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PROTEOMIC STUDY OF PIG'S SPLEEN

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ABSTRACT
This work is devoted to pig spleen proteome study. Spleens were taken from Duroc pigs (females, 145 – 160 days old) and typical two-dimensional electrophoregrams were obtained. On proteomic maps after visualization and image analysis there were detected 600 fractions, including organ-specific proteins – 3 62 fractions. Among the identified constitutive fractions, the highest expression was observed (Vol spots more than 3.0E + 07) four protein spots S1, S9, S12 and S21, which are supposedly Annexin A1 (MW 38.76 kDa), Ectonucleoside triphosphate diphosphohydrolase 1 (MW 57.75 kDa) Pro-cathepsin H CD59 (MW 37.45 kDa) and glycoprotein (MW 13.79 kDa), respectively. Obtained electrophoregrams analysis using information resources made it possible to identify different active compounds in spleen with various functions, mainly immunoregulatory – glycoprotein CD59 (Mm 13.79 kDa) and ATP-dependent RNA helicase (Mm 107.58 kDa); the intensely expressed LIM-domain of the actin-binding protein (Mm 83.99 kDa). The results obtained are a prospect for immunomodulating biologic development based on animal raw materials for farm animals.

Keywords: spleen; two-dimensional electrophoresis; pork; proteomic

INTRODUCTION
Farm animals' organs and tissues are an inexhaustible resource of compounds involved in various regulatory and compensatory oraganism reactions **(Chernukha et al., 2016; Kotenkova, Lukinova and Fedulova, 2017)**. Modern researches are aimed to identifying, studying and isolating proteins that are potentially capable of exhibiting biological activity.

Today, one of the most effective ways to study active compounds derived from animal raw materials is complex tissue-specific proteins analysis at molecular level, which is commonly called the "proteomic approach". The main proteomics method, still relevant today, is two-dimensional electrophoresis, used to study protein changes and identify functional species and tissue-specific compounds **(Chernukha et al., 2017)**. Two-dimensional electrophoresis technology allows us to separate thousands of proteins with high resolution, and characterize the isolated protein fractions using mass spectrometric methods. Obvious advantage proteomic approach over others lies in its ability to detect alternative proteins forms that are the result of co-and/ or post-translational modifications.

Currently, animal tissue researches are aimed at studying biological processes in order to identify product quality and safety markers (for example, species, autolysis, quality defects) **(Mora, Gallego and Toldrá, 2018)**. However, scientific projects devoted to productive animals individual organs proteomic analysis for active protein components isolation and biologics creation based on them have great potential.

This work is devoted to protein composition comparative study of pigs spleen and resulting two-dimensional electrophoregrams analysis in order to identify potential constitutive proteins to create biological preparations of immunomodulatory action.

Scientific hypothesis
Productive animals' organs and tissues are bioactive protein compounds source. Spleen as an immune organ may contain physiologically active proteins with a pronounced immune orientation.

MATERIAL AND METHODOLOGY
Duroc pigs spleen was selected as study object. In order to level the geographic population characteristics, animals were selected from healthy females of 145 – 160 days old on three farms: Lipetsk region (C1); Voronezh region (C2); Tyumen region (C3).

Two dimensional gel electrophoresis (2-DE)
The samples described above were subjected to 2-DE. Proteins were separated by IEF in the first dimension and SDS-PAGE in the second dimension essentially as described by Hirano **(Hirano, 1982)** with slight modifications **(Kimura et al., 2003)**. IEF in the first dimension was performed at 3650 $V.h^{-1}$. The anodic and cathodic electrode solutions used for IEF were 0.02 M H3PO4 and 0.02 M NaOH, respectively, in

