RESEARCH REPORT
PORK QUALITY

Title: Proteomic Analysis of PSE Susceptible Animals - **NPB#02-031**

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Abstract: Meat quality in pigs suffers when there are abnormalities in the regulation of calcium within the muscle cell. This condition is commonly referred to as PSE and results in meat of poor quality. The objective of this study was to identify simultaneous changes in the amount of the sarcoplasmic reticular (SR) proteins association with calcium regulation. The procedure used called proteomics was able to sort out the changes that occur in the following proteins: calsequestrin, two forms of ryanodine receptor and two forms of SR APTase, triadin, dihydropyridine receptor and SR junctional protein. Improvement in both time (< 3 hr) and amount of the sample (< 5g) were developed in isolating SR protein making it possible to analyze an increased number of samples. Early results indicate it was possible to isolate the SR proteins and perform a separation so they could be quantified. Analysis of the SR protein in the pigs that either were normal or contained the napole and halothane gene.showed that the halothane positive pigs had higher ($p < .05$) SR ATPase1 protein and lower ryanodine receptor levels than the normal animal. This work demonstrates a method can be developed that can rapidly identify, using the same samples, the potential of the hog to have PSE problems. Continued work is going on to verify all the possible changes occurring, but the procedures used can be applied to the pork industry

Introduction: The goal of this project is to identify changes in the protein profile (proteome) of skeletal muscle from pigs susceptible to developing PSE. The rationale for using a proteomics approach is that it can provide a broad spectrum of information including the profile and assessment of proteins essential to calcium regulation. Abnormal calcium regulation is the root cause for expression of the PSE condition postmortem. Information obtained from proteomic investigations will provide better understanding of causal factors and enable improved methods for diagnosis and screening. Genomic examinations of PSE have also been extensively used for these purposes. However, tests like the one for a major mutation in the ryanodine gene have not proven adequate for elimination of the PSE problem in meat.

Objectives: The objectives of this project were focused on development of appropriate proteomic analytical tools. Additionally, we have applied the procedures developed to examine essential protein differences in a set of normal and affected animals.

Materials and Methods: Since the development of the methods was a major portion of the discussion, the steps in their development are included in this section.

Extraction and Electrophoretic characterization of SR fractions.

Procedures were developed to prepare SR membrane fractions from small (5g) muscle samples. This protocol is advantageous because 100-200 mg of SR can be isolated in less than 3hr and enables processing of a larger number of samples. Additionally, reduced sample preparation time limits the extent of naturally occurring proteolysis. The procedure yields adequate amounts of protein for subsequent electrophoretic and microarray analysis.

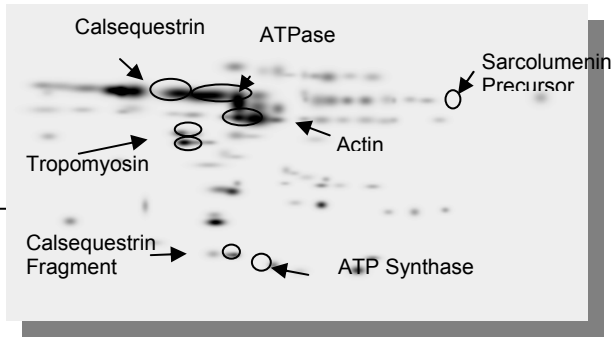

Figure 1

An example of the quality of 2-D separations of SR is shown in figure 1. This figure illustrates the separation of a whole SR fraction (200 µg). Approximately 40 proteins were identified in this separation. Specifically, proteins of importance include; calsequestrin and its fragment, actin, tropomyosin, sarcolumenin and mitochondrial ATPase. While many significant high molecular weight proteins are absent from 2-DE separations, they are of value for comparison of the SR protein profile between normal and affected animals. For example, image analysis was used to quantitatively assess the level of calsequestrin in SR preps from various animal samples.

Recognizing the limitation of 2-DE analysis for larger proteins, alternative approaches (protein microarray analysis) were developed.

Development of Protein Microarray Analysis

During the course of this project, several types of protein microarray analysis were investigated for the purposes of quantitative assessment of protein levels and for protein-protein interactions.

First, microarrays were developed for investigation of the interactions between calsequestrin (CSQ) and other SR proteins. This represents an important biological assay based on the affinity of CSQ for RyR in the SR membrane. In this microarray, 10 ng of purified CSQ was printed onto treated glass slides in 6 x 6 arrays (36 spots in each). The slide was then covered with a mask to isolate each array. The arrays were next incubated with SR extracts in the presence of EDTA or Calcium. After washing away unbound proteins, each set of arrays was probed with anti-RyR1, anti-Triadin and anti-DHPR antibodies. The results showed a strong differential binding of RyR1 to calsequestrin, based on the presence or absence of calcium. Triadin and DHPR also exhibited differential binding to calsequestrin based on the presence or absence of calcium. This microarray approach represents a biological assay for the affinity of RyR1 and other calcium regulatory proteins. It will provide additional information on how

altered calcium regulation occurs.

