MMSL Military Medical Science Letters Since 1925

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2021, 90(3), 120-136
ISSN 0372-7025 (Print)
ISSN 2571-113X (Online)
DOI: 10.31482/mmsl.2021.012

ORIGINAL ARTICLE

MOLECULAR CHARACTERIZATION OF ALCOHOL–ETHER EXTRACT FROM BOVINE TISSUE

Klara Kubelkova [1]✉, Martin Hubalek [2], Pavel Rehulka [1], Helena Rehulkova [3], David Friedecky [4], Jitka Zakova [1], Ales Macela [1]

[1] Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic
[2] Institute of Organic Chemistry and Biochemistry of the CAS, Prague, Czech Republic
[3] Faculty of Science, University of Hradec Kralove, Czech Republic
[4] Institute of Molecular and Translational Medicine, Palacky University, Olomouc, Czech Republic

Received 16th March 2021.
Accepted 7th April 2021.
Published 3rd September 2021.

Summary

It is usual for information to be unavailable regarding the molecular composition of extracts from herbs or animal tissues that are popular in folk medicine. Here, we present analysis of the alcohol–ether extract from bovine tissue analogous to the basic substance used in such commercial products as Retisin, Imuregen, Actovegin, and Solcoseryl. The tested extract contains a whole spectrum of free amino acids, small proteins and oligopeptides of molecular weight up to 10 kDa, various nucleotides, and a small amount of phospholipids. Among the molecules that can explain some biological activities of the extract were identified those of taurine (2-aminoethanesulfonic acid, a derivative of the amino acid cysteine), several defensins, and bactericidal hemoglobin fragments known as hemocidins. All those molecules identified are natural components of bovine tissues, and a substantial number of them might be biologically active *in vivo*. Others are sources of readily available nutrients.

Key words: bovine tissue extract; Juvenil; molecular composition; psychobiotics; biological response modifier; antimicrobial peptides; defensin; hemocidin

Introduction

Extracts from herbs and animal tissues have long been utilized as folk remedies. Their use originated from empirical experience that certain more or less specified products are beneficial to human health. Usually, they had been used in such forms as teas, elixirs, or ointments. Recently, with the domination of Western models of medical care, such preparations are sometimes criticized for the fact that their compositions are not precisely defined. The U.S. National Library of Medicine defines the term "tissue extracts" as follows: "Preparations made from animal tissues or organs (animal structures). They usually contain many components, any one of which may be pharmacologically or physiologically active. Tissue extracts may contain specific, but uncharacterized factors

✉ University of Defence, Faculty of Military Health Sciences, Department of Molecular Pathology and Biology, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
klara.kubelkova@unob.cz
☎ +420 973 255 193

or proteins with specific actions" (1). Despite the fact that their molecular compositions are complex and poorly characterized, utilization of certain tissue extracts as nutritional supplements might be very useful at times when people are under stress, convalescing, or consuming diets consisting mostly of commercially processed foods. Inasmuch as the modern lifestyle is a test of the body's resistance, there may be a place for the use of nutritional supplements positively affecting human immunity or supporting full regeneration. Extracts from animal tissues sold as Retisin, Lyastin, Sangitin, and Silexil, as well as a number of extracts of plant origin, were prepared in the middle of the 20th century in the former Czechoslovakia (2). One of these was an alcohol–ether extract from bovine tissues that was broadly tested to standardize its production, as well as for harmlessness, microbial contamination, and biological activity in model animal systems and on human volunteers. Some data originated also from clinical testing. Taken collectively and comprehensively, these data document the complete harmlessness to human beings of the alcohol–ether extract from bovine tissue prepared according to the original Standard Technology Procedure. It should be noted, however, that due to distrust of preparations having unclear molecular profiles, the test results were only reported in partial documents and research reports. For this reason, there exist only a few publications about them in the Czech language in not very generally available professional journals.

Following is a very brief, critical summary from the original documents concerning the functional profile of the commercial product Juvenil, one of the preparations based on the bovine tissue extract. The hygienic–toxicological evaluation, carried out at the Institute of Hygiene and Epidemiology in Prague, disclosed that experimental animals (white rats) under the Juvenil drinking regime showed no differences in behavior, weight gains, or mortality in comparison with an untreated control group. Motor coordination, grip strength, and endurance in tested animals, all of which are parameters used in measuring toxicological effect of substances, demonstrated the safety of utilizing the Juvenil preparation (3). Histological examination of white rat liver and spleen showed no differences in structure of the evaluated tissues between the control and experimental groups. Testing of human volunteers (conducted at the Institute of Hygiene and Epidemiology in Prague) further demonstrated the harmlessness of Juvenil for humans. Students (girls between 20.7 and 23.9 years of age) were subjected to a test course during which Juvenil was administered every morning for 14 days. The basic parameters of innate immunity (phagocytic activity of neutrophils; levels of lysozyme, transferrin, as well as C3 and C4 complement components in sera) were insignificantly improved after the test. The control of somatometric parameters revealed increased appetite in the test set, but this was not much reflected as increased weight and amount of subcutaneous fat of the students. Both systolic and diastolic blood pressures were favorably reduced, accompanied by a slight increase in heart rate. Students who received Juvenil had improved range of immediate and short-term memory and increased psychomotor performance. The tested parameters of heart resistance, fatigue, and functional insufficiency index were also favorably influenced by Juvenil. Finally, biochemical tests of blood samples taken before and after the Juvenile cure showed no significant changes.

