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BIOLOGICALLY ACTIVE COMPONENTS OF ANTLERS EXTRACTS *(CERVUS NIPPON)* AND RED DEER *(CERVUS ELAPHUS)* PEPTIDE-PROTEIN NATURE

Vladislav N. Karkischenko, Maxim S. Dulya*, Daniil V. Khvostov, Ruslan A. Ageldinov, Stanislav L. Lyublinskiy, Nikolay N. Karkischenko

Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia 143442, Russian Federation, Moscow region, Krasnogorsk district, Settlement Svetlye Gory, building 1

A complex study of the protein composition of the biologically active components of the extracts of the velvet deer antlers (VDA, include *Cervus nippon* and *Cervus elaphus)* using two-dimensional gel electrophoresis (2D-PAGE), size-exclusion chromatography (SEC), and peptide mapping with high-performance liquid chromatography with mass spectrometric detection (HPLC-MS) with the use of fermentation by trypsin was done. A molecular-mass distribution characteristic for protein extracts of VDA has been established. Optimal conditions for extraction, chromatographic separation and relative quantitative determination of the main components have been determined. The results of the identification of the most significant (major and minor) protein components in the extracts of the studied objects are described in detail in accordance with the algorithm of the search program Spectrum Mill MS Proteomics Workbench and the protein database Uniprot. The data of protein profiling are clustered according to molecular and biological functions. The connections of the identified proteins with possible mechanisms of biological action and targets, which can be affected by the protein components of the studied objects, are presented. Based on the results of the study, conclusions about the multicomponent protein composition of extracts of VDA were drawn. The marker protein components in the studied extracts are suggested and the possible interrelationships of the detected proteins in the extracts with biological effects are indicated.

Keywords: 2D-PAGE, HPLC-MS, IEF, peptide mapping, SEC, VDA
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List of abbreviations: VDA — velvet deer antlers, 2D-PAGE — two-dimensional polyacrylamide gel electrophoresis, SEC — size-exclusion chromatography, HPLC-MS — high-performance liquid chromatography with mass spectrometric detection, IEF — isoelectric focusing, RP — regulatory peptides, SDS-PAGE — sodium dodecyl sulfate polyacrylamide gel electrophoresis, PPARGC1A — peroxisome proliferator-activated receptor gamma coactivator 1-alpha, Wnt — acronym from “Wingless/Integrated”, CNR1 — cannabinoid Receptor 1, PFL — pyruvate formate-lyase.

БИОЛОГИЧЕСКИ АКТИВНЫЕ КОМПОНЕНТЫ ЭКСТРАКТОВ ПАНТОВ ПЯТНИСТОГО И БЛАГОРОДНОГО ОЛЕНЕЙ ПЕПТИДНО-БЕЛКОВОЙ ПРИРОДЫ

В.Н. Каркищенко, М.С. Дуля*, Д.В. Хвостов, Р.А. Агельдинов, С.Л. Люблинский, Н.Н. Каркищенко

*ФГБУН «Научный центр биомедицинских технологий
Федерального медико-биологического агентства России»
143442, Российская Федерация, Московская обл., Красногорский р-н, п. Светлые горы, владение 1*

Проведены исследования белкового состава биологически активных компонентов экстрактов пантов пятнистого и благородного оленей (ППО и ПБО) методами двумерного гель-электрофореза (2D ПААГ), гель-размерной эксклюзионной хроматографии (ГРХ) и пептидным картированием методом высокоэффективной жидкостной хроматографии с масс-спектрометрическим детектированием (ВЭЖХ-МС) высокого разрешения с применением ферментативного расщепления трипсином. Установлено молекулярно-массовое распределение, характерное для белковых экстрактов ППО. Найдены оптимальные условия экстракции, хроматографического разделения и относительного количественного определения главных компонентов. Подробно представлены результаты идентификации наиболее значимых (мажорных и минорных) белковых компонентов в экстрактах изучаемых объектов в соответствии с алгоритмом поисковой программы Spectrum Mill MS Proteomics Workbench и белковой базой данных Uniprot. Данные белкового профилирования кластеризованы по молекулярным и биологическим функциям. Отображены связи идентифицированных белков с возможными механизмами биологического действия и мишенями, на которые могут оказывать воздействие белковые компоненты изучаемых объектов. По результатам исследования сделаны выводы о многокомпонентности белкового состава экстрактов ППО, содержащие перспективные для более детального изучения компоненты. Предложены маркерные белковые компоненты в составе изучаемых экстрактов и указаны возможные взаимосвязи обнаруженных белков в составе экстрактов с биологическими эффектами.

