**Investigation of the efficacy of albumin removal procedures on porcine serum proteome profile**

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**Abstract:**

Prediction of livestock performance using biomarkers will provide a benefit for livestock genetic evaluation and improvement. Prediction of growth performance will position livestock producers to customize nutrition and production practices to improve efficiency of food production. The most practical tissue sample to screen for development of biomarkers is serum, due to the ease of collection and ability to screen live animals and thus breeding stock. The goal of the current research is to develop serum-based biomarkers to predict feed efficiency in swine. However, protein profiles in serum are complex and dynamic. Strategies are needed to manage such variation in serum proteins during the biomarker identification. Albumin is the most abundant protein in serum and comprises over 50 percent of the overall protein content. Albumin has historically been depleted from albumin prior to biomarker identification. Removal of albumin permits low abundance proteins to become more readily identifiable for biomarker identification. Albumin is known to bind many proteins in the blood, thus potential biomarkers could be removed during albumin depletion. The objective of this study was to investigate the use off gel-based proteomic techniques to identify effective, efficient, and highly repeatable strategies to screen for serum biomarkers without removing albumin and with it some potential biomarkers. The data obtained show that antibody-based methods are effective in removal of albumin from porcine sera. However, such methods also potentially alter the serum proteome in an unpredictable manner, increasing the level of some proteins but decreasing the level of others relative to the original serum sample. We propose that whole serum may be used in a gel-based proteomics system for the identification of porcine biomarkers.

Keywords: 2D-DIGE, serum, albumin depletion, biomarkers, porcine

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**Introduction:**

Determining an effective, highly repeatable, and efficient protocol for the identification of serum biomarkers will be useful in biomarker development in animal agriculture. Biomarkers have long been considered a valuable tool in the identification of physiological conditions indicating predisposition to a disease state. Determination of protein profile differences of serum is important in establishing the identity of protein biomarkers that predict future growth performance traits. One important trait, known as residual feed intake (RFI), is the difference between observed feed intake and expected feed intake, based on average daily gain and back fat (1-2). . The population of pigs selected for residual feed intake at Iowa State University (1-3) provides a model to investigate the biological differences in feed efficiency and to identify RFI biomarkers.

Multiple proteomic methodologies can be applied to differing aspects of biomarker identification. However, the complexity of the serum proteome presents unique challenges for biomarker identification. Chief among them is the significant range in concentration of serum proteins. The most abundant serum protein is albumin, comprising 50 to 75% of the total protein content (4), and thus albumin contamination or interference can obscure lowly abundant proteins that may be useful biomarkers. Both top-down and bottom-up mass spectrometry proteomic approaches require albumin to be removed from serum for their use as a biomarker identification tool (5). On the other hand, two dimensional difference in gel electrophoresis (2D-DIGE) has been utilized in many published works over the last decade and a half and is an important proteomic tool (6). Gel based proteomic methods, like two dimensional (2D-DIGE), can be used for biomarker discovery potentially without removing albumin from serum because albumin is resolved on the gel to a specific location and is less likely to interfere with biomarker identification.

Albumin must either be removed or resolved on a gel to establish the protein profile of less abundant proteins. Therefore, gel based proteomic approaches may allow the discovery of biomarkers in whole serum without albumin removal with the caveat that proteins resolving close to albumin on a gel may be missed. Removal of albumin and other high abundance proteins from serum is commonplace (7). Methods of depletion have been subject to many investigations using various approaches, either through commercially available kits or in house methods. However, only a limited amount of research has been performed directly comparing enriched or albumin depleted serum to whole serum (8-9).

As albumin is widely known to associate with many other proteins, albumin depletion could have a direct impact on biomarker discovery of such associated proteins by potentially masking differences that would otherwise exist prior to albumin depletion. The objective of this study was to identify and investigate strategies that can be used for the identification of biomarkers without removing albumin from the serum using 2D-DIGE, a gel based proteomic technique.

