

Sample Preparation and Staining Methods for Two-Dimensional Polyacrylamide Gel Electrophoresis of Proteins from Animal Tissues

Levente Czegledi, Gabriella Gulyas, Andrea Radacsi, Szilvia Kusza, Janka Bekefi, Bela Beri, Andras Javor

¹University of Debrecen, Faculty of Agronomy, Institute of Animal Science, H-4032 Debrecen, Böszörményi Street 138, Hungary

Abstract

Proteomics in animal science as well as in other biological sciences is a significant tool in the post-genomic era. In proteomic studies the presence and relative abundance of expressed proteins of a cell, tissue or biological fluid is studied. Recently, the whole genome of more and more domestic animal species is known, but genes and the transcribed mRNA have no direct effect on biological systems as they are regulated by proteins, which explain the importance of proteomics. The most common tool in proteomic approach is the two-dimensional polyacrylamide gel electrophoresis (2D PAGE), when proteins are separated by their isoelectric point followed by their mass separation as a second dimension. In this study authors used different sample preparation and protein staining methods on meat, liver and blood plasma and carried out 2D PAGE experiments. The most appropriate sample preparation methods are described in this paper. We concluded that depletion of major proteins in plasma is required but not necessary for meat and liver samples.

Keywords: animal tissue, protein expression, proteome, 2D PAGE

1. Introduction

The aim of proteomics is to identify new proteins as biomarkers and measure changes in protein expression, modification those were caused by an experimental treatment [1].

The most common tool in proteomic approach is the two-dimensional polyacrylamide gel electrophoresis, when proteins are separated by their isoelectric point followed by their mass separation as a second dimension [2]. This separation technique results hundreds of individual protein spots in a polyacrylamide gel, where left to right separation is by isoelectric point and up and down separation is by molecular weight. There is a similar method to 2D PAGE, named Blue Native polyacrylamide gel electrophoresis (BNPAGE) [3], where protein is separated by molecular weight. In the first

dimension protein complexes are intact and separated by the complex size, electrophoresis based on the amount of bounded charged dye that is proportional to the complex size. Samples are run in a SDS PAGE as the second dimension which separates individual proteins of a complex.

In this study authors used different sample preparation and protein staining methods on meat, liver and blood plasma and carried out 2D PAGE experiments. The aim of this pilot study is to find the most appropriate 2D PAGE method for the identification of differently expressed proteins in nutriproteomic experiments: changes in proteome of animal tissues caused by omega-3 fatty acid rich diet.

2. Materials and methods

Samples

Skeletal muscle of cattle and sheep, liver of pig and blood plasma of cattle were collected. Muscle and liver samples were harvested into cryotubes as an amount of 2 g per sample, 1 day after slaughter and were kept at -80°C until protein extraction. Blood samples of cattle (10 ml per animal) were taken from the jugular vein into ice-cold glass tubes containing EDTA and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Plasma was separated at 3500 rpm for 8 minutes, supernatant was collected into cryotube (2 ml) and snap frozen in liquid nitrogen. Samples were kept at -80°C until subsequent analysis.

Extraction of Proteins

Skeletal muscle and liver

Protein samples were extracted from cattle and sheep muscle tissues, pig liver tissue as follows: tissue samples were placed in liquid nitrogen and ground thoroughly to a fine powder with a mortar and pestle. Tissue powder (100 mg) was transferred to sterile tubes containing 1 ml of sample preparation buffer (Bio-Rad, Hercules, USA) (8 M urea, 2 M thiourea, 2% CHAPS, 50mM DTT, 0.2% 100x Bio-Lyte 3/10 ampholyte). Samples were centrifuged at 15,000 g for 30 min., the supernatant was collected and stored at -80°C until analysis.