Ключевые слова: 2D ПААГ, ВЭЖХ-МС, ГРХ, ИЭФ, пептидное картирование, ППО
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Список сокращений: ППО — панты пятнистого оленя, 2D ПААГ — двумерный гель-электрофорез, ГРХ — гель-размерная эксклюзионная хроматография, ВЭЖХ-МС — высокоэффективная жидкостная хроматография с масс-спектрометрическим детектированием, ИЭФ — изоэлектрическая фокусировка, РП — регуляторные пептиды, SDS-PAGE — электрофорез белков в полиакриламидном геле, PPARGC1A — рецептор пероксисом, активируемый пролифератором гамма-коактиватор 1-альфа, Wnt — акроним от «Wingless/Integrated», CNR1 — рецептор каннабиноидов 1-го типа, PFL — пируват формиат лиаза.

Introduction

VDA is a part of the body, shaped like horns, but consisting of spongy cartilaginous tissue of *Cervus Nippon.* They are penetrated by a large number of blood vessels and have an intensive blood supply. When antlers reach the size of normal horns (on the 120–150th day from the beginning of growth), the process of their ossification begins. It is believed that during this period they are most biologically significant. Blood taken from a vein of an animal at the time of cutting also has activity similar to VDA. The expressed therapeutic properties of VDA are justified by the fact that during the growth of the horns of a young deer, his body actually produces up to 25 kg of bone tissue. Such growth rates are characteristic only for this family. This process requires strong support from all functional systems of the body and high concentrations of substances of a regulatory and protective nature, accordingly.

The use of drugs based on VDA and their blood in traditional medicine has more than two thousand years' history and is known in the past among many nations of the world. However, it is most widely spread as a branch of traditional medicine, and as a culture of maintaining a healthy lifestyle in China, the countries of Southeast Asia, Tibet and, to a lesser extent, among the peoples of southern Siberia and the Far North. At the beginning of XII, in China, for the first time, deer began to be domesticated in order to obtain VDA and their blood for the preparation of medicines [3].

It is known that medical preparations containing VDA products in their composition include a large number of substances of a regulatory nature: kinins, amino acids, phospholipids, growth factors involved in maintaining the activity and stability of the processes of anabolism and catabolism in the body, regulating the processes of inflammation, regeneration and immunity. The main functional difference between VDA and plant adaptogens is that the latter increase the body's resistance, limiting the harmful effects of external factors, while the first drugs increase the capacity of the body's own resistance [3].

Structural and functional information about peptides of plant and animal origin is important for system biology. It can also be used in various application areas: in agriculture, in the manufacture of dietary products, pharmaceuticals, functional foods [1, 6].

The relevance of research

Modern tools of peptidomics and proteomics in biomedical research are aimed at analyzing and developing new active substances, drugs, searching for indicator peptides or proteins and their identification, as well as developing algorithms for identifying and subsequently using effective combinations of various substances of protein-peptide nature for the diagnosis, effective therapies and their use as functional foods [1, 6].

It is paradoxical that, despite the long period of use of extracts and by-products from the tissues of deer-like animals, the composition of their active components remains poorly understood, components associated with a wide spectrum of biological activity of the studied tissues are not reliably identified.

It is known that the composition of extracts from VDA includes fatty acids, waxes, aromatic and steroid compounds, cholesterol esters, as well as micro and macronutrients, free amino acids, oligo and polysaccharides, derivatives of the uracil series, the xanthine group.

Within the framework of the research cycle, the authors' special attention is attracted by the peptide-protein composition of the tissues under the study. Many types of biological activity of a substance and preparations based on extracts and by-products of the studied tissues can be associated with the peptide-protein composition.

The studied objects are multicomponent protein-peptide complexes. The use of a number of methods of sequential sample preparation, separation by 2D electrophoresis, fractionation by chromatography allows obtaining

data on the nature (composition and relative amount) of the protein profile with visualization of the protein pool of the studied extracts.

The purpose of the study was to decipher the protein profile and identify the biologically active compounds of the peptide-protein fraction of antlers from *Cervus nippon* and *Cervus elaphus*. The tasks were to develop methods for sample preparation of extracts of the tissues under different conditions, selection of conditions for further protein profiling and their identification in natural raw materials by the methods of 2D-PAGE, SEC and HPLC-MS after enzymatic hydrolysis by trypsin.

