

COMPARATIVE STUDY OF WEANING PIGS' MUSCLE PROTEINS USING TWO-DIMENSIONAL ELECTROPHORESIS

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ABSTRACT

The proteostasis system of animals, including various types of protein modification during the growth stage, leads to an almost incomprehensible number of possible forms of protein, and each can regulate numerous functions. In the presented work, the composition of muscle tissue protein from different portions of piglets was studied to understand the main muscle protein formation. Comparative analysis of weaned piglets' main muscle protein from *l. dorsi*, *biceps femoris*, and *brachiocephalicus* were analyzed using two-dimensional electrophoresis. Changes in the staining intensity of protein fractions inherent in different muscles were revealed. As part of this work, candidate groups of pig muscle proteins have been selected. Eleven protein spots were revealed for the longest muscle of the back, and seven for the biceps; the muscles of the neck are characterized by indicators of low protein fraction volume. Among the proteins found, myosin light chains, phosphoglycerate mutase, troponins, and adenylate kinase is most likely present. The obtained results of protein identification in muscle tissues, obtained during the intensive growth period, will allow a more detailed understanding of protein regulation, function, and interactions in complex biological systems, which will subsequently be significantly important for biomonitoring health and predicting farm animals productivity.

Keywords: 2-DE; muscle; protein; two-dimensional electrophoresis; pig; piglet

INTRODUCTION

The increase in demand for lean pork has required the selection of carcasses showing increased muscle mass. The selection for this indicator has significantly improved the meat qualities of most domestic and foreign pig breeds. However, an increase in "meat content" without taking into account other economically useful traits led to a decrease in natural resistance and significant shifts in metabolic processes in the animals, which ultimately affected the quality and organoleptic characteristics of pork (Fedulova et al., 2018; Benešová et al., 2019; Gorlov et al., 2020).

It should be noted that the selection efficiency is assessed mainly by identifying genes that control beneficial traits, as well as polymorphic DNA variants in these genes, which directly affect the phenotype of the animal. In this case, genome and proteome interactions are completely ignored. It is known that an animal's proteostasis system, including folding, modification of the primary structure, and protein degradation, are crucial for the realization of phenotypic traits. At the same time, the huge number of possible post-translational modifications, combined with the multitude of amino acid residues, leads to an almost incomprehensible number of possible protein forms, and each can regulate numerous functions (Spoel, 2018). Thus, the use of proteomic methods in animal husbandry for muscle protein identification will allow a more detailed understanding of protein regulation, function, and interactions in complex

biological systems, which will subsequently be vitally important for biomonitoring the health and productivity of farm animals. Also, practical applications in identifying counterfeit meat products could be based on this technique (Mora, Gallego and Toldrá, 2018).

As you know, with the growth and increase in animal live weight, the amount of muscle, adipose, and bone tissue in their body is steadily increasing. However, the growth of these tissues proceeds with unequal intensity. The development of muscle tissue in young animals is due to a high rate of metabolic processes and protein synthesis. Moreover, the main factors that determine the rate of muscle growth are genetic, feeding, muscle activity (exercise), stress hormones, and growth stimulants (natural or synthetic).

As growth slows down and stops, the rates of protein synthesis and degradation are balanced. In conditions of poor nutrition, diseases, including parasitic ones, stress, unfavorable environmental conditions, muscles can atrophy, which means that protein degradation outweighs synthesis (Purslow, 2017).

Until recently, not enough attention has been paid to the development rate of muscle tissue in growing animals to predict the factors that stimulate the growth of animals and determine meat quality.

Scientific Hypothesis

Identifying the composition of muscle proteins of various parts of a growing animal - weaned piglets - will expand the available information on the formation of muscle tissue to identify quality, safety, and authenticity markers in livestock products.

