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COMPARATIVE STUDY OF BIOCORRECTIVE PROTEIN-PEPTIDE AGENT TO IMPROVE QUALITY AND SAFETY OF LIVESTOCK PRODUCTS

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ABSTRACT

Water with modified isotopic composition (D/H = 40 ppm) as a solubilizing agent for biologically active substances extraction from immune organs *Sus Scrofa* increases protein yield in thymus to 20%, in spleen up to 38%, mesenteric lymph nodes up to 35% in comparison with distilled water (D/H = 150 ppm). It was found a significant amount of neurotransmitter amino acids, such as aspartic and glutamic acids, glycine (thymus – 10.5; 13.7; 7.6%; spleen – 12.2; 10.7; 7.8% lymph nodes – 11.0; 13.3; 11.1%, respectively) having the immunological and adaptogenic activity, in extracts of *Sus scrofa* immunocompetent organs (thymus, spleen, mesenteric lymph nodes). Application of water with modified isotopic composition as solubilizing agent (D / H = 40 ppm) for extraction of immunocompetent organs' biologically active compounds led to increased amino acid content, including hydrophilic amino acids, in thymus extracts – up to 22%, in spleen extracts – up to 15% , in lymph nodes exctracts – up to 8%, in comparison to distilled water (D / H = 150 ppm). Peptide profile analysis revealed positive effect of water (D/H = 40 ppm) on a quality protein content extracts in molecular weight range from 10 to 20 kDa and from 30 to 45 kDa and peptide composition (2 kDa), at the same time quantitative content of compounds adaptogenic and immunocorrective action increased. Reducing of deuterium content in the solubilizing agent enhances quantitative amino acid content, i.e. extraction in an aqueous- salt solution based on deuterium depleted water of animal tissues with a high content of amino acids with hydrophilic radicals proceeded more completely.

Keywords: immunocorrection, adaptogen, deuterium depleted water, peptide profole, amino acid

INTRODUCTION

Nowadays, problem of the uncontrolled antibiotic non-medical application causes a sharp challenge because feed antibiotics as growth promoters lead to evolution, selection and accumulation of antibiotic-resistant strains of pathogenic and opportunistic agents in farms **(Sokolov, 2015)**.

Recent studies indicate that antibiotics subinhibitory concentration increases quantity of resistant bacteria by horizontal gene transfer, as well as due to reactive oxygen species stimulation that contribute to the growth of mutant microorganisms with poly-drug resistance **(Khaitov, 1993)**.

These pathogens can be transmitted from livestock to humans through contaminated environment and food products, mainly meat and milk, causing infectious diseases, which treatment is carried out by analogous already inefficient, synthetic antimicrobial agents, resulting in increased doses of medication, the recovery period of the organism and, finally, economic cost **(Carlet, Ben and Chalfin, 2004)**. Thus, the reduction of irrational use of antibiotics in agriculture through the development and implementation of natural antimicrobial and immunomodulating agents, is highly relevant **(Chernukha et al., 2015; Hawkey, 1998)**.

Currently, most of the biotechnology research aimed at obtaining and study of biologically active substances from animal products. Thus, processing secondary raw materials in the food industry presently hardly exceeds 20% by mass formed, despite the fact that bulk of the "waste" for disposal legislation prohibits **(Roca et al., 2015)**. In addition to the environmental aspect, in particular, the reduction of anthropogenic impacts on the environment due to waste recycling, the practical importance is the use of secondary products to create food and additives, high-value pet food, medical and cosmetic products and medicines **(Perron et al., 2015)**.

