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Identification of tissue-specific proteins of immunocompetent organs of *Sus scrofa* isolated in deuterium depleted medium

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The results of studies on the identification of tissue-specific biomolecules of *Sus scrofa* organs isolated in deuterium depleted water are presented. Analysis of the fractional protein composition was carried out using one-dimensional electrophoresis in 12.5% SDS-PAGE in PAGE in a VE-10 chamber. Proteomic analysis of the protein-peptide composition was carried out using two-dimensional gel electrophoresis (DEF) of O'Farrell with isoelectro focusing in ampholin (IEF-PAGE). The detection of proteins on two-dimensional electrophoretograms was carried out by Coomassie R-250 staining and silver nitrate. Further, the corresponding sets of peptides were studied by MALDI-TOF MS and MS/MS on the Ultraflex time-of-flight mass spectrometer (Bruker, Germany). In addition, an analysis was made of the efficiency of producing tissue-specific biomolecules using two methods of fractionation, for which extracts were obtained from the immunocompetent organs *Sus scrofa* (thymus, spleen and mesenteric lymph nodes). Extraction was carried out in a medium with a modified isotopic composition. It has been proved that extraction of proteins, of the studied samples in a solution with a deuterium content low, provides an almost twofold increase in the amount of extracted proteins and peptides. Tissue-specific proteins, directly involved in the formation of innate immunity and responsible for the primary protective reaction of the organism, are revealed.

INTRODUCTION

Immunomodulating preparations of endogenous origin are represented mostly by monopreparations – immunoregulatory cytokines and peptides which are isolated from peripheral immunity organs, for example spleen and central organs of immunity (bone marrow and thymus), predominantly from cattle embryos or its young animals and embryos and young pigs. At the same time spleen contains big quantity of thymus-independent, macrophage and thymus-dependent immune factors, for example non-specific serum tuftsin tetrapeptide, which is included into Fd-fragment of IgG, which affects the functional properties of polymorphonuclear leukocytes and macrophages, components of the complement– C3 and C4-fragments, for example, C3b, factor P etc., which can be considered as basis for development and creation of wide range of medical drugs based on biologically active substances extracted from spleen of young farm animals. On this background we selected tissues of spleen, thymus, and mesenteric lymph nodes of pigs of 8-9 months old as source for biomolecules isolation.

Previously the immunocompetent properties of these protein fractions were researched in a variety of researches [1, 2]. Moreover, it was shown that the influence of protein-peptide compounds, extracted with deuterium water as solubilizer agent from mixture of *Sus scofa* thymus, spleen and lymph nodes extracts, was carried out on Wistar rats with cytostatic-induced immunodeficiency cyclophosphamide model. Intragastric administration of extract fraction with molecular weight more than 30 kDa, did not lead to significant changes. Thus, polypeptide compounds with molecular weight less than 30 kD, extracted from *Sus scofa* immune organs, significantly increased T-lymphocytes amount, affected macrophages system by T-helper (CD4) stimulation and activated cycle of complementary cascade. Differences between the experimental results in T-helper synthesis activation (CD4) in animals treated with the medium and low molecular weight fractions can be explained by the ability of biomolecules having a molecular weight less than 30 kDa (in particular, isolated from the spleen, which, in its cellular structure contains key regulatory factors) to change the speed of cell differentiation [3].

One more pathogenetic mechanism of cyclophosphan impact on body is the increase of free radicals production [4] along with its toxic effect, which also requires antioxidant correction for laboratory animals [5], including use of peptides of different molecular weight isolated in deuterium-depleted liquid medium [6, 7]. The previous researches showed high ability of isotope composition fluctuations in a body to influence the immunological reactivity and functional state of living systems [8-11].

All of the above-listed reasons served as basis for this scientific work, which purpose is to identify tissue-specific proteins of immunocompetent organs of *Sus scrofa* isolated in a deuterium-depleted