MATERIALS AND METHODS

Object

The object of the study was Vietnamese pot-bellied × Wiesenau weaned pigs from Krolinfo LLC laboratory animal resource center (Likino-Dulyovo, Russia), healthy females 60 – 65 days old. Samples were taken from 9 piglets: *l. dorsi* (longest back muscle), *biceps femoris* (hip biceps), and *brachiocephalicus* (neck muscles) muscle.

Materials

Chemical reagents: Urea, Thiourea, Dithiothreitol, Sodium hydroxide (NaOH), Glycerol, Sodium dodecyl sulfate (SDS), Tris, Acrylamide, Ammonium persulfate (APS), 2-Propanol, Acetic acid, Bis-acrylamide, Tetramethylethylenediamine (TEMED), Mercaptoethanol, Bromophenol blue, Glycine, Coomassie Brilliant Blue G-250 (PanReac, Spain); Triton X-100 (Helicon Russia); Ampholyte (Serva, Germany) and Phosphoric (V) acid (H₃PO₄) (Component-reaktiv, Russia).

Samples were taken within 20 minutes after slaughter and placed in dry ice. Frozen muscle tissues (50 mg) were homogenized in 1 mL 7 M Urea, 2 M Thiourea, 1 % Dithiothreitol, 0,4 % Triton X-100, 2% pH 3 – 10 Ampholyte. Homogenates were centrifugated with an acceleration of 20 000 g for 20 minutes. Three samples, obtained from different animals, were studied by two-dimensional electrophoresis in three replicates.

Two-dimensional gel electrophoresis (2-DE)

The samples described above were subjected to 2-DE. Proteins were separated by IEF (isoelectric focusing) in the first dimension and SDS-PAGE in the second dimension. This was completed as described by **Matsumoto et al. (2019)** with slight modifications, IEF in the first dimension was performed at 3650 V.h⁻¹.

2-DE was performed according to the method of O'Farrell (O'Farrell, 1975) with isoelectric focusing on ampholine pH gradient by Glass Tube-Based IEF. The subsequent detection of the proteins was carried out by staining with Coomassie Brilliant Blue G-250 (PanReac, Spain).

For computerized densitometry, two-dimensional electropherograms were used in a wet state. Their full digital images and/or images of individual fragments were obtained using a Bio-5000 plus scanner (Serva, Germany). Scanned images were analyzed with ImageMaster™ 2D Platinum software powered by Melanie 8.0 (GE Healthcare and Genebio, Switzerland). Spots were detected and quantified automatically with minimum thresholds: saliency – 11, min area – 5, and smooth – 3. The relative optical density (OD) and relative volume were computed to correct for differences in gel staining. These measures take into account variations due to protein loading and staining, by considering the total OD or volume over all the spots in the gel. The digitized 2DE images of the cortex were then compared by the matching method (**Grove et al., 2006**).

Protein spots on two-dimensional electropherograms were interpreted following the Swiss-Prot database (**O'Donovan et al., 2002**) and the Muscle organ proteomics database (**Kovaleva et al., 2013**).

Statistical analysis

The experimental data were analyzed using one-way ANOVA (between gels obtained with a different variation of IEF) by ImageMaster™ 2D Platinum software powered by Melanie 8.0 (GE Healthcare and Genebio, Switzerland).

A $p < 0.05$ was considered to indicate a significant difference. All results are presented as mean ±SD from at least three independent experiments.

The Fold change index was calculated. Fold change index is the ratio between the volume of a protein spot with the highest average value and the lowest average value. It is common to select spots with Fold >2, for instance, to identify those proteins in which treatment causes at least a two-fold spot abundance increase or decrease.

RESULTS AND DISCUSSION

A study was carried out on three different muscles of *l. dorsi*, *biceps femoris*, and *brachiocephalicus* by two-dimensional electrophoresis to reveal significant differences in protein composition (**Zia et al., 2020**).

Two-dimensional electrophoretogram fragments with the detected changes are shown in Fig. 1; a total of 18 fractions were selected with Fold index >2 and presented in Table. 1.