Second a sandwich ELISA was developed to measure cytokine levels. Specifically, monoclonal IL-6 antibodies were printed onto amine reactive slides. Homogenates of muscle were then overlayed onto each array. Detection was achieved using anti-IL-6 polyclonal coupled to horseradish peroxidase. Tyramide signal amplification was used for detection. Research indicates that cytokine levels (i.e., IL-6) in muscle are correlated with malignant hypothermia. This microarray approach could be modified to analyze the IL-6 content of sera. Sera microarrays for the assessment of cytokines may prove to be a valuable diagnostic tool in screening of breeding stock for potential development of PSE.

Third, microarrays were developed to quantitatively assess a larger panel of proteins associated with calcium regulation including RyR1, DHPR, calsequestrin triadin, junctin, and SERCA1 & 2- (ATPase). The rational for this approach is based on previous research indicating the ratio of RyR to DHPR was reduced in related abnormal calcium regulation conditions (i.e., malignant hyperthermia).

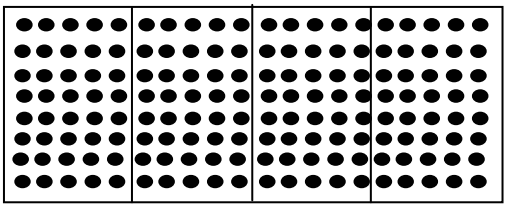

Figure 2 Microarray Print Pattern

We prepared samples for microarray analysis from a group of 32 pigs that were screened for halothane response and genetic defect (R. Napole).

Microarrays were constructed by printing SR protein (5 ng/spot) preparations from each sample in the pattern shown above. In the array each sample was printed 5 times. Four samples were printed per row (20 spots) with a total of 8 rows. Thus each array contained all 32 samples. Arrays were then incubated with monoclonal antibodies to the target proteins. After washing, arrays were incubated with a fluorescently labeled detection antibody. Arrays were subsequently washed to remove unbound antibody, dried and read in a Fluorescence microarray instrument. The results were expressed as fluorescence intensity per ng of SR protein.

Results: Much of the results are in the process of being analyzed and will be included in to manuscripts that are in the process of being prepared. Preliminary results indicate that RYR was lower in the Halothane positive pigs compared to Halothane negative and carrier pigs. The opposite relationship was observed with SR Ca2+ ATPase (SERCA 1) with the Halothane positive pig having a higher amount followed by the carrier and then Halothane negative pigs (Figure 3). Other researchers discovered the *Longissimus* muscle contained only 60% of the Ryanodine receptor content compared to normal *Longissimus* muscles there was also a 12% increase of SR Ca2+ ATPase (SERCA) content in the affected muscles. Our data supports what was observed in other animals, however, with the procedures used it was possible to simultaneously examine these difference along with other proteins that function in controlling calcium levels. The complete results of this investigation will be submitted for publication in the near future.

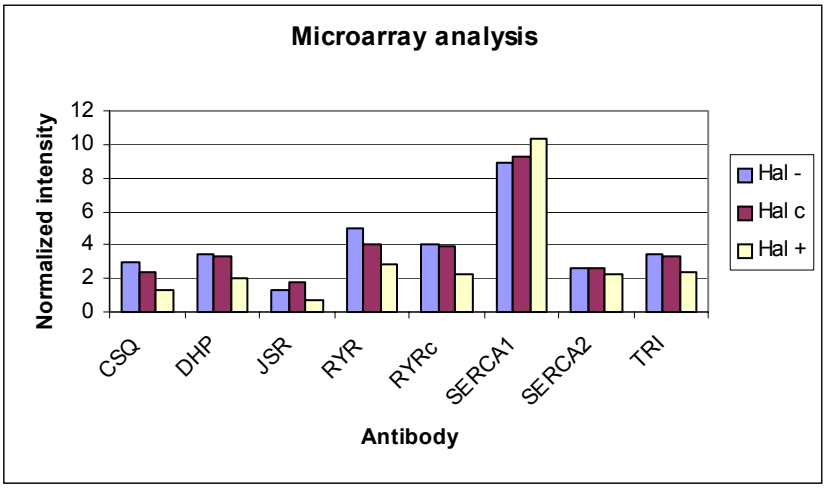

Figure 3

Summary of work:
Accomplishments in developing appropriate proteomic tools include:

- Improved sarcoplasmic reticulum (SR) fraction isolation
- Enhanced electrophoretic methods (both 1-D and 2-D) for SR proteins
- Determination of protein identity via mass spectrometry methods
- Development of protein microarrays, the assessment of key calcium regulatory
  proteins.

Lay Interpretation: With the advent of molecular biology techniques it is possible to examine cellular events that cause problems in the quality of the meat. One such technique is called proteomics. The rationale for using a proteomics approach is it can provide a broad spectrum of information including the profile and assessment of proteins essential to calcium regulation. Abnormal calcium regulation is the root cause for expression of the PSE condition postmortem. This study examined ways in which a large number of muscles could be examined to identify changes in the protein that may be responsible for the calcium regulation. We were able to develop procedures that would rapidly isolate the proteins in question from samples that could be obtained by muscle biopsy. Once the protein could be separated and quantified using procedures a whole host of the proteins could be examined simultaneously. From the results procedures have been developed to determine differences between protein in the ryanodine receptor and another protein, SR Ca+2 APTase, that regulates the calcium level in muscle cells.