Moreover, a combination of Imuregen, an alternative product to Juvenil, with beta glucan positively regulated children's secretion immunity and stress perception (4), and it improved the immune status and physical conditions of children from a Czech region with high level of air pollution (5). Testing of the analogous animal tissue extracts Solcoseryl and Actovegin revealed their wide range of interventions into homeostasis of the human organism (6-10).

How can we explain the multiple effects of animal tissue extracts demonstrated in clinical settings? One explanation can be modulation of the microbiota–gut–brain axis (11, 12). An alternative explanation can be sought in direct ligations of corresponding receptors by individual components of the extracts after their absorption and general distribution throughout the organism by bodily fluids, whereupon their effects would initiate at the cellular level. The affected cells subsequently express their genetically preprogrammed functions. Because it can generally be assumed that knowing the molecular composition is crucial to characterizing the functional dynamics of the extract, we conducted a broader analysis of the molecular composition of the alcohol–ether extract from bovine tissue oriented predominantly toward protein or peptide contents.

Material and Methods

Laboratory testing of the extract was carried out in several labs of different Czech institutions. The testing of amino acid content and identification of proteins as a source of detected peptides was conducted by two independent research labs to verify and, if necessary, supplement the composition of the extract. The laboratory that used a specific technique is identified by a number corresponding to the study authors' affiliation.

Alcohol–ether extract from bovine tissue

The extract was a kind gift of Juvenil Products, a.s. Prezletice, Czech Republic. The extract was prepared according to the expired description of the invention, copyright certificate No. 228 038 CZ, registered 10/07/81 (https://isdv.upv.cz/webapp/!resdb.pta.frm), and using the internal Standard Operating Procedure of this company that specifies more precisely the time and temperature course of the production process. The substance was stored in darkness at room temperature until used for individual experiments.

Mini one-dimensional gel electrophoresis

Protein concentration was defined by bicinchoninic acid assay using Micro BCA Protein Assay kit (Thermo Scientific). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using tricine SDS gels with stacking and resolving gels of 4% and 12%, respectively. Electrophoresis was performed on a Protean II Multi-Cell (Bio-Rad, Hercules, CA, USA) according to the standard running conditions. Blue staining protocol was then used to visualize proteins on a reference protein map. Briefly, for blue staining, the gels were washed once with water for 5 min and then fixed and stained overnight with 50% methanol, 40% acetic acid, and 0.05% Coomassie brilliant blue G250 in water. Blue-stained gels were then washed with 12.5% acetic acid, 5% methanol in water for 1 h. Development was terminated by transferring the gel to fixing solution (7% acetic acid). Silver staining was performed for purposes of further mass spectrometry. Briefly, the gels were washed once with water for 5 min and then fixed with 12.5% glutaraldehyde solution 6 at room temperature for 1 h. Fixed gels were washed with water and then stained with a solution of 1% silver nitrate for 1 h. Stained gels were washed with water and then developed with 6.25% sodium carbonate and 0.25% formaldehyde (developer solution). Development was terminated by transferring the gel to fixing solution (1% acetic acid). The gels were then incubated for 10 min, followed by one quick water wash of 30 s.

Amino acid analysis

Determination of free amino acids was performed by ion exchange chromatography on an AAA 400 automatic amino acid analyzer (INGOS, Prague, Czech Republic). The sample of extract (200 mg/ml) was diluted 200 times with deionized water and then deproteinized with sulfosalicylic acid.

The samples were hydrolyzed with 6 M HCl at 110°C for 20 h, then analyzed on a Biochrom 30 Amino Acid Analyzer (Biochrom, Cambridge, UK). The separation of amino acids was achieved on a high-pressure PEEK column packed with Ultropac 8 cation exchange resin. A proprietary high-performance gradient and temperature program was used with a total run time of 74 mins, utilizing three proprietary sodium citrate buffers.

Mass spectrometry techniques applied to the extract analysis

Source proteins (liquid chromatography–mass spectrometry)

The Coomassie brilliant blue stained piece of electrophoretic gel was cut into smaller pieces. Gel pieces were destained with 25 mM ammonium bicarbonate (Fluka, 40867) in 50% acetonitrile (ThermoFisher Scientific, Massachusetts, USA) at 30°C for 30 min and dried with 200 µl acetonitrile for 5 min at 30°C. Dry gel pieces were treated with dithiothreitol (65°C, 30 min; Sigma-Aldrich, D0632) and iodoacetamide (at room temperature for 30 min in darkness; Sigma-Aldrich, I1148) to reduce and alkylate cysteines. Proteins in gel pieces were digested with 0.1 µg of trypsin solution in 50 mM ammonium bicarbonate at 37°C for 10 h. Peptides were extracted using 50 µl of 2% trifluoroacetic acid (TFA; Thermo Scientific, 28903) and 50 µl of 60% acetonitrile. The peptides were dried in a centrifugal evaporator and dissolved in 15 µl of 0.1% formic acid in water (ThermoFisher Scientific, Massachusetts, USA).