Protein spots indicated by numbers 1 and 2 in Figure 1, and presumably myosin light chains fast (MLC1f) (**Murgiano et al., 2010; Yang et al., 2016**) and slow (MLC1s/v), respectively, were quite well manifested in the longest muscle (**Kim et al., 2017; Zou et al., 2018**). In the *biceps femoris*, only the slow chain was detected, and in the *brachiocephalicus*, a weakly expressed fraction MLC1s/v. **Montowska and Pospiech (2012)** used MLC in their studies as markers in the authentication of meat products made from pork and other meats.

A group of proteins in the range of molecular weights from 50 kDa to 60 kDa, marked with numbers 3 – 7 in Figure 1, were more pronounced in the muscles of the biceps, a smaller amount identified in the longest muscle, and the muscles of the neck trace amounts were detected.

Protein number 8 intensity (Figure 1) decreased evenly from *l. dorsi* to *brachiocephalicus*, the same tendency was observed in fractions 13 – 17, among them troponin is presumably present. The skeletal muscle protein troponin I have already been characterized as a potential thermostable and species-specific biomarker of mammalian muscle tissue in raw meat and meat products (**Zvereva et al., 2015; Mitra et al., 2018; Zuber et al., 2019**), which makes it promising for identifying muscles of different regions of the animal. The lower spot volume in the neck muscles is possibly associated with the biological function of this muscle since it is less active than *l. dorsi* and *biceps femoris*.

Interesting distribution of protein spots was found in fractions 9 and 10, only protein number 9 was found in the biceps, but fraction 10 (adenylate kinase) was present in a small amount, but its intensity in the large, flat muscle on the back was greater than in the neck muscle (**Lee et al., 2011; Scheffler, Park and Gerrard, 2011; Oliván et al., 2015**).