Materials and Methods

The samples of extracts from antlers of the sika deer (VDA) (*Cervus nippon*) and antlers of the red deer (ARD) (*Cervus elaphus*) were received from the collection of the bioanalytical research laboratory (SCBMT FMBA of Russia), obtained under the technology of water-alcohol extraction and subsequent lyophilization.

Materials

In our research, solvents and reagents of analytical degree of purity were used. Solutions for sample preparation were prepared using deionized water. To assess the presence of compounds of peptide or protein nature, an optional go spectrophotometer (Thermo scientific, USA) with 96-well cuvettes 10 mm long (“Corning”, USA) used by spectroscopic method. The degree of proteolysis was assessed using a diode-matrix detector 1260 PAH UG Max-light cell connected to a high-performance liquid chromatograph 1260 infinity II of the laboratory of the OGRN pump equipped with a built-in degasser, equipped with an automatic sample entry system Multisampler 1260, and a unit for thermostating the chromatographic column (“Agilent technologies”, USA).

HPLC-MS/MS HR analysis was performed using liquid chromatograph model 1290 infinity II “Agilent technologies”, equipped with pump 2-channel degasser, equipped with automatic input samples Multisampler 1290 with mass spectrometric detector QTOF 6545XT with ESI-ionisation at atmospheric pressure (“Agilent technologies”, USA), the server station computer (“HP”, USA), chromatographic column columns with different 300SB-C18, length 250 mm and internal diameter 2.1 mm, the size of the sorbent is 5.0 μm with a pore size of 300 Å and it is optimal for peptide mapping with a length of 150 mm and an internal diameter of 2.1 mm, the size of the sorbent is 2.7 μm (“Agilent”, USA).

2D page electrophoresis was performed using a ready-made kit ReadyPrep 2-nd starter kit, strips ReadyStrip IPG strip, 11 cm, pH 4–7 gels criterion TGX any KD dye bio-Safe Coomassie stain, the results were detected on the Gel system Doctor EZ system using the software image Lab bio-rad (“Bio-rad”, USA).

SEC analysis was performed on a chromatographic system of medium pressure to detect NGS (“Bio-rad”, USA), equipped with a multi-wave spectrophotometric detector, a pH sensor of eluent, a conductometric cell and a chromatographic column for high-resolution SEC analysis Enrich SEC-650, as calibration standards with known molecular masses, gel filtration was used-standard (“Bio-rad”, USA).

To identify proteins, a database of polypeptide sequences was used on the Internet (www.ebi.uniprot.org) and the program for the collection and processing of spectrum data Mill MS proteomics workbench (“Agilent”, USA).

Methods

Total protein determination was performed by the Bradford protein assay without prior protein precipitation in accordance with the general pharmacopoeial monograph (1.2.3.0012.15, “Protein Determination”) of the RFSP. The lyophilisate of the extract was pre-dissolved in purified water to a concentration of 150 mg/ml in a volume of solvent. The amount of protein was determined by the cal-

ibration curve for a solution of bovine serum albumin.

All procedures of *two-dimensional separation by electrophoresis* of proteins were performed according to the protocol included in the kit. The amount of the sample was taken at the rate of 250 mg of protein per 185 μl of denaturing solution. In two-dimensional electrophoresis, the first stage of separation is isoelectric focusing. Proteins are separated by electrophoresis based on their pl, the pH value at which the protein does not carry a net charge. The second separation stage is SDS-PAGE, where proteins already separated by IEF are further separated by their size. Gels were previously washed with distilled water and filled with a 200–300 ml paint solution and left overnight for better staining at room temperature. The next day, the gels were washed with distilled water in triplicate and the results were detected on the Gel Doc EZ System.

The molecular weight protein distribution of the product was carried out on a high pressure liquid isocratic chromatograph with a spectrophotometric flow detector with a wavelength of 280 nm.

Analysis on an Agilent Technologies 1260 Infinity II liquid chromatograph was performed in AutoMSMS mode. AdvanceBio Peptide Mapping Chromatographic Column, precursor ZORBAX Extend-C18 Narrow-Bore Guard Column were used. Elution was carried out with a mixture consisting of components A and B in the gradient mode: up to 0.5 min — 5% B, from 0.5 to 15.0 min increased to 35% B, from 17.0 — to 95% and held for 13 min, from 30.01 minutes — returns to initial conditions. The column equilibration time for initial conditions is 5 minutes. Component A was a 0.1% solution of formic acid in deionized water, component B was a 0.1% solution of formic acid and 10% deionized water in acetonitrile. The flow rate was 400 μl/min, the analysis time was 30 minutes. Conditions of mass spectrometric detection are shown in Table 1.