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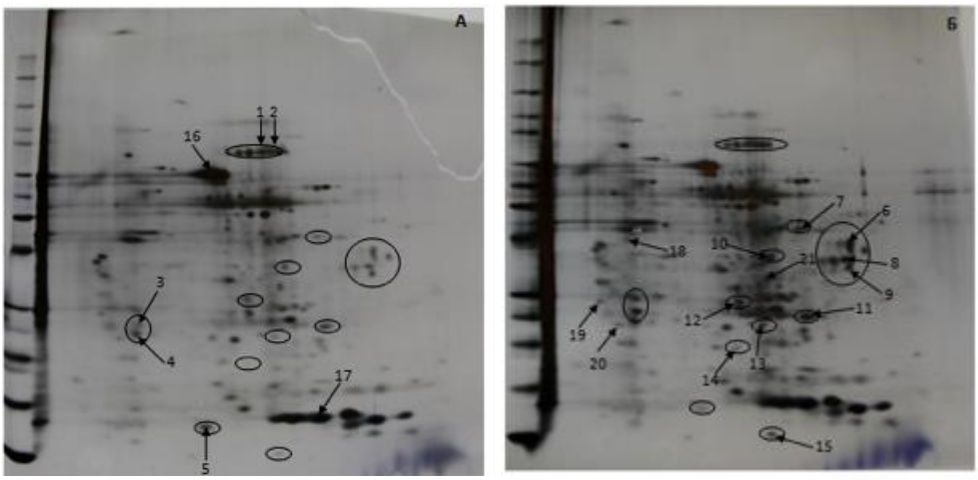

Figure 1 Results of electrophoretic analysis of proteins of extracts of immunocompetent organs of *Sus scrofa* based on distilled water (A) and IMIS (B) by the O'Farrell method, staining with silver nitrate. The numbers in the figure correspond to the Table 1

medium using the methods of two-dimensional electrophoresis and mass spectrometry.

MATERIALS AND METHODS

Within the framework of this scientific work the researches were carried out to identify tissue-specific biomolecules. Analysis of proteins fractional composition in test samples was carried out by the Lemmy method: one-dimensional electrophoresis in 12.5% SDS-PAGE in a PAAG in VE-10 chamber (Helicon, USA) under constant voltage of 160V. When the front reached the separating gel, the voltage was increased to 180V and continued the separation for 4-5 hours. The amount of protein added into the gel was 20 μg for all samples. As electrophoresis standard a marker (Thermo, USA) was used, which marker is a mixture of 11 recombinant proteins (250, 150, 100, 70, 50, 40, 30, 20, 15, 10 and 5 kDa). Staining was performed by Coomassie G-250 dye.

Proteomic analysis of the protein-peptide composition was performed by using two-dimensional gel electrophoresis (DEP) by O'Farrell with isoelectric point electrophoresis focusing in ampholine (IEF-PAGE). The detection of proteins on two-dimensional electrophoregrams was made by Coomassie R-250 staining and nitrate of silver. For computer densitometry two-dimensional electrophoregrams were used in wet state [12]. Its complete digital images and / or images of separate fragments were obtained by scanning on EpsonExpression 1680 scanner. The obtained digital images were edited in a graphic editor and the quantitative content of proteins was counted using the software package ImageMaster 2D Platinum version 7 (GE Healthcare, Switzerland). When determining the amount of protein tehre were used at least 3 electrophoregrams with equal application. The range of values of optical density was no more than ± 1.5%.

To identify proteins some separate fractions were excised from DEP, the excised fragments were cut and trypsinolysis was performed as it was described previously. Then the corresponding sets of peptides were studied by MALDI-TOF MS and MS / MS mass spectrometry on device MALDI, the Ultraflex time-of-flight mass spectrometer (Bruker, Germany) with UV laser (336 nm) in the positive-ion mode within range of masses 500-8000 Da with its calibration according to known peaks of trypsin autolysis [13]. The obtained mass spectra ("peptide fingerprints") were deciphered using traditional bioinformatic technologies.

The analysis of obtained mass spectra of the tryptic peptides was performed using the Mascot software, the Peptide Fingerprint option (MatrixScience, USA) with accuracy of determination of MH + mass equal to 0.01%, searching through databases of the National Center for Biotechnological Information of the USA (NCBI). Comparative analysis of the proteome profiles of presented samples the information modules UniProt and PepBank were used. Further, we analyzed the efficiency of obtaining the tissue-specific biomolecules using two methods of fractionation.

With this purpose the extracts were obtained from the immunocompetent organs of *Sus scrofa* (thymus, spleen and mesenteric lymph nodes). Extraction was carried out using water as extragent with modified isotopic composition of IMIS (IMIS, D/H = 50 ppm). The concentration of deuterium in water was monitored by NMR of Fourier spectrometer JNM-ECA 400 [14, 15]. Fractionation of protein-peptide complexes was performed by the stepwise ultrafiltration method or by gel filtration. Ultrafiltration was carried out at the device (Vlidisart, Russia) by using VivaFlow200 filter modules (Sartorius, Germany). Gel filtration was performed on Sephadex G-100.

RESULTS AND DISCUSSION

It is shown that the main protein components of extracts are only 8 fractions, which according to results of computerized densitometry, contain from 11 to 20% of the total protein material visually detected on DEP when being stained by SVV-250 dye (figure 1).