**Materials and Methods:**

*Animals and Serum Collection*

All animals were treated in accordance with procedures approved by the Iowa State University Animal Care and Use Committee (#11-1-4996-S). Pigs from the Iowa State Residual Feed Intake selection project were used. Pigs have been divergently selected for feed efficiency, pigs with a high residual feed intake consume more feed than expected and pigs with a low residual feed intake consume less than expected (2). Whole blood was collected from pigs in the 8th generation of selection (n = 4 high RFI, n = 4 low RFI). Between 35 and 42 d of age, blood samples were collected from the jugular vein using SST Vacutainer tubes. Samples were allowed to clot and stored in a cooler on ice until centrifugation. Samples were stored no more than 4 hours post-collection prior to centrifugation. Serum was collected and stored at -80C until use. At the time of sampling, pigs were being fed a standard corn-soybean-dried distillers grain solubles diet that met or exceeded nutrient and energy requirements for this size pig (10).

*Sample Selection*

A total of 8 serum samples (n = 4 per line) were selected based on RFI Index. Animals with extremes (greatest and least) RFI index were chosen to test for any potential effects that RFI Index had on albumin depletion. Serum samples were then divided into two aliquots, one for depletion and one for whole serum.

*Albumin depletion and sample preparation*

Albumin removal was accomplished through a commercially available kit (AlbuVoid, Biotech Support Group LLC. Monmouth Junction, NJ) designed for use in one-dimensional and two-dimensional electrophoresis as well as other proteomic methods (Figures 1 and 2). Aliquots of serum, both whole and depleted, were thawed on ice. Protein concentration was determined in each sample using a Bradford Quick Start protein assay (Bio-Rad, Hercules, CA). The samples were adjusted to a final protein concentration of 10 mg/ml by using an extraction buffer (8.3 M urea, 2 M thiourea, 2% CHAPS, and 1% DTT at pH 8.5).

*1-D SDS-PAGE*

One-dimensional SDS-PAGE gels were used to evaluate success of albumin depletion (11). Whole serum, flow-through (albumin enriched), and eluent (albumin depleted) were resolved on 12.5% SDS-PAGE gels. Gels were stained with Colloidal Coomassie stain to confirm albumin was depleted in the serum (Figure 1).

*2-D Difference in Gel Electrophoresis*

To determine the extent to which the protein profiles of whole and depleted sera from the same pigs differ, 2D-DIGE analyses were conducted by comparing whole to depleted serum from the same pig (Table 1). Procedures outlined previously for DIGE (3) were followed, with minor modifications. A pooled sample, to serve as a reference, was created from an equal amount of protein from all samples. Two dimensional DIGE gels were used to resolve protein spots of interest from pooled references representing all samples.

A total of 50 μg of each individual sample was labeled with CyDyes 3 or 5 (GE Healthcare, Piscataway, NJ) according to the manufacturer’s directions. CyDyes were alternated between whole and albumin depleted samples (Table 1)(3). CyDye 2 was used to label the pooled reference sample (containing an equivalent amount from each sample) for each gel. Each strip utilized a total of 45 μg of labeled protein, 15 μg of each CyDye 3 or 5, either albumin depleted or whole sera, and 15 μg of the pooled reference (CyDye 2). DeStreak Rehydration Solution (GE Healthcare, Piscataway, NJ) with 2.5 mM DTT was added to the protein mixture to the volume specified by the strip manufacturer (200 μL). The protein mixture was added to individual wells in a reswelling tray and an immobilized pH gradient (IPG) strip (11 cm, pH 3-10) (GE Healthcare, Piscataway, NJ) was placed on top of the mixture and allowed to rehydrate overnight at room temperature in a humidified chamber. Isoelectric focusing was performed on an Ettan IPGphor isoelectric focusing system (GE Healthcare, Piscataway, NJ) for a total of 11,500 V x h. After isoelectric focusing, strips were equilibrated using two 15 min washes, first with equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and trace amounts of bromophenol blue) with 65 mM DTT and second with equilibration buffer with 135 mM iodoacetamide.