Results and discussion

Total protein determination

Percentage of total protein concentration according to the Bradford method studied extracts demonstrates that the largest amount of protein (30%) is in the VDA sample, and the

Table 1. *Conditions for mass spectrometric detection*
Таблица 1. *Условия масс-спектрометрического детектирования*

Parameter name	Value
Mass spectrometry detector	QTOF 6545XT
Ionization source	Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI)
Resolution	60,000
Type of collisional dissociation cell	Collisional dissociation cell
Ionization energy	(30 ± 15)%
Detectable charge state	2–5
Scan mode	AutoMSMS
Detected mass range	100–1700
Polarity of detected Ions	Positive ion detection
Spray voltage	3,5 kV
Fragmenter voltage	175 V
Flow rate of desiccant dryer gas	13 l/min
Nebulizer gas pressure	35 psig
Spray temperature	250°C

smallest (~20%) in ARD . The coefficient of variation does not exceed 10%.

2D PAGE

Using the method of 2D PAGE held comparative proteome analysis was done on basis results received of samples VDA and ARD. Identification by molecular mass and meaning pI was held with using Image Lab Bio-rad software (Figs 1 and 2).

In the VDA sample, more than 30 marker plots were counted. By the pI values, 3 groups of proteins can be distinguished: 1) pI=4.1. Molecular weight: 12.1, 23.8, 39.5, 50.2 and 66.4 kDa; 2) pI=5.0–6.0. Molecular weights: 18–20 kDa and 70–105 kDa; 3) pI=7.0. This group shows a large accumulation of proteins with a mass about of 15 kDa, which may indicate a significant amount of low molecular weight proteins.

The second sample shows the best separation with the display of about 90 marker plots. According to the IEF more groups can be distinguished than in the first sample, but the molecular mass distribution remains very similar.

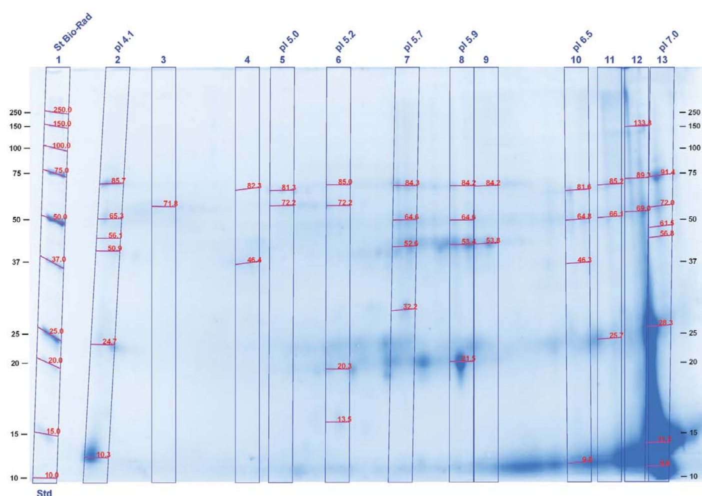

Fig. 1. *2D PAGE of ARD sample stained with Coomassie.*
Рис. 1. *2D-электрофорез ПБО, окрашенного кумасси.*

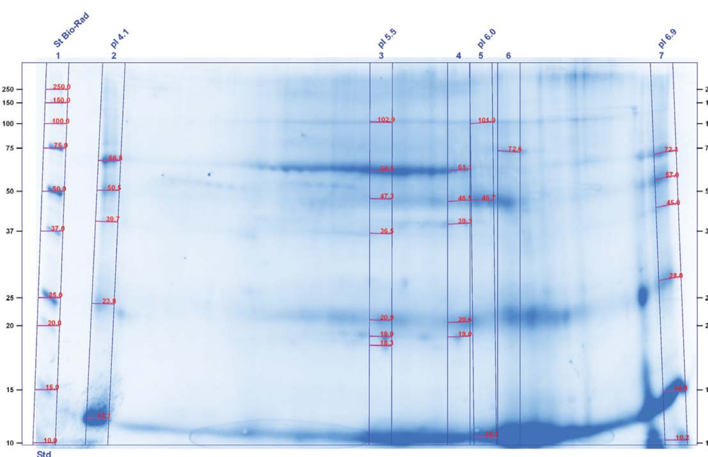

Fig. 2. *2D PAGE of VDA sample stained with Coomassie.*
Рис. 2. *2D-электрофорез ППО, окрашенного кумасси.*

Observed masses in the low area of 11–15, 20 and 25 kDa. In the field of medium weights, this range is from 50 to 75 kDa. No area with large molecular masses of proteins were detected.