Samples were analyzed on the UltiMate 3000 RSLCnano system (Dionex, USA) coupled to a TripleTOF 5600 mass spectrometer with a NanoSpray III source (Sciex, Framingham, MA, USA). The instrument was operated with Analyst TF 1.7 (Sciex). The peptides were trapped and desalted with 2% acetonitrile in 0.1% formic acid at flow rate of 5 µL/min on an Acclaim PepMap100 column (5 µm, 2 cm × 100 µm ID; Thermo Scientific). Eluted

peptides were separated using an Acclaim PepMap100 analytical column (3 µm, 25 cm × 75 µm ID; Thermo Scientific). The 70 min elution gradient at constant flow of 300 nl/min was set to 5% of phase B - 0.1% formic acid in 99.9% acetonitrile (ThermoFisher Scientific, Massachusetts, USA) and phase A 0.1% formic acid (ThermoFisher Scientific, Massachusetts, USA) first for 5 min, then with gradient elution by increasing the content of acetonitrile. Time-of-flight mass spectrometry range was set to 350–1500 m/z in MS/MS mode. The instrument acquired fragmentation spectra with m/z ranging from 100 to 2000.

Protein Pilot 4.5 (Sciex) was used for protein identification from raw (*.wiff) spectra using a database consisting of *Bos Taurus* proteins (Uniprot, April 2018) and common contaminants. The search was set to choose iodoacetamide as alkylation substance, trypsin as digestion agent, and TripleTOF 5600 as instrument. All samples were evaluated by Paragon algorithm in the "Thorough" regime allowing mass spectrometry (MS) precursor ion deviation up to ± 0.05 Da and ± 0.1 Da for MS2. Charge of fragments was set between +2 and +5.

In parallel, the extract was electrophoretically separated using standard SDS-PAGE (as described in the previous section) and protein bands were visualized using Coomassie brilliant blue G-250 staining. The protein-containing region was excised from the polyacrylamide gel and cut into small pieces. The gel pieces were processed using standard in-gel digestion procedure involving dithiothreitol for reduction of disulfide bridges and iodoacetamide for cysteine modification. Enzymatic digestion was done overnight at 37°C. The peptide extracts acidified by addition of 5% TFA were chromatographically purified using short custom-made reversed phase microcolumns (15 mm length, 250 µm ID) packed with 2.6 µm Kinetex EVO C18 resin (Phenomenex, Torrance, CA, USA) in fluorinated ethylene propylene (FEP) tubing (VICI AG, Schenkon, Switzerland) blocked with a small piece of Whatman® glass microfiber filter (GE Healthcare, USA). Peptides were eluted with nonlinear gradient formed in a 50 µl SGE gas-tight microsyringe with cone needle (Trajan Scientific and Medical, Melbourne, Victoria, Australia) with gradually increasing acrylonitrile (ACN) content (2–40% ACN/0.1% TFA) from the microcolumns and dried using a vacuum concentrator (Eppendorf, Hamburg, Germany) as described previously (13).

The prepared tryptic peptide samples were dissolved in 20 µl of 2% ACN/0.1% TFA. Each sample was first analyzed using the UltiMate 3000 HPLC system (Dionex). This system coupled with UV detection included a µ-Precolumn (300 µm × 5 mm, C18PepMap 5 µm 100 Å particles; Dionex) connected to the analytical NanoEase column (100 µm × 150 mm, Atlantis C18 3 µm 100 Å particles; Waters, Milford, MA, USA). The peptides were separated using the bilinear gradient of 5–45% ACN/0.1% TFA over 81 min under a flow rate of 360 nl·min^{-1} and UV detection set to 215 nm. The data were collected and visualized using Chromeleon software (v. 6.80, Dionex).

In the case of liquid chromatography–mass spectrometry (LC-MS) analysis, peptides were separated using the UltiMate 3000 RSLC-nano HPLC system (Dionex) with a trap column (75 µm × 20 mm) packed with 3 µm Acclaim PepMap100 C18 particles and a separation column (75 µm × 150 mm) packed with 2 µm Acclaim PepMap RSLC C18 particles. The separation was performed with dual linear gradient using 3–44% ACN in 0.1% formic acid over 89 min for the non-fractionated sample and over 63 min for the obtained fractions under the flow rate of 300 nl·min^{-1}. The separation was monitored using the UV detection system at 214 nm and further directly coupled to MS analysis with the QExactive system (Thermo Fisher Scientific) in positive mode with full MS scan (350–1650 m/z) at 70,000 full width at half maximum (FWHM), maximum filling time 100 ms, normalized collision energy (NCE) at 27% and automatic gain control (AGC) target value of 1E6. The top 10 precursors with isolation window 1.6 m/z were selected in MS/MS at 17,500 FWHM for purified and fractionated samples, respectively, with maximum filling time 100 ms and AGC target 1E5.

