Proteomics in Muscle-to-Meat Conversion

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INTRODUCTION

Variation in meat quality traits is a well-known problem. Although extensively researched, the underlying mechanisms of many of the different meat quality traits are not fully understood. Basic knowledge of these mechanisms is essential to reduce the variation in meat quality traits such as tenderness, water-holding capacity, and color, and new research tools must be applied in meat science to obtain this knowledge. The great progress in biotechnology in recent years has resulted in the development of new scientific research areas such as genomics and proteomics, which are used to study the complex patterns of gene and protein expression in cells and tissues. The technologies developed within genomics and proteomics have great potential for application within food science because the gene expression and protein composition of plants and animals have a major impact on the yield and quality of the final food products.

Proteomics is the study of the proteome, which is defined as the protein complement expressed by the genome of an organism. The term "proteome" refers to all the proteins produced by the genome of an organism, just as the genome refers to the entire set of genes. However, unlike the genome, the proteome is dynamic and varies with the physiological state of the organism. Because encoded proteins carry out most biological functions, application of proteomics is essential to understand how the organism works. However, the composition of the proteome also has a major influence on the biophysical characteristics of protein-based food products such as meat, and several meat quality traits, such as tenderness, water-holding capacity, and color, are influenced by the protein composition of the muscle or meat. Hence, proteomics can provide valuable information on the mechanisms influencing the different quality traits, contributing to a better understanding of these mechanisms. This information can be used to optimize meat production and improve meat quality.

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TECHNOLOGY IN PROTEOMICS

Proteomics is a very challenging task because of the wide-ranging biochemical heterogeneity of the proteins. An animal genome (pig, cow, or chicken) contains approximately 20,000 genes, each of which, on average, may produce 5 or 6 different messenger RNA. Each of these messenger RNA species is, in turn, translated into proteins that are processed in various ways, generating on the order of 8 to 10 different modified forms of each protein. Thus, the genome may potentially produce approximately 1.8 million different protein species (Jensen, 2004). Furthermore, in muscle cells, myosin heavy chain, actin, titin, and nebulin make up almost 80% of the total myofibril protein, whereas other proteins are present in only a few copies, resulting in a large dynamic range, on the order of 10^6. An ideal proteomics methodology combines high-throughput capability with detection of as many protein products as possible in a sensitive, reproducible, and quantifiable manner and provides detailed information about the modifications of the individual detected proteins.

The classical method in proteomics is 2-dimensional gel electrophoresis (**2DE**), in which the proteins are separated according to their isoelectric points in the first dimension, followed by separation according to their molecular weights in the second dimension. The proteins of interest are then identified by mass spectroscopy (**MS**). The combination of 2DE and MS is termed a "gel-based" proteomics approach. The basic principle of the gel-based proteomics approach is that the proteins are first separated with 2DE, and then the individual proteins are quantified and matched by using of special software. The proteins of interest are finally identified by using MS.

Protein detection and quantification are essential issues in proteomics because the main purpose of differential proteomics is to study the expression level or amount of proteins. Coomassie blue or silver staining is normally used for protein detection in 2DE. However, these stains have a limited dynamic range; consequently, one can accurately quantify only the subset of proteins that happens to fall within the linear-range region on the 2D gel. In recent years, fluorescent detection of proteins has gained popularity in proteomics research because of its large dynamic range and high sensitivity. Moreover, fluorescent probes have been developed that are covalently linked to

the proteins before 2DE analysis. With this approach, it is possible to conduct dual- and multiple-label proteome analyses, in which the samples are labeled with different fluorescent probes and then mixed together and analyzed on the same 2DE gel. This approach is also known as 2D-difference gel electrophoresis (Tonge et al., 2001).