The large dynamic range of concentrations of the studied extracts, as well as the wide variability of the physicochemical properties of the components in their composition, necessitates the use of multistage separation technologies with predominant use of SEC, 2D PAGE, specialized chromatographic chips, and all their possible combinations with technological methods (solid phase extraction, precipitation, exhaustion, etc.).

Size-exclusion chromatography

VDA sample demonstrated similar profile at low molecular weight areas as well as ARD sample. Also the meanings received from 2D PAGE result are confirmed by SEC at more high areas of molecular mass distribution. Received chromatograms are presented in pictures 3 and 4 and relevant values of molecular masses are demonstrated in the Table 2.

HPLC-MS

Peptide mass spectrometry is currently rapidly improving as the methodological basis of proteomics, both in terms of qualitative and quantitative analysis [2]. Unlike proteomics, based on controlled protein cleavage into peptides with “predictable” terminal amino acids, peptide — mass spectrometry is aimed at analyzing native peptides.

According to the results of previous analyzes, indicating a closely related component protein composition of the extracts and based on the most pronounced results for subsequent analysis, VDA extracts were used in this step. The result of the decoding of the protein profile of the fraction from the VDA and their classification became the annotated groups of proteins presented below (Figs 5 and 6). HPLC-MS analysis after enzymatic hydrolysis with trypsin demonstrated the identification of a large number of protein components in both fractions. For a more convenient perception, the obtained data was analyzed in the UniProt

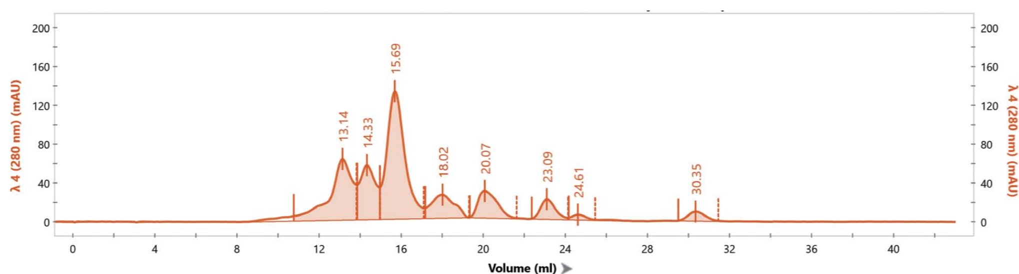

Fig. 3. *SEC water solution of the ARD extract.*
Рис. 3. *ГРХ водного раствора экстракта ПБО.*

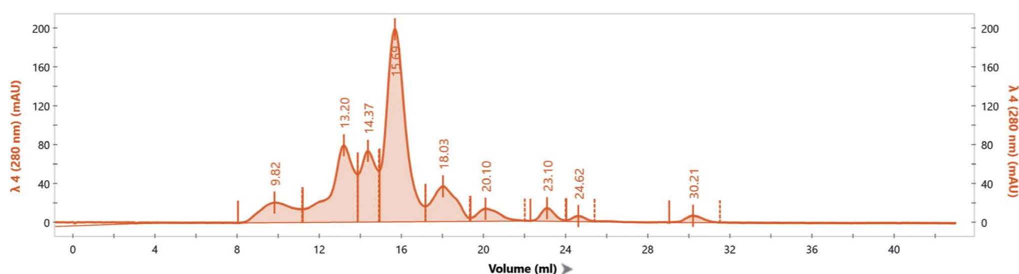

Fig. 4. *SEC water solution of the VDA extract.*
Рис. 4. *ГРХ водного раствора экстракта ППО.*

Table 2. *The results of SEC from VDA and ARD extracts**
Таблица 2. *Результаты ГРХ по экстрактам ПБО и ППО**