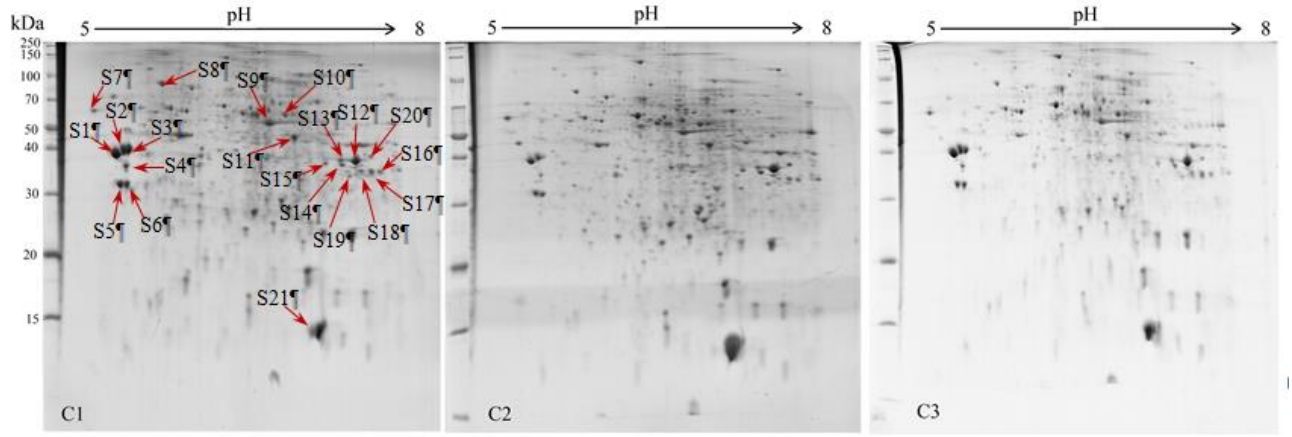

Figure 1 2D PAGE of pig's spleen.
Note: C1 – AgroEko, C2 – SGC, C3 – Tumen. Spots showing differential expression were marked excised.

2.4 mm × 160 mm tube gels. After IEF, the extruded tube gels were incubated for 10 min, in 2.5 mL of equilibration buffer I (6 M urea, 20% w/v glycerol, 2% w/v SDS and 1% w/v DTT in 50 mM Tris-HCl buffer, pH 8.8) followed by equilibration buffer II (6 M urea, 30% w/v glycerol, 2% w/v SDS and 4% w/v iodoacetamide in 375 mM Tris/HCl buffer, pH 8.8). For SDS-PAGE, the equilibrated tube gels were transferred to a 12.5% polyacrylamide gel (170 mm × 180 mm × 1.5 mm). Electrophoresis was carried out with a gel running buffer containing 25 mM Tris-HCl, 192 mM glycine, and 0.1% w/v SDS at 30 mA per gel until the bromophenol blue front had reached the bottom ofthe gel.

Protein visualization and image analysis

Protein spots were visualized by staining with Coomassie Brilliant Blue G-250. For computerized densitometry, two-dimensional electrophoregrams were used, which were 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. 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 cortex were then compared by the matching method.

Protein spots interpretation on spleen two-dimensional electrophoregrams was carried out in accordance with the **Swiss-Prot database (2002)**.

Statisic analysis

The experimental data between three organs were analyzed using student's t-test, and data among several groups were analyzed by one-way ANOVA by ImageMaster™ 2D Platinum software powered by Melanie 8.0 (GE Healthcare and Genebio, Switzerland). A p value <0.05 was considered significantly different. All results are presented as mean ±SD from at least three independent experiments.

RESULTS AND DISCUSSION

As a result of spleens 2-DE gels quantitative proteomic study images from pigs at three different farms (Figure 1) using ImageMaster ™ 2D Platinum software, it was found that, on average, there are about 597 spots on each gel. At the same time, the most pronounced protein expression was detected in samples C2 – 624 fractions, the least pronounced protein expression was observed in samples C3 – 570 fractions. Two-dimensional electrophoregrams comparative analysis in all samples revealed 362 major (constitutively present) fractions.

