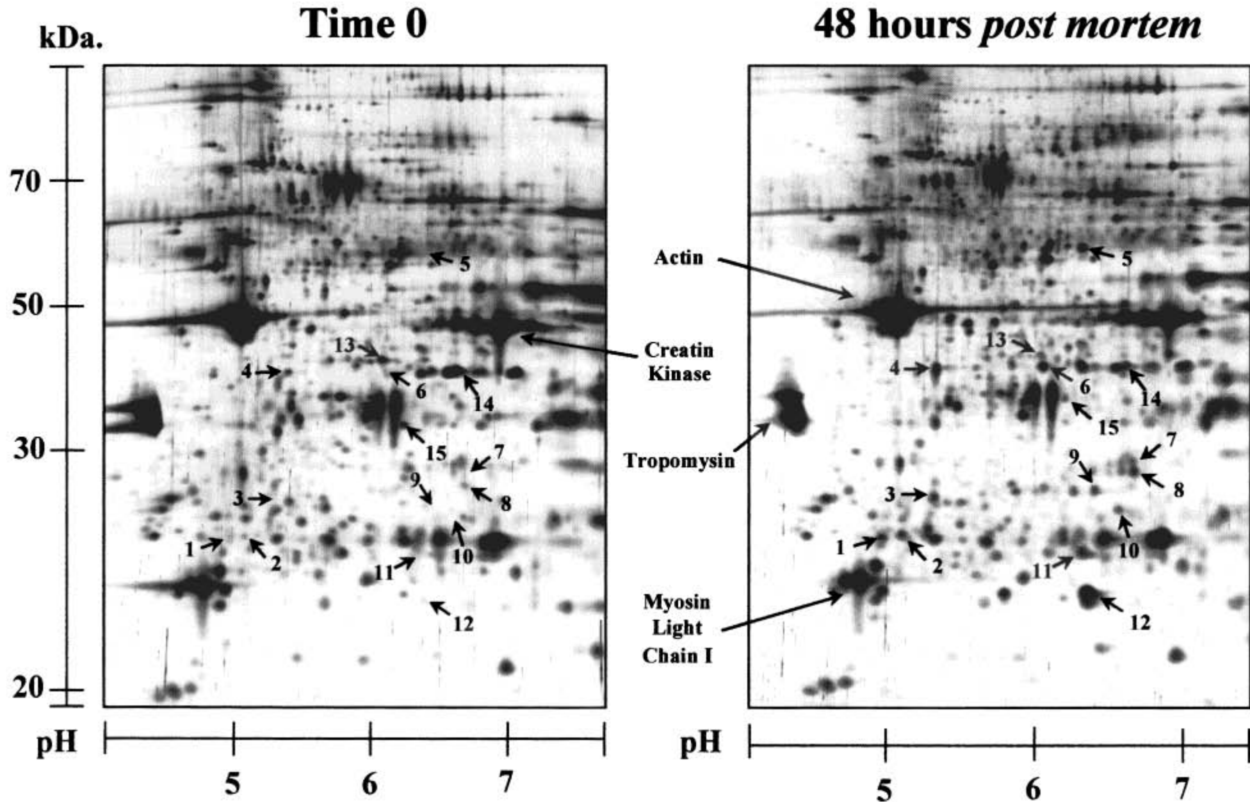

Figure 5. Comparative *post mortem* analysis. Representative 2DE analyses of porcine muscle samples taken immediately after exsanguination (time 0) and 48 h *post mortem*. Protein (50 μg) was loaded on each gel, and separation was performed in the pH region of 4−9. This figure shows only the parts of the 2DE images that were useful for computer-assisted image analysis. Arrows show 15 spots that change in intensity during *post mortem* storage. Major muscle proteins were annotated by comparing the 2DE patterns of the porcine muscle to the mouse gastrocnemius muscle 2DE database published on the ExPASy website.