Molecular weight distribution in ARD extract						
Peak number	The volume of output, ml	Column volume, ml	Column dead volume, ml	Kav, distribution coefficient	Mol. weight, Da	The contribution of the peak, %
1	13.14	23.56	8	0.33	166725	20.02
2	14.33	23.56	8	0.41	75638	12.27
3	15.69	23.56	8	0.49	30651	33.41
4	18.02	23.56	8	0.64	6522	7.91
5	20.07	23.56	8	0.78	1671	7.57
6	23.09	23.56	8	0.97	225	4.32
Molecular weight distribution in VDA extract						
Peak number	**The volume of output, ml**	**Column volume, ml**	**Column dead volume, ml**	**Kav, distribution coefficient**	**MW, Da**	**The contribution of the peak, %**
1	9.82	23.56	8	0.12	1512382	7.87
2	13.2	23.56	8	0.33	160211	20.44
3	14.37	23.56	8	0.41	73655	12.5
4	15.69	23.56	8	0.49	30651	41.82
5	18.03	23.56	8	0.64	6478	9.7
6	20.1	23.56	8	0.78	1638	3.26
7	23.1	23.56	8	0.97	223	2.04

Note: ** — In these calculations, when building a calibration curve, the coefficient of determination is 0.99.*
Примечание: ** — В данных расчетах при построении калибровочной кривой коэффициент детерминации равен 0,99.*

database, which reflected the enormous number of functions and biological roles of protein components from the studied fractions. In total, 435 protein components were found in extracts from VDA, including 75, which were also not experimentally established in the deer antlers. The diagrams demonstrate general directions of functions and processes involving the identified proteins. Table 3 shows the brightest representatives of the studied profiles with the highest index of similarity.

In the classification according to molecular functions of protein components from the VDA fraction, four directions predominate: transferases, hydrolases, DNA/RNA-binding. Proteins that realize transport functions dominate involvement in biological processes.

The results given in Table 3 constitute a representatively selected part of the analyzed protein components of the tissues under study; however, they are among the known and significant among all. Particular attention is caused identified in the extracts from the VDA: PPARG1A, Wnt protein, CB1, PFL.

Peroxisome proliferative activated receptor, gamma, coactivator 1 (PPARGC1A) is a coactivator of nuclear receptors and other transcription factors that regulate metabolic processes, including biogenesis and respiration of mitochondria, hepatic gluconeogenesis and switching between types of muscle fiber [7].

Wnt proteins are a large number of secreted molecules involved in cell-to-cell signaling. The influence of the Wnt protein controls many cellular processes in many different organisms. Signal transmission based on the interaction with this molecule is largely associated with tumor genesis, early embryonic mesodermal pattern, brain and kidney morphogenesis, regulation of breast proliferation and Alzheimer’s disease [5, 8].

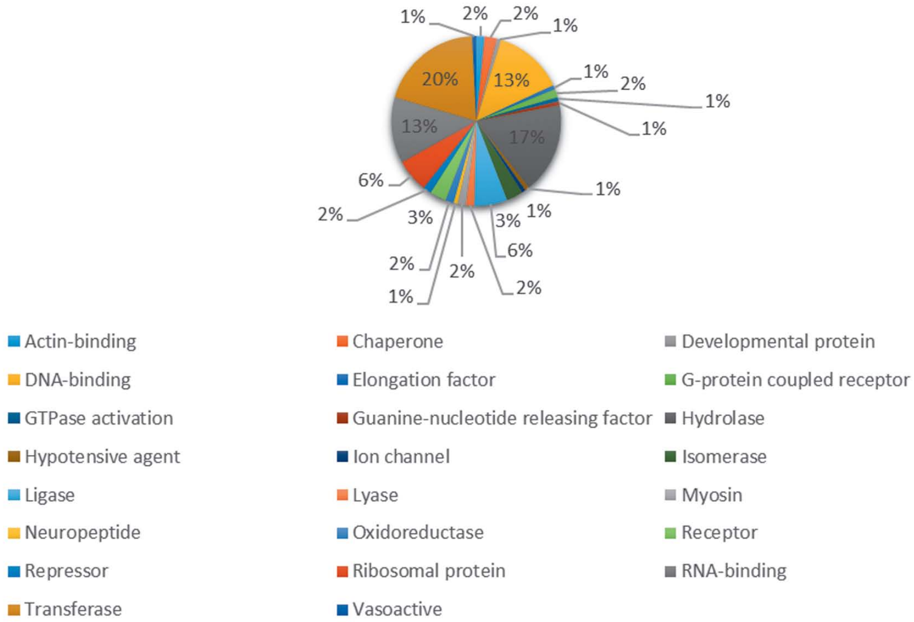

Fig. 5. *Groups of proteins from VDA extracts, distributed by molecular functions.*
Рис. 5. *Группы белков из экстрактов ППО, распределенные по молекулярным функциям.*