There was a second purification procedure for cattle and sheep muscle tissues. Supernatant from previous extraction was purified with ReadyPrep 2-D Cleanup Kit (Bio-Rad) following manufacturer's protocol. The kit concentrates proteins from samples, allowing for higher protein loads that can improve spot detection. The procedure works by quantitatively precipitating and concentrating proteins in a sample while leaving behind and washing away substances like ionic detergents, salts, nucleic acids and lipids, all of which are known to interfere with isoelectric focusing (IEF).

Blood plasma

Protein samples were prepared from cattle blood plasma. Proteins of plasma were extracted with ReadyPrep 2-D Cleanup Kit. 200µg protein was loaded for Biosafe Colloidal Coomassie Blue G-250 staining and 100 µg for silver staining procedure, as silver staining is a more sensitive protocol.

Protein concentrations were measured in a nanodrop spectrophotometer at a volume of 2 µl using Bradford assay and BSA as standard (Bio-Rad).

2-dimensional electrophoresis

For the first dimension (isoelectric focusing), IPG strips, 17 cm, pH 3 to 10, were rehydrated in 300 µl of rehydration/sample preparation buffer containing 300 µg muscle/liver protein, 100 µg and 200 µg plasma protein for 15 h at room temperature.

Isoelectric focusing was conducted in Protean IEF Cell (Bio-Rad). Low voltage (250 V) was applied for 20 min. The voltage was gradually increased to 10,000 V over 2.5 h, and was maintained at that level until a total of 50,000 Vh.

Focused IPG strips were equilibrated for 10 min. in 6 M urea, 20% glycerol, 2% SDS, 50 mM Tris pH 8.8 and 2% DTT, and then for an additional 10 min. in a similar buffer containing 2.5% iodoacetamide instead of DTT. After equilibration, proteins were separated in the second dimension using Protean II XL vertical electrophoresis system (Bio-Rad). Second dimension was performed on 160 x 200 mm, 13% polyacrylamide gels (37.5:1 acrylamide:bis ratio). A cooling system provided constant 20°C running temperature.

Gels of cattle muscle and plasma samples were stained with Biosafe Colloidal Coomassie Blue G-250 (Bio-Rad), documented by scanning, washed twice with MilliQ water (2 x 10 min.) and the same gels were stained with silver stain procedure. The applied silver staining is compatible with subsequent MALDI-MS analysis.

Biosafe Colloidal Coomassie Blue G-250 stain

Staining was carried out following manufacturer's instruction: gels were washed thoroughly (3 x 5 min.) with MilliQ water and incubated in the staining solution on an orbital shaker platform for overnight. Finally gels were washed 2 x 20 min. with MilliQ water.

Silver stain

Silver staining protocol was based on Shevchenko method [4]. Gels were fixed in 50% methanol, 5% acetic acid for overnight. Then gels were rinsed in 50% methanol for 20 min. and in MilliQ water 2 x 15 min.. The sensitize step was in 1.27 mM Na₂S₂O₃ for 3 min., then gels were washed with MilliQ water for 2 x 1 min., stained with 0.15%

silver nitrate for 30 min., rinsed with MilliQ water for 2 x 2 min.. Gel staining was developed in 0.04% formaldehyde, 2% Na₂CO₃ to the degree desired, with intensive shaking and stopped in 5% acetic acid for 5min.. Gels were stored in 1% acetic acid until analysis.

2D PAGE was performed in duplicate of each sample as a technical replication of procedure.

3. Results and discussion

The higher sensitivity of silver staining compared to Coomassie dye is shown on Figure 1 and 2.