It was defined that extraction of studied proteins into solution with reduced deuterium content provides almost twofold increase of amount of extracted proteins and peptides, which can be caused by change in ionic strength of the solution when the amount of deuterium changes.

The DEP-detectable proteins were representatives of families of α and β hemoglobin chains, and only in one case the presence and profilin 1 was found (actin-binding protein, which is present in many types of cell). Moreover, the hemoglobins fractions turned out to be post-translationally modified. In fraction of β chains of hemoglobin Deamination of amino acid residues (Q) of glutamine or (N) asparagine was observed, and its combination was different for different fractions. In case of fractions of α chains of hemoglobin, another modification of Lys-Alllysine (lysine oxidation to aminoadipic semialdehyde) was found - with formation of allysin aldehyde. The catabolism of L-lysine starts

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Table 1 List of protein fractions in extracts of immunocompetent organs *Sus scrofa* identified by mass spectrometry (MALDI-TOF MS and MS / MS).

№	Protein; some synonyms, (gene symbol)	Numbers in Protein NCBI	Mm/pI (exper.)	Mm/pI (calcul.)
1	Transferrin (*TF*)	833800	77.0/7.80	77.0/6.73
2	Transferrin (*TF*)	833800	77.0/7.80	77.0/6.73
3	rho GDP-dissociation inhibitor 1 (*ARHGDIA*)+ Acetyl (Protein N-term)	31940195	25.0/5.15	23.4/5.12
4	rho GDP-dissociation inhibitor 2 (*ARHGDIB*)	346716314	24.0/5.10	22.8/5.08
5	Cystatin-B (*CSTB*) + Acetyl (Protein N-term)	2494023	11.5/6.00	11.1/5.87
6	Homolog fructose-bisphosphate aldolase A [Bostaurus] (*ALDOA*)	156120479	39,0/8,40	39,4/8,45
7	phosphoglyceratekinase 1 (*PGK1*)	153792027	44.0/8.00	44.5/8.02
8	glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	329744642	36.0/8.40	35.8/8.51
9	malate dehydrogenase, mitochondrial (*MDH2*)	346421415	36.5/8.35	35.6/8.93
10	glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	329744642	36.5/7.90	35.8/8.5
11	flavin reductase (*NADPH*) (*BLVRB*) + Acetyl (Protein N-term)	335289705	22.0/8.00	22.2/6.35
12	glutathione S-transferase P (*LOC100739163*)	927100908	22.5/7.40	23.7/7.66
13	phosphoglyceratemutase 1 isoform 1 (*PGAM1*)	350592969	24.0/6.70	28.8/6.77
14	rho GDP-dissociation inhibitor 2 (*ARHGDIB*)	346716314	19.0/6.30	22.8/5.08
15	Homolog Ubiquitin [Camelus dromedaries etc.]	51701999	9.0/6.60	8.60/6.56
16	Albumin (*ALB*)	833798	69.0/6.10	69.4/5.92
17	hemoglobinsubunitbeta (*HBB*)	261245058	14.5/8.00	16.2/7.10
18	serpin B9 (*SERPINB9*)	162139836	41.5/5.10	42.5/5.37
19	Homolog proteasome subunit alpha type-5 [Leptonychotesweddellii] (*PSMA5*)	58519750	28.0/4.65	25.6/4.65
20	Homolog translationally-controlled tumor protein isoform 2 [Homo sapiens] (*TPT1*)	4507669	22.0/4.95	19.6/4.84
21	S-formylglutathionehydrolase (*ESD*)	47522936	30.5/7.70	31.5/6.54

from condensation of lysine with ketoglutaric acid, followed by reduction of the Schiff base formed with participation of dehydrogenase in sugaropine.

The combinations of positions of modified amino acid residues (a.r.) of lysine (K) also differed in different fractions. This effect can be caused by increased content in immunocompetent organs of enzymes value that cause these types of modification or changes in the physicochemical properties of the medium.

In Uniprot database there is no information on post-translational modifications of hemoglobines of *Sus scrofa* [ref. to records P02067 (HBB_PIG) and P01965 (HBA_PIG)]. There is indicated only a set of supposed variants of post-translational modifications of other types. In body of a human, as the most studied object [ref. to records P68871 (HBB_HUMAN) and P69905 (HBA_HUMAN)], some posttranslational modifications were confirmed experimentally, but also of the other types. The obtained results showed that in the extracts of immunocompetent organs the non-traditional hemoglobin modification is induced, proceeding along different variants of the a.r., and, consequently, biologically active peptides with different properties can be formed. The role of such modifications for functional peptides is completely unclear and requires further research.