Proteome Discoverer software (Thermo Fisher Scientific, v. 2.4.0.305) was used for proteomic identification of the MS/MS spectra. The raw files from the QExactive mass spectrometer were processed within the processing workflow containing spectrum selector, non-fragment filter, top N peaks filter, precursor detector, SequestHT search engine, target decoy PSM validator, and IMP-ptmRS nodes. The parameters for SequestHT database searching were the following: protein database – UniProt bovine reference proteome UP000009136 (30 November 2020); enzyme – trypsin; maximum missed cleavage sites – 1; min. peptide length – 7; precursor mass tolerance – 15 ppm; fragment mass tolerance – 0.02 Da; weight of b- and y-ions – 1; static modifications – carbamidomethyl / +57.021 (C); dynamic modifications – oxidation / +15.995 Da (M), dynamic modifications (protein terminus) – acetyl / +42.011 Da (N-terminus), met-loss / −131.040 Da (M), met-loss+acetyl / −89.030 Da (M); dynamic modifications (peptide

terminus) – Gln->pyro-Glu / −17.027 Da (Q). The search results obtained in the msf file were further processed via the consensus workflow containing PSM Grouper, Peptide Validator, Protein and Peptide Filter, Protein Scorer, Protein FDR Validator, Protein Grouping and Protein in Peptide Annotation nodes.

Peptides

The extract (10 mg) was dissolved in 200 µl 1% TFA (v/v), briefly vortexed and sonicated, then centrifuged for 1 min at 10,000 × g. The supernatant was transferred into a clean vial and it was 6 times extracted by addition of 200 µl ethyl acetate, brief vortexing, centrifugation for 1 min at 10,000 × g, and removal of top ethyl acetate phase layer. At the end, the bottom layer without sediment was collected (approximately 200 µl) and 2 µl of this extract was mixed with 18 µl of 2% ACN/0.1% TFA (v/v) for microcolumn reversed phase purification. The purified sample was 20× diluted with 2% ACN/0.1% TFA (v/v) prior to LC-MS analysis. The sample purification and LC-MS analysis were done in the same way as was described in the preceding section about protein identification from in-gel digestion. The peptide identification settings in Proteome Discoverer software were also identical to those for tryptic peptide identification with the exception that enzyme specificity was set to "No-Enzyme (Unspecific)" for the non-specific database searching.

Phospholipids

The lipid extraction was done using methyl-tert-butyl ether (MTBE) as described previously (Matyash et al. 2008). Briefly, the extract (9.9 mg) was first dissolved in 240 µl methanol, 800 µl were then added and the mixture was vortexed for 10 min. Next, 200 µl water was added, the mixture was briefly vortexed, and then centrifuged for 5 min at 10,000 × g. The upper layer containing lipids was collected as the lipid-containing solution for further analyses.

High-performance thin-layer chromatography (TLC) was carried out according to the protocol of Matyash et al. and Stübiger et al. (14, 15), with slight modifications. Silikagel 60 F_{254} plates (Merck, Darmstadt, Germany) were used for separation of extracted lipids in the mobile phase composed of ethyl acetate/2-propanol/chloroform/methanol/0.25% aqueous potassium chloride (25:25:25:10:9, v/v/v/v/v). Visual detection was carried out by staining the plates in 0.03% Coomassie brilliant blue R-250 in 20% methanol (10 min) and destaining in 20% methanol (5 min). For comparison, a lipid extract from human plasma (50 µl) was separated along with the lipids from bovine tissue alcohol–ether extract. The same lipid separation without Coomassie staining was carried out in parallel for subsequent mass spectrometry analysis after extraction of the lipid fraction from chromatography plates with methanol/2-propanol (1:1, v/v) mixture.

LC-MS analysis of isolated lipids was done both for unfractionated lipid extract and the isolated fractions from high-performance TLC separation. For this purpose, a simple microgradient device with a short custom-made reversed phase microcolumn (27 mm length, 250 µm ID) packed with 2.6 µm Kinetex EVO C18 resin (Phenomenex) in FEP tubing (VICI AG) blocked with a small piece of Whatman® glass microfiber filter (GE Healthcare, USA) was coupled online to an ESI-MS instrument (QExactive system, Thermo Fisher Scientific) similarly as described earlier for peptide separations (Lenobel et al. 2015). The gradient was formed in a 100 µl SGE gas-tight microsyringe with cone needle (Trajan Scientific and Medical, Melbourne, Victoria, Australia) from 6 different mobile phases. These mobile phases were mixed from mobile phases A (acetonitrile/isopropanol/water/1M ammonium formate/formic acid = 30:30:39:1:0.1, v/v/v/v/v) and B (acetonitrile/isopropanol/1M ammonium formate/formic acid = 10:89:1:0.1, v/v/v/v) and aspirated into the microsyringe as follows: 30 µl B, 12 µl A/B = 1:4 (v/v), 10 µl A/B = 2:3 (v/v), 10 µl A/B = 3:2 (v/v), 10 µl A/B = 1:4 (v/v), 8 µl A, 4 µl sample, 16 µl A. The sample loading and separation were done in a single step taking 24 or 12 min (flow rate was 4 or 8 µl/min, respectively). Samples were appropriately diluted with mobile phase A for loading on reversed phase separation. The chromatographic zones from unstained TLC separations were eluted with isopropanol:methanol (1:1, v/v) mixture. Mass spectrometry analysis was performed with the QExactive system (Thermo Fisher Scientific) in positive mode with full MS scan (350–1650 m/z) at 70,000 FWHM, maximum filling time 100 ms, NCE at 27%, and AGC target 1E6. The top 6 precursors with isolation window 1.6 m/z were selected in MS/MS at 17,500 FWHM for purified and fractionated samples, respectively, with maximum filling time 100 ms and AGC target 1E5. Lipid identification was based on m/z values of lipid species using tools (e.g., ALEX^{123} lipid calculator accessible via LipidMaps database homepage [lipidmaps.org]).