Among identified constitutive fractions (Figure 2), there were noted high expression protein spots S1, S9, S12, and S21, presumably Annexin A1 (MW 38.76 kDa), Ectonucleoside triphosphate diphosphohydrolase 1 (NTPDase 1, MW 57.75 kDa), Pro-cathepsin H CD59 (MW 37.45 kDa) and glycoprotein (MW 13.79 kDa), respectively. The biology of protein Annexin A1 functions, as revealed by studies **D'acquisto, Perretti and Flower (2008)** using transgenic animals, peptide mimetics and neutralizing antibodies, speaks to its role as a key modulator of both innate and adaptive immune systems. **Lemmens et al. (2000)** found that NTPDase 1 possesses both immunological identity and functional characteristics of vascular ATPDase. It is also known that Prokatepsin H regulates the signaling pathway of immune response **(Gladue et al., 2014)**, and Glycoprotein is a strong inhibitor of membrane attack complex and nonspecific immune response **(Maher et al., 1998)**.

In accordance with the information databases resources, the following functional compounds were found in spleen samples: the leptin receptor (Mm 132.52 kDa), which acts as factor regulating appetite, causing decrease in food intake and an increase in energy consumption, also regulates bone mass and secretion of hypothalamic-adrenal pituitary hormones **(Ruiz-Cortés et al., 2000)**; in small quantities, the transmembrane glycoprotein 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Mm 97.15 kDa), which limits the rate of cholesterol biosynthesis and participates in the biosynthesis of isoprenoids necessary for the normal functioning of cells **(Chen et al., 2012)**.

Fractions, involved to innate immune response mechanisms, were identified, such as glycoprotein CD59 (Mm 13.79 kDa) and ATP-dependent RNA helicase (Mm 107.58 kDa) **(Zhang et al., 2000)**; the intensely expressed LIM-domain of the actin-binding protein (Mm 83.99 kDa),