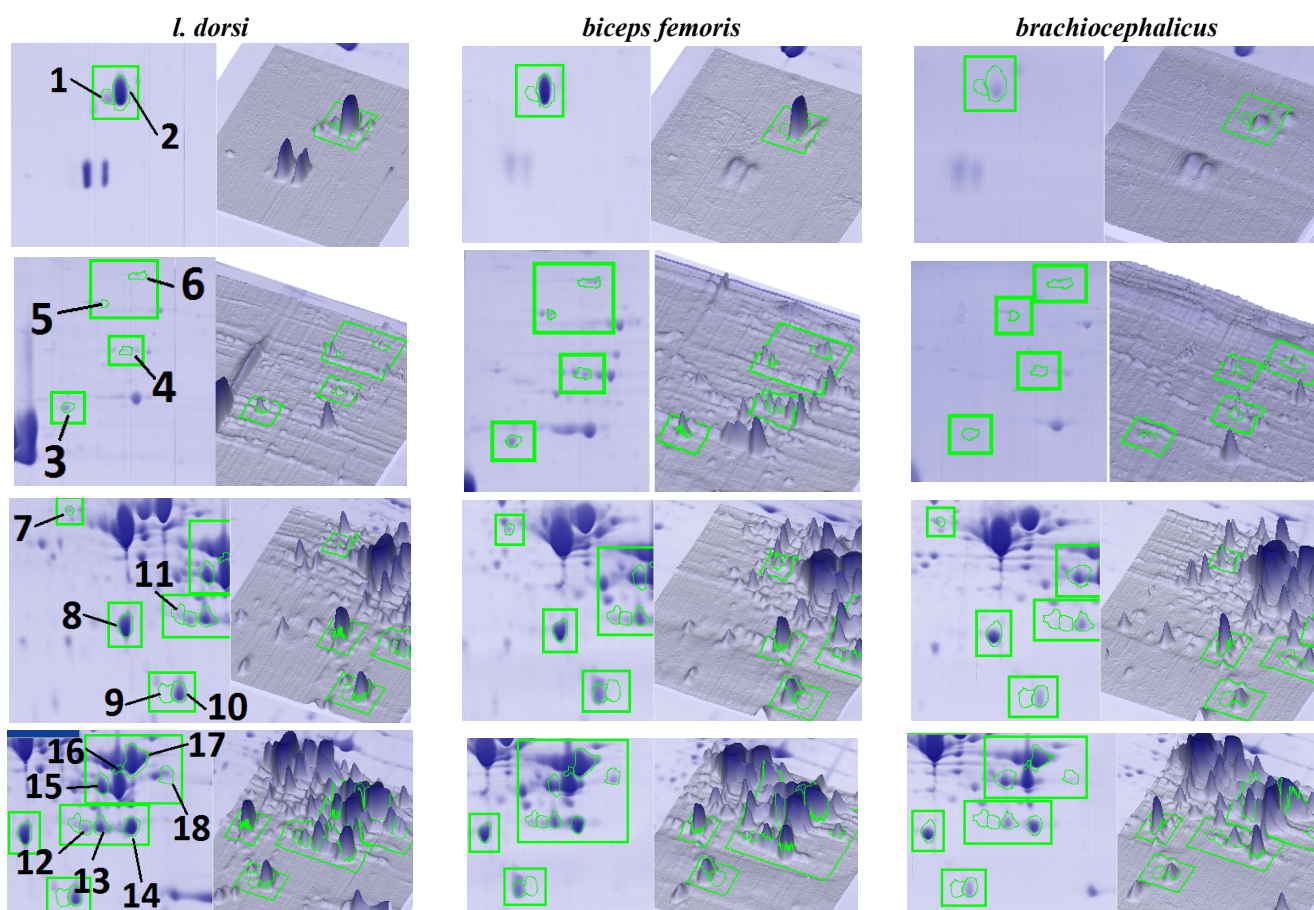


Figure 1 Fragments 2-DE gels of pig muscle tissue.

Table 1 Results of the densitometry analysis.

No of Spot	Muscle tissue			Fold
	<i>l. dorsi</i>	<i>biceps femoris</i>	<i>brachiocephalicus</i>	
1	9.07 ±1.19 x 10 ⁶	2.60 ±1.63 x 10 ⁶	1.46 ±0.36 x 10 ⁶	6.20
2	0.11 ±1.35 x 10 ⁷	8.93 ±1.13 x 10 ⁷	2.78 ±1.77 x 10 ⁷	4.18
3	3.84 ±0.67 x 10 ⁶	2.74 ±1.15 x 10 ⁶	1.02 ±0.37 x 10 ⁶	3.78
4	1.15 ±0.34 x 10 ⁶	2.63 ±0.52 x 10 ⁶	1.18 ±0.57 x 10 ⁶	2.29
5	2.55 ±0.63 x 10 ⁵	1.18 ±0.29 x 10 ⁶	2.72 ±0.67 x 10 ⁵	4.62
6	6.88 ±3.57 x 10 ⁵	2.42 ±0.40 x 10 ⁶	1.42 ±0.47 x 10 ⁶	3.52
7	1.49 ±0.79 x 10 ⁶	5.96 ±1.67 x 10 ⁶	3.49 ±0.22 x 10 ⁶	4.00
8	6.21 ±0.29 x 10 ⁷	5.16 ±0.83 x 10 ⁷	1.75 ±1.52 x 10 ⁷	3.55
9	4.90 ±2.28 x 10 ⁶	1.86 ±1.21 x 10 ⁷	2.08 ±0.16 x 10 ⁶	8.94
10	3.33 ±0.48 x 10 ⁷	1.27 ±0.92 x 10 ⁷	8.07 ±5.80 x 10 ⁶	4.12
11	5.93 ±1.10 x 10 ⁶	5.78 ±1.72 x 10 ⁶	2.03 ±0.49 x 10 ⁶	2.93
12	1.77 ±0.45 x 10 ⁷	8.42 ±2.46 x 10 ⁶	1.69 ±0.54 x 10 ⁶	10.50
13	4.37 ±0.32 x 10 ⁷	3.38 ±1.30 x 10 ⁷	1.24 ±0.34 x 10 ⁷	3.15
14	5.99 ±0.19 x 10 ⁷	5.19 ±1.02 x 10 ⁷	1.84 ±1.53 x 10 ⁷	3.25
15	5.31 ±0.88 x 10 ⁷	4.18 ±0.62 x 10 ⁷	2.52 ±0.99 x 10 ⁷	2.11
16	1.56 ±0.28 x 10 ⁷	7.86 ±2.20 x 10 ⁶	3.90 ±2.56 x 10 ⁶	3.98
17	1.40 ±0.12 x 10 ⁸	1.16 ±0.05 x 10 ⁸	6.86 ±1.51 x 10 ⁷	2.04
18	1.07 ±0.23 x 10 ⁷	1.21 ±0.17 x 10 ⁷	3.71 ±1.17 x 10 ⁶	3.27