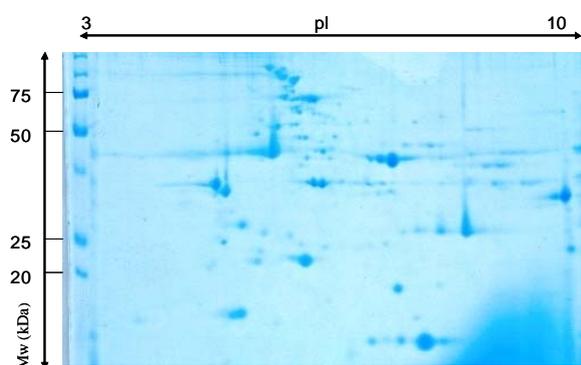


Figure 1. 2D PAGE image of cattle muscle, Biosafe Colloidal Coomassie Blue G-250 stain

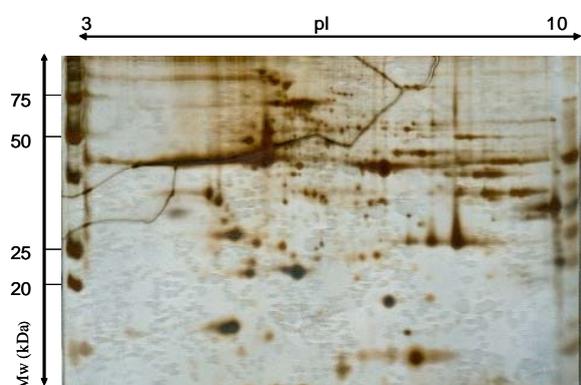


Figure 2. 2D PAGE image of cattle muscle, silver staining

In proteomic studies hundreds of individual proteins are analysed in one gel, the probability to find the protein of which expression has changed by treatment could be increased by staining methods. Three times more protein spots are detected using silver staining method than colloidal Coomassie dye, but it should be considered that each stain interact differently with different proteins [5], thus there is no one stain

which is adequate in all 2D PAGE experiment. Polyacrylamide gels should be stained with two or more different dyes to perform a very accurate analysis/biomarker discovery.

The protein extract of sheep skeletal muscle was stained by silver staining (Figure 3). The figure shows an example on the effect of a not optimal condition at isoelectric focusing step on protein spot pattern and streaking. IEF was run for total of 38,000 Vh instead of the optimal 50,000 Vh. Similar streaking at the basic side of gel was also observed in the case of using broad range (pH 3-10) carrier ampholytes.

Proteins of pig liver were extracted by the same protocol as used for muscle samples. Figure 4 shows the pattern of spots of described method.

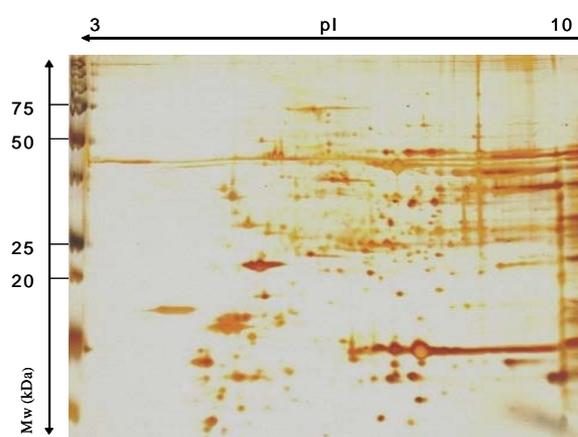


Figure 3. 2D PAGE image of sheep muscle, silver staining

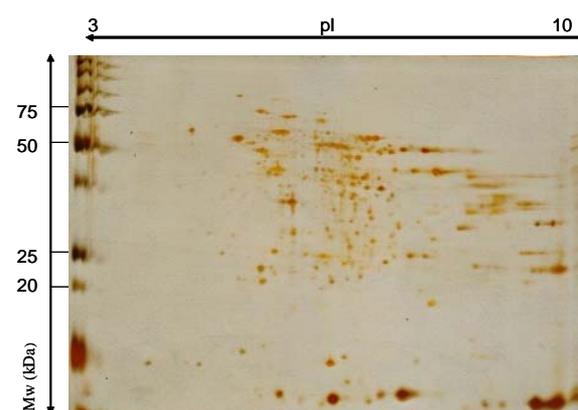


Figure 4. 2D PAGE image of pig liver, silver staining

Cattle blood plasma proteins were purified by ReadyPrep 2-D Cleanup kit and stained by Biosafe Colloidal Coomassie Blue G-250 stain and silver staining method as well (Figure 5, 6).