Natural veterinary products industry with high biological activity based on animal raw for farm animals is on the stage of development in Russia. This is a very perspective and dynamically developing direction in the field of deep processing of secondary raw materials. Moreover, meat industry has a great potential for its implementation due to the diverse and unique composition of the main and

secondary products of the industrial slaughter of animals **(Kim and Yung, 2015)**. Ongoing research suggests the development of veterinary drugs manufacturing technology and isolating bioactive compounds from low-value raw materials of animal origin, which may result in higher quality livestock products **(Dzhimak et al., 2014)**. The scientific concept of innate immunity origin confirms the fact that survival possibility of any species including humans under the environment abundant in potentially pathogenic microorganisms is evolutionary process of mechanisms development ensuring resistance to infections **(Kokryakov, 1999)**. Resistance (immunity) is divided into the general and local, innate and adaptive one. Reactions providing resistance are divided into antibacterial and antitoxic **(Pozdeev, 2004)**. Immunemodulating bioactive substances extracting from endocrine glands, thymus and spleen of reindeers featuring have been shown to possess a strong stimulating impact on main characteristics of the immune system **(Vladimirov, 2001, Matveev, 2001, Lebedeva and Jamsaranova, 2004, Bondarenko and Bezborodov, 2009)**. Extracts of pigs immune organs potentially contain large amounts of protein and peptide compounds that are actively involved in immune defense reactions of the organism **(Vasilevskaya and Fedulova, 2015)**.

We suppose that water with modified D/H isotopic composition (WMIC) can be used for optimization the biocatalysis process as a solubilizing agent and also it can lead to significant quantitative and qualitative changes in the composition of protein-peptide extracts.

The aim of the study was investigation of bioactive components contained in water-salt extracts obtained from Sus scrofa immune organs involed in immune system functioning.

MATERIAL AND METHODOLOGY

The subjects was water-salt extracts obtained from *Sus Scrofa* organs (spleen tissue extracts; thymus tissue extracts; extracts of mesenteric lymph nodes and a combined mixtures of the extracts) based on distilled water (DW) and water with modified D/H composition (WMIC).

Sample preparation

Sample preparation consisted in the separation of organs from related tissues, grinding to a particle size of 3 ±1 mm, extraction physiological solutions DW and WMIC (Hydromoduls 1 : 5, speed 400 rev / min for 3 hours) at 4 °C in laboratory dispersing system (Laboteks, Russia). Extracts sampling for determination of protein concentration was carried out punctually, prior to extraction, and then every 15 minutes. After extraction finished samples were centrifuged in a centrifuge CM-6M (ELMI, Latvia) at 3500 rev / min for 5 min, supernatant was collected. Protein concentration was determined in a photometer BioChem SA (HTI, USA) by biuret method. The mathematical treatment of the data including calculation of averages with standard errors (M ±m) was carried out.

Amino acid composition

The study of the amino acid composition was carried out on “BiotronikLC-2000” amino acid analyser (Germany). Amino acids determination was carried out in automatic ion exchange resin “DC-6A” (USA) analytical column using three sodium citrate buffer system (Sigma-Aldrich, USA): buffer A – 0.18 M, pH 3.25; buffer B – 0.3 M, pH 3.9; buffer C – 1.6 M, pH 4.75. High of the resin in the column was 22 cm, buffer solution flow rate - 32 $mL.h^{-1}$. Ninhydrin reaction was used for amino acids detection, ninhydrin flow rate was 20 $mL.h^{-1}$.

Electophoresis

One-dimensional (1D) electrophoresis was performed according to the method of Laemmli (Laemmli U.K., 1970) under denaturing conditions in 12.5% polyacrylamide gel with the presence of SDS. The marker was used comprising of eleven standards (recombinant proteins) “Thremo” (USA).

Two-dimensional (2D) electrophoresis was performed according to the method of O'Farrell **(O'Farrell, 1975)** with isoelectric focusing in ampholine pH gradient (IEF-PAGE). The subsequent detection of the proteins was carried out by staining with Coomassie Brilliant Blue R-250. Identification of protein fractions was performed on DE after trypsinolysis by MALDI-TOF/MS and MS/MS mass spectrometry on Ultraflex MALDI-TOF mass spectrometer (Bruker, Germany) with UV laser (336 nm) in the positive ion mode in molecular weight range of 500 – 8000 Da with calibration according to known peaks of trypsin autolysis. Analysis of obtained tryptic peptides mass spectra was performed using Peptide Fingerprint option in Mascot software (Matrix Science, USA) with MH+ mass determination accuracy of 0.01%; search was performed in databases of the National Center for Biotechnology Information, USA (NCBI).