Equilibrated strips were loaded onto 12.5% SDS-PAGE gels (acrylamide: N,N’-bis-methylene acrylamide 100:1, 0.1% SDS, 0.05% TEMED, 0.05% ammonium persulfate, and 0.5 M Tris-HCL, pH 8.8) using agarose as an overlay. Isoelectric focused proteins were resolved on an Ettan DALT SIX system (GE Healthcare, Piscataway, NJ). Each gel was run in duplicate, with separate isoelectric focusing steps and SDS-PAGE steps. Gels were imaged using an Ettan DIGE Imager (GE Healthcare, Piscataway, NJ). Images were processed and analyzed using DeCyder 2D software version 6.5 (GE Healthcare, Piscataway, NJ).

To compare variation between protein abundance measures derived from depleted and whole serum samples, normalized data were analyzed using separate linear mixed models for each spot and serum type (depleted or whole). Each linear mixed effects model included an intercept term, fixed effects for dyes (Cy3 or Cy5), fixed effects for lines (high or low RFI), and random effects for animals. The standard error of the linear mixed effects model estimate of spot abundance (averaged over dyes and lines) was compared across serum types for each spot. A one-sided Wilcoxon signed-rank test was then performed to determine if the standard errors tended to be greater in depleted vs. whole serum.

**Results:**

A total of 372 spots were resolved and detected in the broad pH range 2D-DIGE analysis of whole versus albumin-depleted sera for 8 independent serum samples. Of these, 239 were present in at least half of the images of which 204 were found to be changed in abundance between the depleted and whole serum samples (Figure 3). Of these 204 spots, 179 had a change in abundance of over 50%. In the depleted serum, 86 protein spots were increased in abundance compared to whole serum, and 118 spots were decreased (P ≤ 0.05) (Figure 3). A total of 59 spots had a change in abundance over 400 percent between the whole and albumin depleted serum (30 and 29, increase in whole and depleted, respectively).

Analysis for variation of the normalized data from the 239 spots present in 50 percent of gels indicated that 59.4% of spots had a larger standard error in the depleted serum samples than in the whole serum samples. Based on a one-sided Wilcoxon signed-rank test, the standard errors tended to be larger in albumin-depleted samples than in whole samples (P = 0.0004) (Figure 4).

In addition to 2D-DIGE with the pH 3-10 IPG strip, the feasibility of utilizing a narrower pH range to enhance resolution and minimize the impact of albumin on the gel based protein profile of serum was explored. Comparison of the single broad pH range and two more narrow pH ranges (pH 4-7 and pH 6-11) demonstrates the impact of albumin on the protein profile can be managed (Figure 5). By selecting a narrower pH range, proteins are better resolved in the gel allowing for protein(s) of interest to be more readily identified.

**Discussion:**

Albumin removal and sample fractionation is required with almost all off gel based proteomic approaches. High abundance proteins such as albumin, transferrins, and immunoglobulins can interfere with detection of low abundance proteins. Therefore it has been common practice to remove these proteins from the serum or plasma in the course of identifying biomarkers (8). Investigations to develop applications to remove albumin have been undertaken (8, 12-13). It has been suggested that depletion of high abundance proteins could induce variation within the samples potentially leading to biased results (8). Even after depletion, albumin often remains in the sample (Figures 1 and 2). The purpose of this study was to investigate the feasibility of using whole serum for the identification of serum biomarkers using a gel-based proteomic approach. Gel-based approaches provide approaches to reduce the impact albumin has on identification of biomarkers (Figure 5).

While albumin was successfully depleted in this study, a majority (~74%) of the 240 protein spots present on half of the gels were significantly impacted by albumin depletion (Figure 3). An increase in abundance relative to whole serum was not unexpected given that albumin depletion allows low abundance proteins to represent a greater proportion of the protein amount applied to the gel. However, a majority of the affected proteins were actually decreased in abundance due to albumin depletion. We interpret this finding to indicate these proteins have also been depleted in this process, likely due to a physical interaction with albumin or to non-specific effects. Using 2D-DIGE and DeCyder provides the direct comparison of only protein spots found in both depleted and whole serum, not protein spots found in only one of the treatments. Spots detected in only one treatment are not included in the DeCyder analysis. This direct comparison of spots shows that the protein profile between whole and albumin depleted serum is different.