Deamination of the amino acid residues of glutamine (Q) or asparagine (N) is referred to the non-enzymatic cleavage of ammonia from the amide group of asparagine or glutamine through formation of intermediate product – cyclic imide. The rate of this product formation is determined by the local amino acid environment, the properties of solution (pH and composition). The asparagine residue in proteins is deaminated 40 times more often than the glutamine residue, while the asparagine deamination rate is 100 times faster than the deamination rate of glutamine, which was also observed in the extracts where deamination of asparagine was detected from 4 to 7 a.r., and glutamine deamination was from 1 to 4 a.r.

The deamination reaction results to creation of additional ionizable carboxyl group with negative charge at physiological values of pH, which negative charge changes the total charge of the protein and its spatial structure, as was observed in this case, where the HBB (NVV) fractions were shifted to acidic side in reference to pI depending on amount of N / Q modified residues. Deamination is often associated with subsequent transamination (the interaction of the ε-amino group of the lysine residue in protein with the side chain of glutamine residue in the same protein), which is one of the types of cross-links characteristic of post-translational modification.

Modification of Lys-Alllysine (formation of allysine aldehyde) is carried out by extracellular lysyloxidase in presence of variety of vitamins, it is usually associated with the maturation of collagen. But in this case, under *in vitro* conditions, it happened targetedly with the fractions of α chains of hemoglobin, which shows certain specificity of proteins modification of immunocompetent organs in *in vitro* conditions.

When studying extracts of immunocompetent organs at the level of short peptides spectrum, no significant differences were found. It was possible to construct an amino acid sequence and to identify by two tryptic peptides present in short peptides spectra.

In its amino acid sequence these peptides turned out to be fragments of α-hemoglobin and α-thymosin. The presence of peptide of hemoglobin derivative is caused by use of the spleen tissues, as spleen is involved in destruction of red blood cells, which leads to a significant increase in the products of its degradation in the extract. The fact of its discovery in the extracts of immunocompetent organs of the α-thymosin

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peptide is interesting - it was first discovered in our studies at peptide level.

With the method of proteomic analysis, the maximum density of potential biomolecules identified by nitrate of silver within the range less than 30 kDa was established. 20 protein fragments were mass-spectometrically identified (table 1).

Tissue-specific proteins (Table 1) were found, which are directly involved in the formation of innate immunity and responsible for the primary defense response of the organism: interferon beta protein, signal peptide with antiviral and immunomodulating activity; gamma interferon inducible lysosomal thiol reductase, the main enzyme that affects the antigen presentation of macrophages; myeloid differentiating protein of primary immune response, cytosolic adapter protein; pro-inflammatory cytokine interleukin-12 beta subunit; annexin A1, one of the signaling molecules that activates immunosuppressive, anti-inflammatory, antiallergic effects; antigen of the main complex of histo-compatibility of the 1st class, involved in the recognition of foreign body and development of immune response; signaling immune peptide chemokine-like receptor 1. Also, the presence of compounds that participate in the secondary immune response and responsible for adaptation processes in a body was found: transferrin, that influences hematopoiesis and plays an important role in cell division; phosphoglycerate mutase B; inhibitor of dissociation Rho GTPase involved in activation of oxygen of superoxide-generating NADP oxidase of phagocytes; glutathione S-transferase, which is one of the most important means of protection from toxins; serpin B9, which controls the processes of coagulation and inflammation. Range of proteins involved in metabolism-glyceraldehyde-3-phosphate dehydrogenase, cystatin-B malate dehydrogenase, aldolase, was identified.

CONCLUSION

Thus the obtained results show that use of fragmented cells of immunocompetent pig organs isolated in a medium with a modified D / H composition as base matrix, allows creating an innovative immunostimulating active complex that corresponds to the concept of environmentally safe sustainable industrial development at the expense of reduction of consumption and the production of abiotic components of the environment and improving the quality and safety of livestock products.

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Authors' contributions

Lilia Fedulova – conducting an experiment, writing an article
Anna Elkina – writing an article, translation of article
Ekaterina Vasilevskaya – conducting an experiment
Ekaterina Barysheva – writing an article

Conflict of interests

All Authors declare that:
- The manuscript is original work of author. All data, tables, figures, etc. used in the manuscript are prepared originally by authors, otherwise the sources are cited and reprint permission is attached.
- The manuscript has not been and will not be published elsewhere or submitted elsewhere for publication.
- Authors mention that there is no conflict of interest in this study.

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