Cleanup was required to eliminate substances those may interfere isoelectric focusing. High abundant proteins as albumin, globulin were not depleted, thus quantitatively, the major part of proteome remained in sample. Depletion would be needed to increase the number of detected proteins in case of both staining methods.

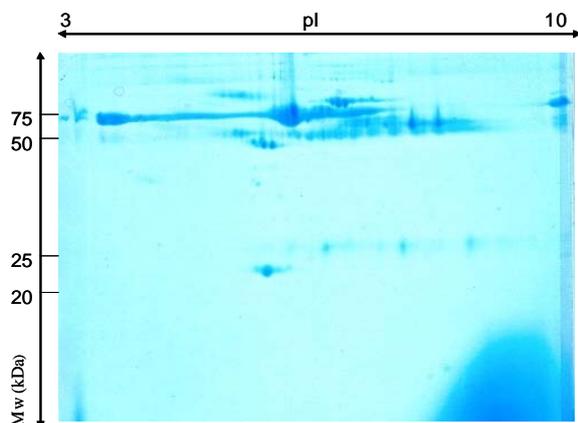


Figure 5. 2D PAGE image of cattle blood plasma, Biosafe Colloidal Coomassie Blue G-250 stain

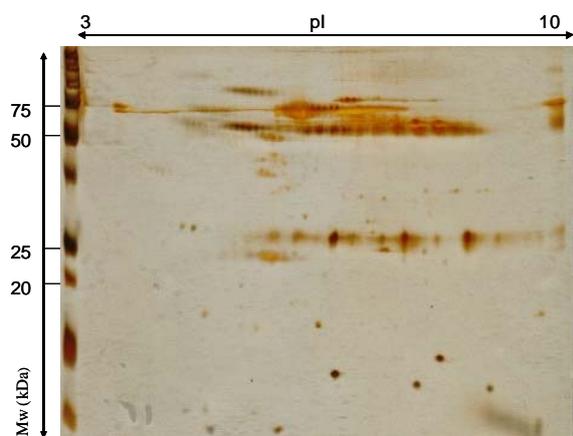


Figure 6. 2D PAGE image of cattle blood plasma, silver staining

4. Conclusions

There are several sample preparation, protein extraction, concentration methods for gel based separation of proteome of different tissues, cells and subcellular fractions.

In the present study animal tissues of our interest, as samples of cattle, sheep and pig from different trials/experiments, were used for 2D PAGE analysis. The protein extraction method described in detail in materials and methods, was the most adequate for skeletal muscle and liver samples. Blood plasma 2D PAGE analysis requires not only cleanup, but depletion of major proteins as well. Coomassie and silver staining were tested for protein spot analysis and results show that the higher sensitivity of silver staining is significant.

Acknowledgements

This work was financed by EEA & Norwegian Financial Mechanisms and Research and Technology Innovation Fund, grant name: "Longlife", "Goodmeat", "LactoSel".

References

1. Lippolis, J, D, Reinhardt, T, A, Centennial Paper: Proteomics in Animal Science, *Journal of Animal Science*, 2008, 86, 2430-2441
2. Stasyk, T, Huber, L, A, Zooming in: Fractionation strategies in proteomics, *Proteomics*, 2004, 4, 3704-3716
3. Schagger, H, von Jagow, G, Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form, *Analytical Biochemistry*, 1991, 1999, 223-231
4. Shevchenko, A, Wilm, M, Vorm, O, Mann, M, Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels, *Analytical Chemistry*, 1996, 68, 5, 850-858
5. Carroll, K, Ray, K, Helm, B, Carey, E, Two-dimensional electrophoresis reveals differential protein expression in high- and low secreting variants of the rat basophilic leukaemia cell line, *Electrophoresis*, 2000, 21, 2476-2486