RESULTS AND DISCUSSION

It was shown solubilizing agent effect on extraction time and protein content in extracts (Table 1). While spleen extraction, protein content maximum was reached in 75 minutes, and obtained in WMIC solution 25.5 ±0.2 $g.L^{-1}$, in DW solution – by 20 % lower (20.4 ±0.4 $g.L^{-1}$). While thymus extraction with WMIC solution, maximum protein concentration obtained 18.9 ±0.2 $g.L^{-1}$ in 30 min, whereas while extraction with DW solution maximum protein content was by 38% lower (11.7 ±0.3 $g.L^{-1}$) in 45 min. While lymph nodes extraction in WMIC solution maximum protein concentration obtained 19.5 ±0.47 $g.L^{-1}$ in 75 min, in solution based on DW protein level peaked at 45 minutes and was by 35% lower (12.6 ±0.38 $g.L^{-1}$). Protein content difference between "zero samples" of respective extracts with WMIC and DW before the experiment did not exceed 0.1 $g.L^{-1}$.

It revealed a high content of amino acids in the extract of the lymph nodes based on WMIC. All extracts showed a significant amount of neurotransmitter amino acids such as aspartic and glutamic acid, glycine (thymus – 10.5; 13.7; 7.6%; spleen – 12.2; 10.7; 7.8%; lymph nodes – 11.0; 13.3; 11.1%, respectively) having the immunological and adaptogenic activity. Thus, it was detected high content of arginine and lysine residues involved in the normalization of the immune response; tyrosine and threonine adaptogenic mechanisms involved in stress leveling and depressive reaction; serine, alanine, valine involved in the

Table 1 Protein content in extracts, (g.L^{-1} ±SD).

Time, min	Spleen		Thymus		Lymph Nodes	
	WMIC	DW	WMIC	DW	WMIC	DW
0	3.77 ±0.23	3.75 ±0.30	4.44 ±0.27	4.46 ±0.26	6.69 ±0.09	6.58 ±0.16
15	22.10 ±0.30	18.00 ±0.41	17.70 ±0.21	10.30 ±0.58	16.00 ±0.73	11.70 ±0.37
30	23.30 ±0.13	18.10 ±0.19	**18.90 ±0.15**	10.80 ±0.10	18.60 ±0.14	11.90 ±0.12
45	24.40 ±0.41	19.50 ±0.43	18.10 ±0.32	**11.70 ±0.25**	19.10 ±0.67	**12.60 ±0.38**
60	23.80 ±0.58	20.30 ±0.43	17.60 ±0.43	11.00 ±0.64	19.00 ±0.56	11.00 ±0.23
75	**25.50 ±0.15**	**20.40 ±0.40**	17.50 ±0.70	11.10 ±0.11	**19.50 ±0.47**	11.70 ±0.80
90	24.00 ±0.63	19.50 ±0.54	17.30 ±0.61	11.00 ±0.76	19.50 ±0.20	11.50 ±0.67
120	24.10 ±0.24	20.10 ±0.17	17.50 ±0.43	11.20 ±0.57	19.40 ±0.16	11.60 ±0.46

formation of active sites of several enzymes and isoleucine, leucine and proline involved in energy metabolism.