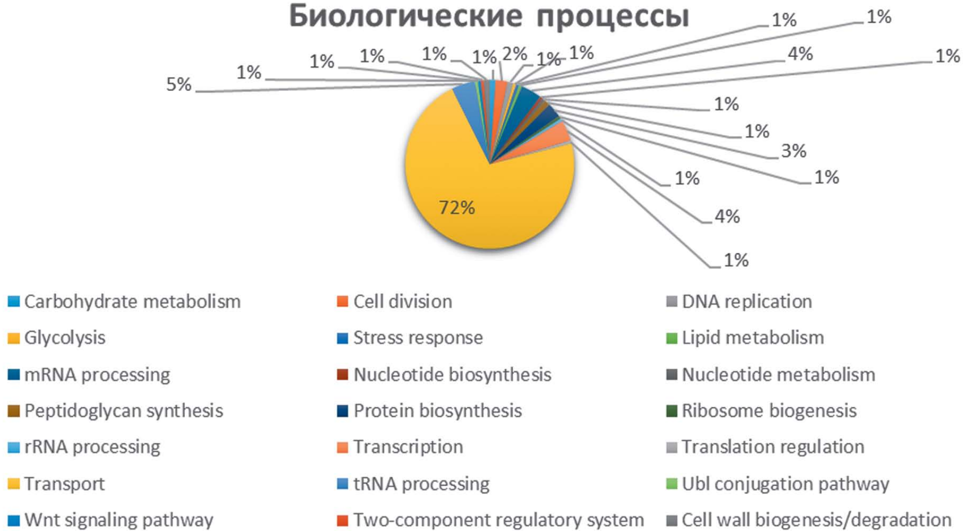

Fig. 6. *Protein groups from VDA extracts, distributed by biological processes.*
Рис. 6. *Группы белков из экстрактов, распределенные по биологическим процессам.*

Table 3. *Molecular mass characteristics of some typical annotated proteins in the composition of the VDA extract*
Таблица 3. *Молекулярно-массовые характеристики некоторых типичных аннотированных белков в составе экстракта ППО*

Protein number	MS/MS Score	Amino acid coverage, %	Total spectral intensity	MW, Da	Protein name
1	55.61	6.8	1.54E+06	66149	Serum albumin (Fragment)
2	48.51	4.9	1.49E+06	69080	ALB
3	43.71	12.3	3.09E+06	42765	HBA
4	42.4	30.3	5.21E+06	15953	Adult beta-globin
5	33.18	23.4	3.26E+06	15824	Hemoglobin subunit beta-3
6	21.95	10.3	2.12E+05	19244	HBB
7	21.47	14.2	3.43E+06	15952	Beta-globin subunit
8	12.44	6.1	1.86E+05	16522	HBG-T2
9	12.60	4.1	3.13E+05	30235	APOA1
10	12.31	2.5	3.90E+04	39576	FETUB (Fragment)
11	10.65	2.8	6.47E+04	32307	Vps23 core domain protein
12	10.36	4.9	6.50E+04	26317	HSD11B1L
13	10.27	2.8	9.22E+04	46667	GABRD
14	10.25	3.8	2.16E+05	34418	MDH1B (Fragment)
15	9.98	2.5	1.04E+05	34364	MRPL2
16	9.68	2.8	4.49E+05	23404	Glycerol-3-phosphate acyltransferase
17	9.22	3.4	2.77E+05	60607	DNA topoisomerase
18	8.95	2.2	1.53E+05	34361	Iron ABC transporter substrate-binding protein
19	8.93	5.9	8.06E+04	26292	GntR family transcriptional regulator
20	8.06	7.5	2.91E+05	16021	BUB3
21	7.80	4.7	9.29E+04	23302	WRN
22	7.47	2.2	9.41E+04	55725	Phosphoglucomutase
23	7.44	5.0	1.46E+05	19653	Histone H4
24	7.40	8.7	7.39E+04	23879	Arginine/serine-rich 7 splicing factor (Fragment)
25	7.23	6.1	6.51E+04	10838	Rhodanese-like domain-containing protein
26	7.20	2.3	1.17E+05	38297	Methionine import ATP-binding protein MetN
27	7.17	3.9	5.85E+05	27897	HAD family hydrolase
28	7.08	3.7	7.83E+04	15634	HTH-type transcriptional regulator LrpC
29	6.72	2.6	8.52E+04	41870	Beta sliding clamp
30	6.45	3.9	1.44E+05	20987	PPEF2 (Fragment)
31	6.19	5.0	1.07E+05	35138.7	Protein Wnt
32	4.02	1.3	3.03E+04	83831	Formate C-acetyltransferase
33	3.82	3.6	3.93E+03	37282.6	Cannabinoid receptor 1 (Fragment)
34	3.0	4.2	4.86E+05	25684.1	PPARGC1A