Determination of nucleotide content

Purine and pyrimidine nucleotide contents of the extract were tested using capillary electrophoretic method (16, 17). Briefly, the final background electrolyte buffer consisted of 40 mM citric acid with addition of 0.8 mM cetyltrimethylammonium bromide titrated by g-aminobutyric acid to pH 4.4. The electrophoretic separations were carried out in an uncoated silica capillary (ID/OD – 75/375 mm; effective/total length – 90/97 cm). Sample preparation was optimized in order to shorten worktime and prevent analyte degradation.

Results

The original crude extract obtained from Juvenil Products was used for all analytical procedures without further modification. The basic stock solution of the extract was prepared by dissolving the weighed batch in deionized water. Further manipulation of the samples was performed according to the protocols of the methods used.

Free amino acids as components of the extract

In view of the technology applied to preparation of the extract, we had assumed that the resulting molecular complex would contain free amino acids. The four independent testings based on four independently prepared extract samples revealed substantial content of free amino acids in an almost representative composition (Table 1).

Table 1. Composition of free amino acid content in the extract. The content of cysteine and methionine is distorted by the technology used, as it hydrolyses these two amino acids. The content of amino acids is given in mg per 100 g of extract ± standard error. The analyses were done in biological quadruplicates at three different Czech institutions (Palacky University, Olomouc; Institute of Hygiene and Epidemiology, Prague; and Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague).

Amino acid		mg/100 g
Asparagine	Asp	5.04 ± 2.42
Threonine	Thr	2.97 ± 0.85
Serine	Ser	4.52 ± 1.05
Glutamine	Glu	13.62 ± 2.25
Proline	Pro	4.83 ± 2.31
Cysteine	Cys	0.00
Glycine	Gly	5.84 ± 2.87
Alanine	Ala	10.77 ± 3.51
Valine	Val	7.34 ± 2.16
Methionine	Met	3.59 ± 4.51
Isoleucine	Iso	0.74 ± 0.23
Leucine	Leu	8.46 ± 2.78
Tyrosine	Tyr	2.41 ± 1.31
Phenylalanine	Phe	2.85 ± 1.20
Histidine	His	2.60 ± 0.72
Lysine	Lys	3.54 ± 1.35
Arginine	Arg	1.00 ± 0.41
Tryptophan	Trp	1.45 ± 0.02
Taurine	Tau	1.21 ± 0.04*

*Taurine content is expressed as the average of just three technical replicates.

The content of methionine is understated, because it partially decomposes during hydrolysis. Cysteine is missing for the same reason. The last amino acid analysis, carried out at Medical Faculty of Palacky University, Olomouc, Czech Republic, revealed a presence of taurine. Taurine (2-aminoethanesulfonic acid) is an amino acid derivative of cysteine and is therefore sometimes classified among the amino acids even though it lacks a carboxyl group. The total amino acid content in the extract corresponds to the general amino acid composition of bovine tissues (data not shown) and, due to the significant proportion of small proteins and peptides in the extract (see next paragraph), we focused subsequent experiments directly upon identifying proteins and peptides.

Extract contains proteins and peptides of only low molecular weight up to 10 kDa

To obtain a basic understanding of protein (peptide) content, we used mini one-dimensional polyacrylamide gel electrophoresis with tricine SDS gels. Although tricine–SDS-PAGE is commonly used to separate proteins in the mass range 1–100 kDa, it is preferentially recommended for the resolution of proteins smaller than 30 kDa. We chose this method both because we had assumed hydrolysis of large proteins by the technology of the extract preparation and also because indicative classical SDS-PAGE had given no signal after silver staining. The blue staining and silver staining was used to visualize separated proteins, labeled proteins or peptides at the lower half of the gels, predominantly below 10 kDa (Figure 1). This result thus documents the fact that the technology used to prepare the extract hydrolyzes large protein molecules to form protein fragments, some of which may be biologically active peptides.