Detection of variation in protein modification is very simple with the use of 2DE because one of the separation parameters, the isoelectric point or molecular weight, is altered in the modified form. If the proteins are phosphorylated, the isoelectric point will change and the protein will migrate differently during isoelectric focusing in the first dimension. Furthermore, different types of florescence staining for 2D gels have been developed for the specific detection of protein modifications such as phosphorylation and glycosylation (Wu et al., 2005). If the proteins are modified by cleavage, the molecular weight of the resulting fragments will change and migrate differently in the second dimension. In the subsequent MS analysis of the proteins separated in 2DE, the modification can be characterized and the modification site and type can be identified (Jensen, 2004). It is also possible during MS analysis to predict the cleavage site if the protein is degraded (Larsen et al., 2001; Lametsch et al., 2002).

Despite the apparent advantages of 2DE for the separation of complex protein mixtures, the technique has several major drawbacks. The first drawback is linked to the physicochemical properties used for protein separation, molecular weight and isoelectric point. Proteins with high (>150 kDa) and low (<10 kDa) molecular weights and proteins with extreme isoelectric points, in particular basic proteins, are usually not detected in standard 2DE. Another drawback concerns the hydrophobic proteins that are not extracted in the buffers used for sample loading or are lost because of precipitation during the electrophoretic process. These limitations of 2DE make it very difficult to investigate many structural proteins in the muscle, because many of them, such as myosin heavy chain, titin, and nebulin, are high molecular weight proteins with a molecular weight above 150 kDa. Two-dimensional gel electrophoresis also has some technical limitations. Briefly, the process is time-consuming, labor-intensive, and requires significant technical expertise to generate quantitatively and spatially reproducible gels.

Recent advances in MS technology have led to the development of several different MS-based proteomic methods. These methods have the advantage over 2DE that they allow examination of high- or low-abundance proteins in the same analysis and are unbiased with respect to molecular weight, isoelectric point, and hydrophobicity of the proteins. Furthermore, all steps may be automated for high-throughput analysis. The developments in MS-based proteomics are enormous, and new and smarter methods are constantly introduced (Pan et al., 2009). In a typical MS-based proteomics workflow, the proteins are first enzymatically cleaved into peptides and then further fractionated by single- or multidimensional separation. The fractionated peptides are then analyzed by MS analysis and annotated through a database search. Quantification of the peptides in MS-based proteomics has predominantly relied on the differential isotopic labeling of 2 or more samples, which were combined and subjected to MS analysis. The most frequently used methods for isotopic labeling in MS-based proteomics are iTRAQ (isobaric tag for relative and absolute quantitation) and SILAC (stable isotope labeling with amino acids in cell culture). The iTRAQ method is based on the covalent labeling of the N terminus and side-chain amines of the peptides with tags that produce reporter fragments of varying masses in the MS/MS spectrum. Currently, iTRAQ exists as 4-plex or 8-plex so that 4 or 8 different samples can be labeled with a different iTRAQ reagent, mixed, and quantified in a single MS run. In the SILAC approach, a heavy nonradioactive isotope is added during cell growth, typically a modified arginine, which leads to a mass shift in the peptides that contains a modified arginine. A sample that contains the heavy isotope is then mixed with a control sample without the heavy isotope. The peptides are then quantified during MS analysis by comparing the intensity of the same peptides with and without the isotope. In addition to the labeling methods, several label-free methods have been developed that use comparative measurement of precursor ion currents or spectral counting (Bantscheff et al., 2007). Another MS-based proteomic strategy is targeted protein quantification, in which defined peptides from specific proteins can be quantified by using the multiple reaction monitoring technique. It has been demonstrated that more than 50 proteins can be simultaneously targeted in a single measurement by using the multiple reaction monitoring technique (Pan et al., 2009). This development in MS-based proteomics has made it possible to reproducibly, accurately, and quantitatively measure any protein or the whole proteome (Ahrens et al., 2010). However, different MS-based proteomic strategies have to be combined, which makes it very complex and challenging to obtain complete information about a specific proteome. The limitations of most of the MS-based proteomic strategies are that the proteins are first digested with a specific protease, normally trypsin, so not all the resulting peptides are identified during MS analysis, and that information about different protein modifications or degradations may be lost. One strategy that has been used to obtain information about the changes in protein modifications is affinity enrichment of the peptides containing the modifications. This strategy has been used especially for monitoring changes in protein phosphorylation (Thingholm et al., 2009).