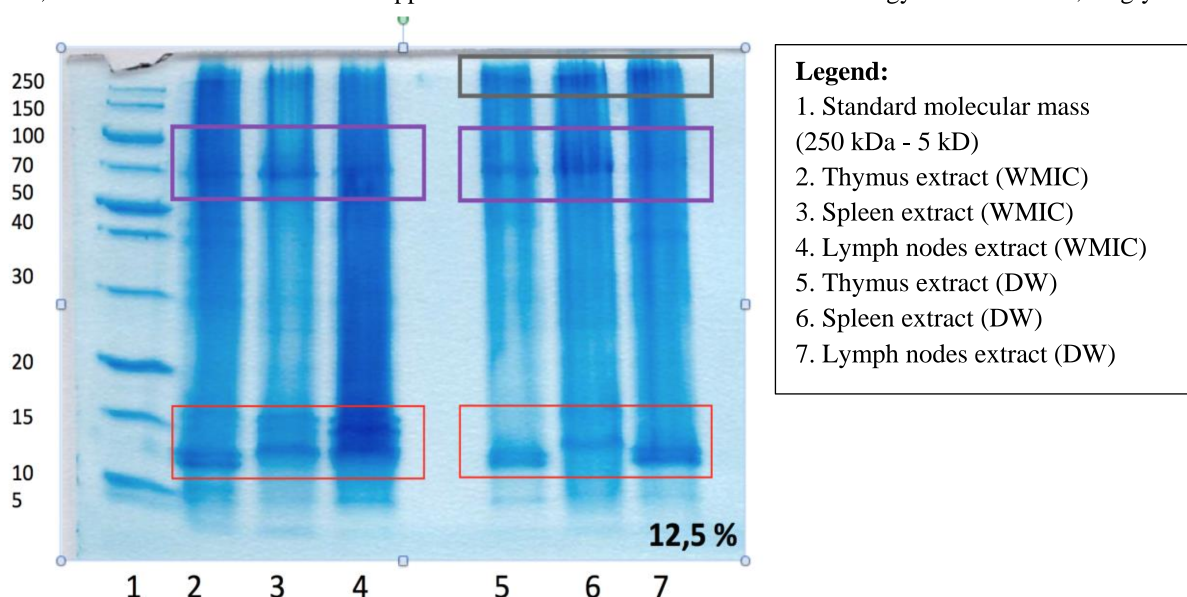
It was shown expressed increase of quantitative hydrophilic amino acids content in WMIC extracts in relation to DW extracts: thymus extract – up to 22%, spleen extracts – up to 15%, lymph nodes extract – up to 8%, respectively. This difference in the amino acid content of extracts with different isotopic D/H composition demonstrates positive impact of reduced deuterium content of the solubilizer in the extraction process in an water-salt solution.

The analysis of the protein-peptide profile by 1D electrophoresis revealed qualitative differences between WMIc and DW extracts (Figure 1). The tracks of the thymus extracts (2,5) found qualitative differences in the area of 10 kDa – 3 additional bands observed in the WMIC extract; in the range from 30 to 50 kDa protein contains two new fractions. We can expect that low-molecular weight fraction of thymus extract (WMIC) contain thymosin beta-10, capable of activating wound healing with protective effect (4.9 kDa) **(Kim and Jung, 2015)**; annexin A1 (38.8 kDa), involved in the innate immune response, like glucocorticoids effector and inflammatory processes regulator **(Gao, Li and Yan, 1999)**.

The spleen extracts track (3), based on WMIC solution were detected two additional bands – in area from 12 to 15 kDa; also there were observed appearance of the peptide pool in the area below 5 kDa. Presumably, the proteins in the 12 – 13 kD are antigen MHC-1 (12.7 kDa) **(Koopmann, Albring and Hüter, 2010)**, beta-2-microglobulin (13.4 kDa), involved in the presentation of peptide antigens in the immune system **(Huang W.C., 2008)** and C-1 lysozyme (14.7 kD), with antibacterial properties **(Kajla et al., 2011)**.