Two dimensional DIGE has been previously utilized to show depletion of high abundance proteins is an efficacious approach to detect biomarker, especially those of low abundance (9).

However, the current results also show that depletion of albumin may result in an unintentional increase in the variation of abundance of individual proteins. Estimates of protein spot abundance within depleted samples tended to have a greater standard error than the corresponding estimates for whole serum spots. This indicates evidence of increased variation in response to albumin depletion. The current results demonstrate that using a gel based system, such as 2D DIGE, provides a method to resolve proteins and determine the abundance of proteins that are not masked by albumin. This also has the benefit of removing a sample preparation step prior to analysis.

Other strategies can be utilized to mitigate the impact the presence albumin has on biomarker discovery. The current comparison utilized a broad pH range (3-10). Porcine albumin has an isoelectric point of 6.08, indicating that on a 3-10 pH range 2D-SDS-PAGE gel it will resolve in the center portion of the gel. Using a multi-gel fractionation (14) or “proteomic contigs” (15) approach with more narrow pH ranges provides the ability to preferentially select pH ranges to isolate albumin towards the edges of the gel. The current study utilized pH ranges of 4-7 and 6-11 to demonstrate albumin can be successfully managed in analysis of whole sera to ameliorate any negative effects in the majority of the resolving gel (Figure 5). Further gel fractionation by molecular weight, through the use of gradient or changes to the percentage of acrylamide, though not used in this study, is an additional avenue of reducing the effect albumin has on biomarker discovery (16).

Whole serum can be used to identify potential biomarkers. No proteomic methodology is ideal in all situations however, the vast number of different methodologies allow researchers to tailor one to their focus. Two shortcomings of gel based approaches in biomarker discovery are the decreased ability to determine the identity of low abundance proteins and the lack a high throughput spot identification methods (12). This may be an area of concern in the human medicine applications. Conversely, in animal production low abundance proteins may not be cost effective to develop into suitable biomarkers strictly due to steps required to analyze samples. It is the ultimate goal of all biomarker searches to identify an easily accessible biomarker using an effective highly repeatable methodology. These data show that 2D-DIGE is an effective method when using whole serum for biomarker identification.

**Conclusion:**

These data demonstrate whole serum can be used to resolve large numbers of proteins without albumin depletion. Furthermore, depletion of high abundance proteins such as albumin may contribute to a greater variation in data produced during biomarker identification. Gel based proteomic techniques like 2D-DIGE are useful in the detection and identification of biomarkers, particularly with protein samples with extremely broad ranges of protein abundance such as whole serum.

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Table 1: Experimental design for the 2D Difference in Gel Electrophoresis. Dyes were alternated between whole and albumin depleted samples.

|  |  |  |  |
| --- | --- | --- | --- |
| Pig ID | Whole Sera | Depleted | Reference |
| 1807 | Cy3 | Cy5 | Cy2 |
| 4810 | Cy5 | Cy3 | Cy2 |
| 1209 | Cy3 | Cy5 | Cy2 |
| 1906 | Cy5 | Cy3 | Cy2 |
| 3908 | Cy3 | Cy5 | Cy2 |
| 3106 | Cy5 | Cy3 | Cy2 |
| 2107 | Cy3 | Cy5 | Cy2 |
| 2712 | Cy5 | Cy3 | Cy2 |

Figure 1: One-dimensional SDS-PAGE analysis of albumin depletion from whole serum using a commercially available kit. Representative samples of the albumin depletion process were separated using a 1D- SDS-PAGE gel and stained with Colloidal Coomassie. (A) whole serum, (B) flow through from the albumin removal step, and (C) elutant (80 μg of protein per lane, 12.5% SDS-PAGE gel).