In general, both the MS- and gel-based proteomic strategies have some limitations, and the selection of a proteomic strategy should be carefully considered in each project. It is not necessarily an advantage to use the most advanced proteomic strategy that provides a large amount of information about the selected proteome because it makes the interpretation of the results difficult; it is often

of benefit to use a simpler proteomic strategy that is more focused. However, the developments in technology, especially bioinformatics, make it increasingly easy to perform proteomic analyses and also to interpret the outcomes. These developments are occurring especially in the area of MS-based proteomics.

PROTEOMICS IN MEAT SCIENCE

The quality of raw pig meat is influenced by changes in the muscle or meat proteome caused by different factors, such as animal growth, age, rate of glycolysis, and postmortem protein degradation. Meat scientists have performed a substantial amount of research on these factors, which has led to considerable improvements in quality and composition. However, the underlying biochemical and physiochemical mechanisms behind the influence of these factors on meat are, to some extent, still not fully understood. The recent application of proteomics in the field of meat science has provided some interesting and promising results.

Postmortem protein changes in muscle have been investigated with proteomics (Lametsch and Bendixen, 2001; Hwang, 2004; Morzel et al., 2004). These studies revealed that a large part of the proteome changes postmortem. The mechanisms behind these postmortem changes are, to some extent, still unclear. However, the main cause of protein changes postmortem is probably protein degradation because many of the identified changes are protein fragments that increase in spot intensity postmortem (Lametsch et al., 2002). Changes in protein modification, such as phosphorylation or oxidation, which change the isoelectric point of the proteins or their release from protein complexes, most likely also contribute to postmortem protein changes. Even protein expression may, to some extent, cause some changes postmortem; however, it is unlikely that protein expression causes major changes after the muscle has entered the state of rigor mortis because protein expression is an energy-requiring process and the energy is almost depleted after the muscle has entered the state of rigor mortis (Henckel et al., 2002).

At slaughter, the blood supply stops and a source of oxygen is no longer available. This results in a change in energy metabolism from aerobic oxidative metabolism to anaerobic glycolytic metabolism, causing an increase in the formation of lactate and hydrogen ions, which results in a decrease in the pH of the muscle cell. Two-dimensional gel electrophoresis has been applied to characterize pale, soft, and exudative (**PSE**) zones in pig muscle, and 16 protein spots were found to be affected by PSE. Myosin light chain (**MLC**) I, fragments of creatine kinase, and troponin T were identified as proteins with a higher intensity in PSE meat compared with control meat, and this was suggested to be a consequence of a decrease in postmortem proteolysis (Laville et al., 2005). Another interesting observation was that the 2 heat-shock proteins HSP27 and α-crystallin were absent in the PSE meat (Laville et al., 2005). Another study showed that the postmortem intensity profiles of HSP27 and α-crystallin in non-PSE meat increased to maximum intensity during the first 4 h postmortem and remained unchanged in the following period (Lametsch et al., 2001). These results show that the postmortem change in the 2 proteins was probably a result of protein expression or modification. Heat-shock protein 27 and α-crystallin are both believed to participate in the organization and protection of the myofibrils, and the expression and modification of these proteins are affected by conditions such as stress. The results obtained on HSP27 and α-crystallin by proteomic analysis indicate that these proteins may affect meat quality by stabilizing the myofibrils postmortem and that they could also be useful biomarkers for PSE or stress. Protein phosphorylation is a key regulator of biological processes and is known to be involved in the regulation of energy metabolism in the cell. In a recent work, (Lametsch et al., 2011) we investigated the postmortem changes in pork muscle protein phosphorylation in relation to the RN genotype. We found that the protein phosphorylation levels of several proteins were affected by the RN genotype and were changed during postmortem development. Glycogen phosphorylase, phosphofructokinase, and pyruvate kinase were identified in protein bands affected by the RN genotype, and the protein phosphorylation profile indicated that the increased rate and extended pH decline of the RN genotype could be a consequence of phosphorylation of these key enzymes during postmortem metabolism. This result illustrated that the protein phosphorylation level of the metabolic proteins in the muscle could be interpreted as a global metabolic fingerprint containing information about the activity status of the enzymes in postmortem metabolism (unpublished data; Lametsch et al., 2011).