Note: Spot Vol* were normalized by total valid spot volume and mean of value from duplicate analytical gels from three replicates. Data represented are means ±SD of three independent experiments. *Vol: The volume of a spot is the sum of the background-subtracted gray values of all pixels delimited by the spot.

The volume of spot number 11 in *brachiocephalicus* was three times less than in *l. dorsi* and *biceps femoris*. The highest Fold value was in fraction 12, which is probably phosphoglycerate mutase, corresponded to 10.5, and is maximally expressed in *l. dorsi* (Welzenbach et al., 2016; He et al., 2016; Lepczynski et al., 2019). Protein number 18 is more strongly expressed in the muscles of the biceps, less intensely noted in *l. dorsi* and a small amount in the neck.

The changes described above in the staining intensity of selected protein fractions may reflect the dynamics of muscle tissue formation in growing animals (Chen et al., 2018; Li et al., 2020). For example, in *l. dorsi*, the most powerful muscle of the spinal column, which determines the movement of the spinal column and head, the maximum amount of intensely colored protein fractions was revealed (Liu et al., 2014; Paredi et al., 2019). Candidate markers for *l. dorsi* can be fractions 1, 3, 8, 10 – 17 (Figure 1, Table 1) (Zou et al., 2017). For the *biceps femoris*, which functionally exhibits actin, the hip extensor, and hock joints, and the flexor of the knee joint, muscle fractions 2, 4 – 7, 9, and 18 can serve as markers (Figure 1, Table 1). The thigh muscle and its protein profile are quite well studied, since various versions of jerky ham are made from pork, such as ham and prosciutto, it is already known that the peptides formed in this muscle have biological functionality (some peptides from MLC1, CK, MYO, TNT, and MHC7 proteins were the most influential) (Bermúdez et al., 2014; Mora et al., 2016; Chernukha et al., 2018; Zhou et al., 2020).

For *l. dorsi* composition, 11 proteins were identified (Kim et al., 2008; Grujić and Savanović, 2018), and 7 proteins for *brachiocephalicus*, besides, the latter showed a tendency of lower intensity protein fractions, some of which are smaller in comparison to *l. dorsi*.

The lowest intensity of staining of protein fractions was noted in *brachiocephalicus*, which is possibly associated with low metabolic processes in this muscle, due to a low functional load.

CONCLUSION

As part of this work, data were obtained on the variations in muscle protein groups in the muscles of different regions in piglets. For the longest muscle of the back, eleven protein spots with a pronounced intensity of staining were revealed, for the biceps - seven, for the muscles of the neck, low indicators of the spot volume are characteristic. These protein fractions vary across the entire spectrum of intercellular proteins, from heat shock proteins and enzymes of energy metabolism to structural proteins. The identified protein spots can be used as molecular markers of muscle tissue development. However, much remains to be clarified on a structural and molecular basis. This study further revealed the markers of productivity and adaptation of animals, as well as the quality, safety, and authenticity of animal products.

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The authors declare no conflict of interest.

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