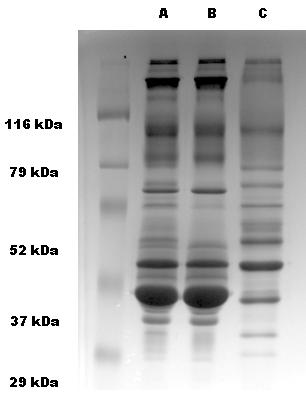


Figure 2: Albumin depletion from whole serum using a commercially available kit designed for both one dimensional and two dimensional electrophoresis. Red indicate the location of albumin. Comparison on a two dimensional SDS-PAGE gel of a single sample of (A) Whole sera, (B) albumin enriched sera, and (C) albumin depleted sera. First dimension; 11 cm, pH 3-10 immobilized pH gradient strip. Second dimension: 12.5% SDS-PAGE gel.

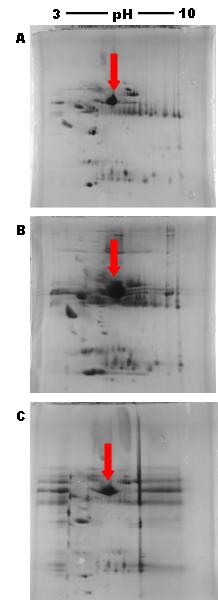


Figure 3: Representative two dimensional difference in gel electrophoresis (2D-DIGE) images from the comparison of whole and albumin depleted samples (A) all 372 spots identified to be present by DeCyder, (B) 78 spots found to be increased by ≥50% (P ≤ 0.05) in albumin depleted samples, and (C) 99 spots found to be increased by ≥50% (P ≤ 0.05) in the whole serum. Each 2D-DIGE gel was loaded with 15 μg of protein labeled with Cy2, Cy3, or Cy5, for a total of 45 μg of protein per gel. First dimension; 11 cm, pH 3-10 immobilized pH gradient strip. Second dimension: 12.5% SDS-PAGE gel.

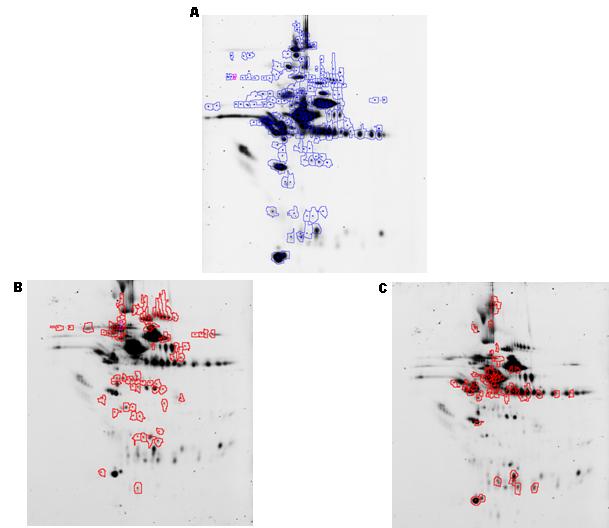


Figure 4: Albumin depleted samples tended to exhibit greater variation in abundance when compared to whole serum samples. Boxplots of the standard errors of estimated abundance of all 236 spots identified in DeCyder. Standard errors were determined using normalized data from DeCyder. A spot-specific linear mixed model for depleted and whole data with fixed effects of dyes (Cy3 or Cy5), fixed effects of lines (high or low RFI), and random effects of animals was used. Standard errors tended to be greater in depleted versus whole serum (one-sided Wilcoxon signed-rank test).



Figure 5: Comparison of the resolution of the serum protein profile on a 7 cm, pH 3-10 immobilized pH Gradient (IPG) strip; 7 cm, pH 4-7 IPG strip; and 7 cm, 6-11 IPG strip. Second dimension was a 12.5% SDS-PAGE gel. Circled areas located on the pH range 3 to 10 gel correspond with the same circles on the 4 to 7 and 6 to 11 pH range gels below. This demonstrates the increase in resolving ability through the use of a more narrow pH range.