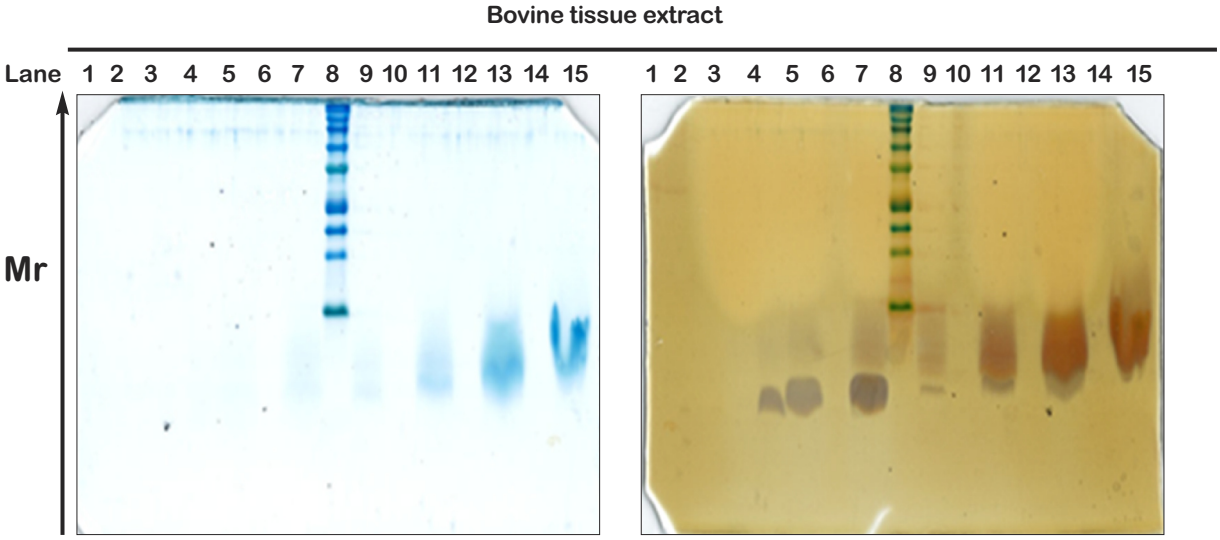


Figure 1. Blue staining (left) and silver staining reference gels with visualized low molecular weight proteins or peptides. Molecular markers with marked positions of 10 and 15 kDa (on left gel) are visualized vertically in the center of the gels. Marker lane (**8**), sample lane – 1 ug (**2**), 10 ug (**3**), 50 ug (**4**), 100 ug (**5**), 200 ug (**7**), 100 ug (**9**), 500 ug (**11**), 1000 ug (**13**), and 1000 ug (**15**) of alcohol–ether extract from bovine tissue (position 1, 6, 10, 12, and 14 left unused.

The MS/MS analysis was used for the identification of proteins or their peptide fragments in the extract. Peptides belonging to 138 bovine proteins with known function and subcellular localization were identified by the methods used. Their identification and characterization were carried out according to the UniProt protein knowledgebase for organism *Bos taurus* (bovine) at www https://www.uniprot.org/uniprot (Table 2). Also found were peptides belonging to uncharacterized bovine proteins having the Ig-like domain as well as peptides assigned to completely unidentified proteins, the majority of which with reverse sequences corresponding to known proteins. The data for peptides corresponding to the sequence of uncharacterized and Ig-like domain proteins are not listed in Table 2.

Considering the image of one-dimensional mini electrophoresis, it cannot be assumed that the proteins in the extract could be in complete sequences. The molecular weights of most proteins in the extract exceed the 10 kDa limit given by the result of 1D-mini electrophoresis, so they cannot be assumed to be in full length and thereby ensuring their biological function. Table 2 shows that defensins (m.w. 4 to 6 kDa), apolipoprotein C-III (8 kDa), and basal body orientation factor 1 (8 kDa) could be represented in the extract in complete sequence. A dominant number of peptides were derived from proteins falling into the categories “extracellular region / secreted” and “cytosolic proteins.” Other peptides were derived from nuclear, membrane and cytoskeletal proteins (Figure 2). Peptides derived from proteins of such other subcellular structures as mitochondria, cell membranous or filamentous organelles, endoplasmic reticulum, Golgi complex, centrosome, or desmosome were present in smaller numbers. Rather striking is that we identified all four core histones (H2A, H2B, H3, and H4), but we did not identify H1 linker histones. Attempting to explain this disproportion can only be speculative at the moment.

Table 2. Bovine proteins identified by presence of their peptides in the extracts. The proteins information was obtained from the UniProt protein knowledgebase for organism Bos taurus (bovine) (https://www.uniprot.org/uniprot)