In lymph nodes extracts (tracks 4,7) were identified differences in area up to 15 kDa – significant intensification of the protein bands in the area of 12 – 100 kDa (WMIC extract), appearance of a protein band in area of 15 – 20 kDa. Probably identified set of bands correspond interleukins: IL-2 (15.2 kDa), interleukin 15 (18.4 kDa), interleukin 6 (21.1 kDa), interleukin 23 subgroups 19 (21.13 kDa), which are directly involved in the immune response **(Liao, Lin and Leonard, 2011; DePaolo et al., 2011; Heinrich et al., 2003; Yen et al., 2006)**.

Proteomic study (2D electrophoresis) revealed in a mixture of extracts based WMIC (thymus, spleen and lymph nodes extracts, 1:1:1) tissue-specific proteins: transferrins, affecting blood formation and plays an important role in the division of cells involved in innate immunity provision; dissociation inhibitor of Rho GTPases, involved in activation process of oxygen superoxide-generating NADPH oxidase of phagocytes; cystatin-B, involved in intracellular proteolysis; aldolase, involved in energy metabolism; glyceraldehyde



Legend:
1. Standard molecular mass (250 kDa - 5 kD)
2. Thymus extract (WMIC)
3. Spleen extract (WMIC)
4. Lymph nodes extract (WMIC)
5. Thymus extract (DW)
6. Spleen extract (DW)
7. Lymph nodes extract (DW)

Figure 1 1D electrophoresis of WMIC and DW extracts.

Table 2 The results of mass spectrometric identification (MALDI-TOF/MS and MS/MS) of protein fractions in complex extracts.

Protein name; (Gene symbol)	S / M/ C *	mM/pI** (calculation)
Transferrin (TF)	143/27/39	77.0/6.73
Transferrin (TF)	305/35/53	77.0/6.73
rho GDP-dissociation inhibitor 1 (ARHGDIA) +Acetyl (Protein N-term)	179/14/71	23.4/5.12
rho GDP-dissociation inhibitor 2 (ARHGDIB	141/5/43	22.8/5.08
Cystatin-B (CSTB) +Acetyl (Protein N-term)	140/9/94	11.1/5.87
Homologue fructose-bisphosphate aldolase A (ALDOA)	299/29/67	39.4/8.45
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2)	108/20/63	35.8/8.51
Malate dehydrogenase, mitochondrial (MDH2)	274/27/66	35.6/8.93
Glutathione S-transferase P (LOC100739163)	193/20/61	23.7/7.66
Phosphoglycerate mutase 1 isoform 1 (PGAM1)	352/30/87	28.8/6.77
Serpin B9 (SERPINB9)	119/13/35	42.5/5.37

Note: *S/M/C – traditional identification indicators adopted in the English literature: Score – indicator of conformity or "scorecard"; Match peptides –the number of matched peptides; Coverage – % coverage of the entire amino acid sequence of the protein by identified peptides.
**mM/pI (calculation) – estimates made based on amino acid sequence data with consideration of signal peptide removal, but with no consideration of other post-synthetic modifications using the ExPASy Compute pI/Mw tool software.

3-phosphate dehydrogenase, involved in activation transcription initiation apoptosis axonal transport processes; malate dehydrogenase catalyzed the last step of Krebs cycle; glutathione S-transferraza, one of the most important defenses against toxins; phosphoglycerate mutase B, participated in adaptation processes; serpin B9, controlled a plurality of biological processes, including inflammation and coagulation; as well as several proteins involved in the metabolism (Table 2).

CONCLUSION

Obtained data revealed that water-salt extracts obtained from *Sus scrofa* organs with WMIC as solubilizing agent, can lead to significant quantitative and qualitative changes in the composition of protein-peptide extracts.

WMIC as a solubilizing agent affects the qualitative and quantitative composition of the protein in extracts from 10 to 20 kDa and in the molecular weight range of 30 to 45 kDa. Physico-chemical interactions during the extraction of WMIC don’t affect macromolecular compounds, thus it is affecten on selection of proteins and peptides that are directly or indirectly involved in the immune response.

Perspective material for further research are protein-peptide fractions obtained with WMIC, characterized by the presence of nonspecific immunity stimulant and adaptogenic immunocorrective action.

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