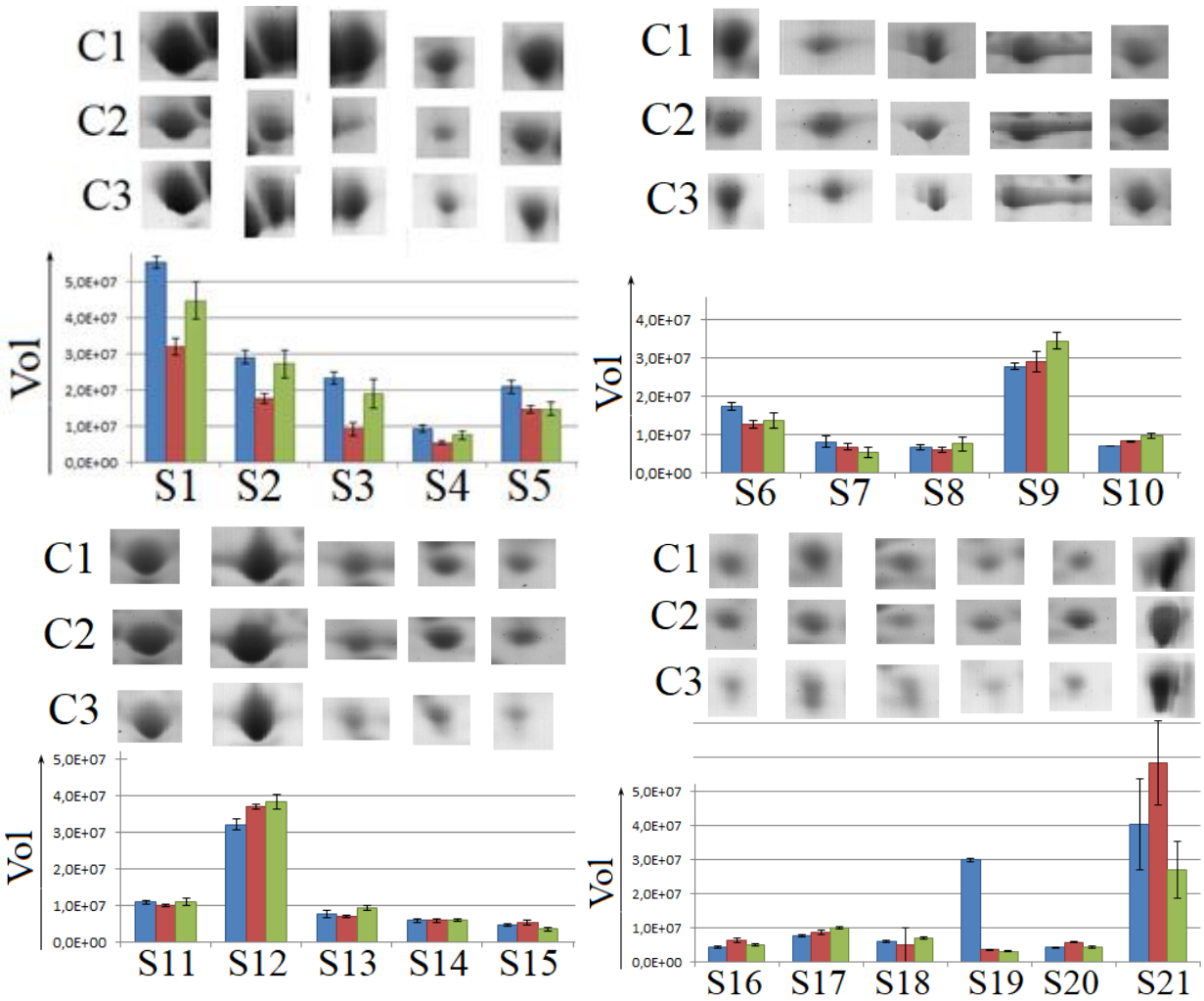

Figure 2 Relative vol change in differentially expressed proteins in spleens (blue – C1, red – C2, green – C3).
Note: Spot intensities were normalized by total valid spot intensities and mean of values from duplicate analytical gels from three replicates. Data represented are means ±SD of three independent experiments.

which is involved in the regulation of the cytoskeleton of actin, which increases the number and size of actin stress fibers, as well as inhibits the depolymerization of actin filaments **(Wang et al., 2007)**.

Also on two-dimensional electrophoregrams, a proteins group was identified that is characteristic of all analyzed spleens and plays an important role in innate immune response and inflammatory processes regulation: interferon stimulator protein (Mm 41.8 kDa), chemokine-like receptor 1 (Mm 41.38 kDa), platelet activating receptor (Mm 39.43 kDa); TYRO tyrosine kinase binding protein (Mm 11.61 kDa), activating macrophages and neutrophils, directly involved in immune response **(Xie et al., 2010; Huang et al., 2010;. Yang, Diehl and Roudebush, 2003; Yang et al., 2003; Yim et al., 2000).**

CONCLUSION

Studies of pigs spleens two-dimensional maps made it possible to establish proteins wide range presence — up to 600 fractions, of which 362 fractions are structural.

Detected fractions on the obtained maps are predominantly physiologically active, wherein their activity consists mainly in participation in various immunoregulatory reactions. Thus, in spleen samples there are found both compounds with immunoregulatory function (glycoprotein CD59, ATP-dependent RNA-helicase, LIM-domain of actin-binding protein), and factors involved in metabolism regulation (leptin receptor, transmembrane glycoprotein 3-hydroxy- 3-methylglutaryl-coenzyme A-reductase). It can be explaned by spleen biological role in organism and, as a result, a special structure – the division into red and white pulp and marginal zone, which produce compounds of various specificities.

This work is the first step to development of immunomodulating biologic based on animal raw materials for farm animals.

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