Accession number	Name	Gene	Peptides*	MW (kDa)	Function
P68032	Actin, alpha cardiac muscle 1	ACTC1	1	42	cell motility
F1MLQ4	AA_permease_C domain-containing protein	LOC789418	1	62	transmembrane transport
P68138	Actin, alpha skeletal muscle	ACTA1	1	42	cell motility
P62736	Actin, aortic smooth muscle	ACTA2	4	42	gene expression - positive regulation
P60712	Actin, cytoplasmic 1	ACTB	15	41	cell motility
P63258	Actin, cytoplasmic 2	ACTG1	3	41	signaling pathway - Fc gamma receptor
P63267	Actin, gamma-enteric smooth muscle	ACTG2	1	41	gene expression - positive regulation
A0A3Q1N1Z7	Actin-like protein 6B	ACTL6B	1	76	vesicular transport, spindle orientation, chromatin remodeling
P34955	Alpha-1-antiproteinase	SERPINA1	1	± 54	protease inhibitor - can limit acute phase response
Q7SIH1	Alpha-2-macroglobulin	A2M	2	±720	protease inhibitor - can inhibit inflammatory cytokines
A0A3S5ZP98	α-aminoadipic semialdehyde dehydrogenase	ALDH7A1	6	59	oxidoreductase activity
Q3MHH8	Alpha-amylase	AMY2A	1	58	hydrolyze intact starch granules
B6V3I5	Alpha-lactalbumin	N/A	2	±14	regulatory subunit of lactose synthase
B5B3R8	Alpha-S1-casein	CSN1S1	3	23	transport - calcium
P02663	Alpha-S2-casein	CSN1S2	4	25	transport - calcium
A2VE25	Ankyrin 1	ANK1	1	171-206	cytoskeleton organization
P04272	Annexin A2	ANXA2	4	35-40	cellular growth / signal transduction pathways
P41361	Antithrombin-III	SERPINC1	1	58	inactivates several enzymes of the coagulation system
F1MV51	APC, WNT signaling pathway regulator	APC	1	300	antagonist of the Wnt signaling pathway
P15497	Apolipoprotein A-I	APOA1	7	28	cholesterol transport
P81644	Apolipoprotein A-II	APOA2	11	17	cholesterol transport / may stabilize HDL
P19035	Apolipoprotein C-III	APOC3	1	8	multifaceted role in triglyceride homeostasis
F1N7L8	Basal body orientation factor 1	BBOF1	1	8	cilia orientation in response to flow
P02666	Beta-casein	CSN2	1	24	negative regulation of inflammatory response
P46171	Beta-defensin 13	DEFB13	1	4	potent antimicrobial activity
P46160	Beta-defensin 2	DEFB2	1	4	potent antimicrobial activity
P46162	Beta-defensin 4	DEFB4	1	6	potent antimicrobial activity
G3N0C6	Beta-defensin 4 precursor	DEFB4	1	6	potent antimicrobial activity
P46163	Beta-defensin 5	DEFB5	1	4	potent antimicrobial activity
E1BL29	Bleomycin hydrolase	BLMH	1	53	response to toxic substance
P18892	Butyrophilin subfamily 1 member A1	BTN1A1	1	59	T cell receptor signaling pathway
P00921	Carbonic anhydrase 2	CA2	1	29	reversible hydration of carbon dioxide
P00432	Catalase	CAT	2	232	decomposition of hydrogen peroxide to water and oxygen
P00760	Cationic trypsin	N/A	1	24	serine protease
E1BK91	Centrosomal protein 78	CEP78	1	78	G2/M transition of mitotic cell cycle
F1MW44	Coagulation factor XIII A chain	F13A1	3	82	metal ion binding, protein-glutamine γ-glutamyltransferase activity
P62894	Cytochrome C	CYCS	32	12	mitochondrial electron transport, apoptotic process
G3N0V2	Cytokeratin-1	KRT1	2	63	complement activation, lectin pathway
E1BCF2	Cytokine receptor like factor 3	CRLF3	1	50	negative regulation of cell cycle progression
Q01107	Desmocollin-1	DSC1	1	100	cell - cell adhesion / homophilic / neutrophil degranulation
A6QR67	Desmocollin-1	DSC1	1	100	calcium ion binding
Q03763	Desmoglein-1	DSG1	2	150	cell-cell adhesion / neutrophil degranulation
E1BKT9	Desmoplakin	DSP	19	225-285	adherens junction organization / neutrophil degranulation
A0JNF4	DNA primase large subunit	PRIM2	1	58	synthesizes small RNA primers during discontinuous DNA replication
A8E641	DPYSL5 protein	DPYSL5	1	61	hydrolase activity
F1MN61	Early endosome antigen 1	EEA1	1	134	fusion of early and late endosomes, sorting at the early endosome level
Q32PH8	Elongation factor 1-alpha 2	EEF1A2	2	50	translation factor activity / RNA binding
A4IFN5	Fat storage-inducing transmembrane protein 2	FITM2	4	30	cytoskeleton organization, lipid storage
P55052	Fatty acid-binding protein 5	FABP5	1	15	intracellular carrier for long-chain fatty acids
P10790	Fatty acid-binding protein, heart	FABP3	1	15	intracellular transport of long-chain fatty acids
Q3SZZ9	FGG protein	FGG	5	49	metal ion binding, protein polymerization
P02672	Fibrinogen alpha chain	FGA	13	95	signaling receptor binding, platelet aggregation
P02676	Fibrinogen beta chain	FGB	4	53	signaling receptor binding, cell adhesion, platelet aggregation
A0A3S5ZPP8	Fibrosin	FBRS	2	103	fibrogenic cytokine
Q2T9N0	Fibrous sheath CABYR-binding protein	FSCB	1	20	negative regulation of protein sumoylation
Q32LC6	G protein pathway suppressor 1	GPS2	1	56	suppress G-protein / mitogen-activated signal transduction
P10096	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1	36	catalyzes the sixth step of glycolysis
F1N726	Glycoprotein 2	GP2	1	68	receptor structure / binds pathogens such as enterobacteria