The cannabinoid receptor type 1 (CB1) is a cannabinoid G-protein coupled receptor. The CB1 receptor is expressed predominantly in the peripheral and central nervous systems and is activated by: endocannabinoids, a group of retrograde neurotransmitters; plant phytocannabinoids, such as tetrahydrocannabinol and its synthetic analogues [4].

Pyruvate formate-lyase (PFL) or formate C-acetyltransferase is an enzyme in the transferase family that helps regulate anaerobic glucose metabolism. It catalyzes the reversible conversion of pyruvate and coenzyme-A to formate and acetyl-coenzyme A. This enzyme is involved in the metabolic pathways of pyruvate, propionate and butyrate [9].

Conclusions

Using two-dimensional gel electrophoresis and size-exclision chromatography, protein profiling and molecular-mass distribution data were obtained from proteins in VDA extracts under denaturing conditions.

Using the Bradford method, the content of total water-soluble proteins in the extracts was evaluated. The effect of detergent additives and extraction conditions on the total protein level in lyophilisates was revealed.

The HPLC-MS assay of extract samples identified extensive and representative groups of proteins that characterize the features of a wide spectrum of biological activity of the objects under study. The identification of the most significant (major and minor) protein components in the extracts was carried out in accordance with the search software Spectrum Mill MS Proteomics Workbench and the Uniprot protein database. As a result of the interpretation of the protein composition of the studied VDA extracts, all the detected proteins are clustered according to molecular and biological functions. Relationships of the identified proteins with possible mechanisms of biological actions and targets that can be affected by the protein components of the objects under study are displayed. According to the results of the study, conclusions about the multicomponent protein composition of VDA and ARD extracts were drawn. Marker protein components were proposed as part of the extracts under study, and possible relationships of the detected proteins in extracts with biological effects were indicated.

The proteins are of main future interest, based largely on involvement in life processes: PPARGC1A, CB1, Wnt and PFL. In total, more than 400 endogenous animal proteins in the VDA extract were detected and 66 annotated proteins were identified from among them.

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INFORMATION ABOUT THE AUTHORS | СВЕДЕНИЯ ОБ АВТОРАХ

Vladislav N. Karkischenko, Dr. Sci. (Med.), Prof., Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia;
e-mail: scbmt@yandex.ru

Maxim S. Dulya*, Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia;
e-mail: mdulya@gmail.com

Daniil V. Khvostov, Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia;
e-mail: daniil_hvostov@mail.ru

Ruslan A. Ageldinov, Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia;
e-mail: ageldinov@gmail.com

Stanislav L. Lyublinskiy, Cand. Sci. (Biol.), Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia

Nikolay N. Karkischenko, Dr. Sci. (Med.), Prof., Corresponding Member of the Russian Academy of Sciences, Academician of the Russian Academy of Rocket and Artillery Sciences, Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia;
e-mail: niknik2808@yandex.ru

Каркищенко Владислав Николаевич, д.м.н., проф., ФГБУН «Научный центр биомедицинских технологий Федерального медико-биологического агентства России»;
e-mail: scbmt@yandex.ru

Дуля Максим Сергеевич*, ФГБУН «Научный центр биомедицинских технологий Федерального медико-биологического агентства России»;
e-mail: mdulya@gmail.com

Хвостов Даниил Владиславович, ФГБУН «Научный центр биомедицинских технологий Федерального медико-биологического агентства России»;
e-mail: daniil_hvostov@mail.ru

Агельдинов Руслан Андреевич, ФГБУН «Научный центр биомедицинских технологий Федерального медико-биологического агентства России»;
e-mail: ageldinov@gmail.com

Люблинский Станислав Людвигович, к.б.н., ФГБУН «Научный центр биомедицинских технологий Федерального медико-биологического агентства России»

Каркищенко Николай Николаевич, д.м.н., проф., чл.-корр. РАН, акад. РАРАН, ФГБУН «Научный центр биомедицинских технологий Федерального медико-биологического агентства России»;
e-mail: niknik2808@yandex.ru

* Corresponding author / Автор, ответственный за переписку