The effect of preslaughter handling was also investigated with the use of gel-based proteomics (Morzel et al., 2004). Two preslaughter handling procedures were used; in the first, the pigs were transported the day before slaughter, and in the other, the pigs were transported immediately before slaughter. The intensities of 8 spots were significantly affected by the preslaughter conditions. Two of the spots were identified as F1-adenosine triphosphatase (**F1-ATPase**) chain B, and one was identified as MLC II. The intensities of the 2 F1-ATPase chain B and the MLC II spot were greater in the muscle of pigs that were transported immediately before slaughter. The reason for the increase in intensity of F1-ATPase chain B is most probably related to accelerated postmortem metabolism, whereas the increase in intensity of MLC II may be a consequence of changes in phosphorylation of MLC II (Morzel et al., 2004). It has been reported that phosphorylation of MLC affects the Ca^{2+} sensitivity of muscle contraction (Szczesna et al. 2002), and it can be speculated that postmortem changes in the phosphorylation of MLC may affect meat texture.

It is well known that meat tenderizes during postmortem storage, and it is believed that postmortem degradation

of the myofibrillar proteins, such as desmin, titin, and nebulin, is the main reason for this improvement in meat tenderness. Proteomics has proved a powerful tool to investigate postmortem protein degradation. More than 100 protein spots have been found to change postmortem, probably as a consequence of protein degradation (Lametsch and Bendixen, 2001; Hwang et al., 2005). Several reports have claimed that neither actin nor myosin heavy chain is degraded postmortem (Bandman and Zdanis, 1988; Koohmaraie, 1994; Huff-Lonergan et al., 1996). However, Hwang et al. (2005) and Lametsch et al. (2003) found both proteins to be degraded postmortem. Moreover, some of the actin fragments and the myosin heavy chain fragment have been found to correlate significantly with meat tenderness (Lametsch et al., 2003; Hwang et al., 2005), indicating that postmortem actin and myosin heavy chain degradation contribute to meat tenderization. However, it is estimated that only a minor proportion of the α-actin and myosin heavy chain are degraded because the amounts of actin and myosin heavy chain fragments were much lower compared with the amount of full-length actin, and degradation of full-length actin was not detected. Mass spectroscopy analysis of the myosin heavy chain fragment revealed that the fragment is part of the globular myosin (Lametsch et al., 2002). It can be speculated that myosin heavy chain degradation leads to disruption of the myosin-actin interaction, which may result in more tender meat. Even if only a minor part of actin is degraded, it is reasonable to believe that it could have an effect on the integrity of the thin filament. Several other structural or structurally related proteins, such as MLC, troponin T, desmin, capping protein α1 subunit, cofilin 2, F-actin capping protein, CapZ, and titin, were found to degrade postmortem. Many of them have not previously been reported to degrade postmortem (Lametsch et al., 2002, 2003; Hwang, 2004).

FUTURE PERSPECTIVES

The studies that have been performed in meat science with the use of proteomics clearly illustrate the great potential for proteomics in meat research. Proteomics has especially proved to be a powerful tool to investigate postmortem protein degradation in meat and has provided new and valuable information about the complex mechanisms behind postmortem proteolysis in meat. Proteomics can provide information on the cleavage sites and degradation patterns of the proteins degraded postmortem. Proteomics has been applied to study postmortem metabolism to provide further knowledge of undesirable meat characteristics, such as PSE, and recent results have shown that proteomics has the potential of providing a global metabolic fingerprint containing information about the activity status of the enzymes in postmortem metabolism. Proteomics is also an effective research tool to investigate the relationship between meat quality and postmortem protein modifications, such as protein oxidation, especially in relation to texture and color.

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