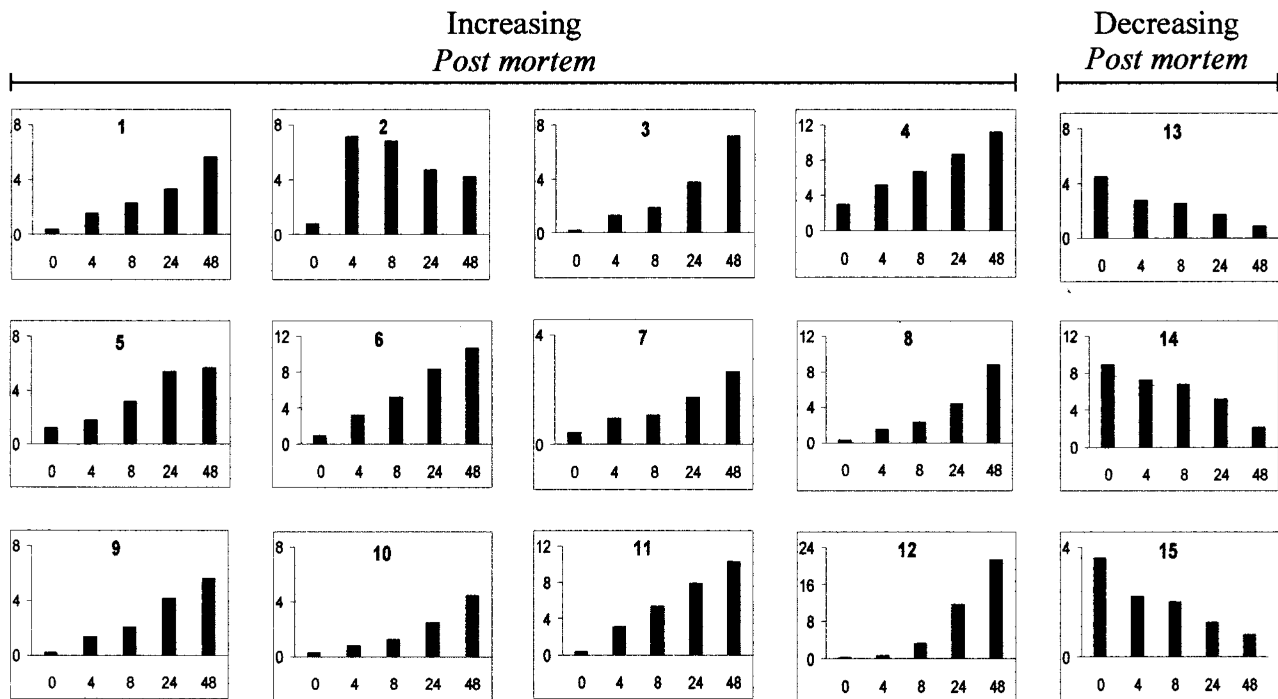

Figure 6. Expression profiles of 15 *post mortem* changes. Each subfigure shows the relative abundance of a protein spot altered *post mortem*. The 15 individual spots are the ones indicated in Figure 5. Times of sample removal are indicated on the *X*-axes (0, 4, 8, and 24 h *post mortem*), while the *Y*-axes show the relative spot intensity of the individual spots. The bars represent the average intensity based on observations in seven animals.

locus has been observed to give a higher meat content, but also less tender meat (*27*).

During the past few years, there has been a rapid development of methods for studying the expression of proteins and genes, with the focus on characterizing molecular markers for cellular pathways and physiological conditions. Such molecular markers in muscle and meat will be useful both as indicators of physiologi-

cal processes (e.g., disease markers and genetic variation markers) and as leads to be further characterized in revealing technological properties and potentials of the raw product (which would be useful, for example, for the meat processing industry). By analyzing the presence and function of single proteins and genes and their functions in the living muscle as well as during the *post mortem* period, we will realize better breeding, handling, and production of animals as well as optimizing meat quality.

Proteolysis during the *post mortem* process of meat has been extensively studied by SDS−PAGE and immunodetection in normal and Callipyge lamb muscle (*28*), and in calf muscle (*29*, *30*). The study we present in this paper is not, in aim and design, very unlike these studies. Yet the advantages of using 2DE for comparing protein displays are that 2DE technology allows more detailed observations to be made than was the case in the studies based on 1DE gel analysis. This is primarily due to the greatly enhanced resolving power of the 2DE analysis, enabling the display of 1000 well-separated proteins in a single display. By manipulating the pH and acrylamide gradients, we can greatly exceed this resolution by making zoomed pH and molecular mass windows that will allow separation of several thousand proteins. Moreover, this enhanced separating power allows direct identification of proteins by removing spots from the 2DE matrix and analyzing them by mass spectrometry-based peptide mass fingerprinting (for a recent review of this technology, see ref *31*). Another important advantage of using 2DE analysis is that the frequency of assay-induced artifactual protein modifications is greatly reduced, especially in comparison to that from in vitro enzyme assays. This means that the proteome changes we observe using 2DE analysis reflect the actual events occurring *post mortem* in the pig carcass.

Many previous studies of proteolysis during tenderization of meat have focused on the importance of μ-calpain (*10*, *28*), but many yet unrecognized peptidases and proteases could also be important. Even if these are low-abundance proteins, they might nevertheless play key roles in cascades of protein modifications, and their activities will easily be overlooked by using assays with less-than-optimal sensitivity. With proteome technolgies, we have the ability to do large-scale identifications of protein fragments. If several of these fragents have cleavge sites in common, we may even gain information about the nature of the proteolytic activities in *post mortem* pork meat.

Proteome technology allows the comparison of proteins expressed by cells or tissue types, and is useful for characterizing coherent expressions of cellular proteins. Yet there are also serious limitations in the contemporary technologies that must be considered when planning and performing experiments, and even more importantly when interpreting proteomic data. To reduce the occurrence of artifactual proteome changes, it is necessary to keep the experimental variations to a minimum during sampling and processing of tissues, as well as during the separation and staining of proteins. It has been a major issue throughout this study to reduce intersample variations that do not relate to the *post mortem* processes, and in all steps and processes to keep them at a minimum.

Data interpretation must also be handled with careful consideration, taking into account that 2DE analysis, like all other electrophoretic protein analysis, is still of a semiquantitative nature; hence, the relative levels of expression of the spots that we observe might to some extent be influenced by technical variablity. To perform a reliable computer-assisted 2DE analysis, all analyzed spots have to be well-separated, with a reproducible migration behavior and a similar staining property. Great variations in, for example, the silver-stained background will result in seriously misinterpreted relative intensities of otherwise comparable spots; hence, the quality of the image analysis is highly dependent on the reproducibility of the 2DE analysis. On the other hand, the great sensitivity of the 2DE technology implies that some of the spots we interpret as markers are due to factors other than the ones under investigation; they might, for example, be due to individual genetic variation, state of health, age, sex, or feed conditions. Therefore, one of the dilemmas in this study has been to select the markers that are the ones that strictly relate to the *post mortem* metabolism of pig muscle, and therefore are worthy of further study. The 15 markers presented in this study have been restricted to ones that have a very similar expression profile in all animals, but also by the criteria of being so markedly altered during *post mortem* storage that their expression profiles could not be mistakenly interpreted. Many other markers, rejected from this first screening, will be reinvestigated under refined separating conditions and in a larger animal material.

In conclusion, we have presented data that show that 2DE analysis is well-suited to studying the protein changes that occur in pig muscle during *post mortem* storage of the carcass. The separation conditions we used here allowed detection of 15 notable *post mortem* changes that were neither gender nor RN-gene specific.

Finding these changes is an initial step in understanding the molecular mechanisms of *post mortem* metabolism of muscle. In forthcoming work, the protein sequences of the *post mortem* changes will be obtained, and also, correlation studies of the observed expression profiles and meat quality traits will be performed. This work is presently in progress in our laboratory.

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