Accession number	Name	Gene	Peptides*	MW (kDa)	Function
P80195	Glycosylation-dependent cell adhesion 1	GLYCAM1	4	16	ligand for the receptor L-selectin / lymphocytes exit the bloodstream
P19120	Heat shock cognate 71 kDa protein	HSPA8	1	71	chaperone function / disassembly of clathrin-coated vesicles
E1BLD1	HECT domain E3 ubiquitin transferase	HECTD1	1	289	tagging of proteins to subcellular localization or proteasomal degradation
P01966	Hemoglobin subunit alpha	HBA	62	15	iron-containing metalloprotein / main protein for oxygen transport
P02070	Hemoglobin subunit beta	HBB	10	16	iron-containing metalloprotein / main protein for oxygen transport
F6QMU7	Histone deacetylase	HDAC5	1	55	remove acetyl groups from an ε-N-acetyl lysine amino acid on a histone
Q17QG8	Histone H2A	H2AFX	1	14	involved in the structure of chromatin in eukaryotic cells
P0C0S9	Histone H2A type 1	N/A	1	14	involved in the structure of chromatin in eukaryotic cells
A1A4R1	Histone H2A type 2-C	H2AC20	2	14	involved in the structure of chromatin in eukaryotic cells
F2Z4F9	Histone H2B	H2BC11	1	18	involved in the structure of chromatin in eukaryotic cells
E1BGN3	Histone H3	HIST2H3D	1	15	involved in the structure of chromatin in eukaryotic cells
P68432	Histone H3.1	N/A	1	15	involved in the structure of chromatin in eukaryotic cells
P84227	Histone H3.2	N/A	2	15	involved in the structure of chromatin in eukaryotic cells
Q5E9F8	Histone H3.3	H3F3A	1	15	involved in the structure of chromatin in eukaryotic cells
A5PK61	Histone H3.3C	H3-5	1	15	involved in the structure of chromatin in eukaryotic cells
P62803	Histone H4	N/A	1	11	involved in the structure of chromatin in eukaryotic cells
Q9XSA7	Chloride intracellular channel protein 4	CLIC4	1	29	form poorly selective ion channels that may also transport chloride ions
G3N3D3	Chromosome 3 C1orf68 homolog	C3H1orf68	1	28	uncharacterized protein
G3MXS3	Chromosome 6 C4orf54 homolog	C6H4orf54	1	189	uncharacterized protein
M0QVY0	IF rod domain-containing protein	KRT6A	1	61	epidermis-specific type I keratin
F1MKE7	IF rod domain-containing protein	KRT6C	1	61	epidermis-specific type I keratin
F1MX21	Inositol 1,4,5-trisphosphate receptor type 1	ITPR1	2	313	intracellular channel that mediates calcium release from the ER
Q8WN96	Inositol 1,4,5-trisphosphate receptor type 2	ITPR2	1	308	receptor for inositol 1,4,5-trisphosphate / release of intracellular calcium
Q8SPJ1	Junction plakoglobin	JUP	8	82	submembranous plaques organization / cell - cell adhesion
P02668	Kappa-casein	CSN3	2	19	stabilizes micelle formation, preventing casein precipitation in milk
A6QNZ7	Keratin 10 (Epidermolytic hyperkeratosis)	KRT10	12	55	protein heterodimerization activity
F6S1Q0	Keratin 18	KRT18	3	48	scaffold protein binding, extrinsic apoptotic signaling pathway
G3MZ71	Keratin 2	KRT2	7	64	intermediate filament organization
P06394	Keratin, type I cytoskeletal 10	KRT10	2	55	plays a role in the establishment of the epidermal barrier on plantar skin
P08728	Keratin, type I cytoskeletal 19	KRT19	7	44	important for cell-cell communication
P04262	Keratin, type II cytoskeletal 68 kDa, comp. IB	N/A	3	16	may regulate the activity of kinases
Q5XQN5	Keratin, type II cytoskeletal 5	KRT5	5	62	structural constituent of cytoskeleton
Q8N1N4	Keratin, type II cytoskeletal 78	KRT78	3	57	structural molecule / respond to external signals
Q148H7	Keratin, type II cytoskeletal 79	KRT79	1	57	structural molecule / enzyme binding
Q17QL7	KRT15 protein	KRT15	2	49	scaffold protein binding
Q3ZBL4	Leucine zipper transcription factor-like protein 1	LZTFL1	1	35	identical protein binding
A0A3Q1M701	Lipocln_cytosolic_FA-bd_dom	PAEP	7	26	retinol binding, milk protein
Q6B411	Lysozyme C, milk isozyme	N/A	1	16	bacteriolytic function / enhance the activity of immunoagents
F1MD29	Methyltransferase like 3	DNMT3B	1	70	posttranscriptional methylation of internal adenosine residues in mRNAs
G3MY07	Microtubule crosslinking factor 1	MTCL1	1	201	regulation of autophagy
F1N6H4	Microtubule-actin cross-linking factor 1	MACF1	1	827	posttranslational protein targeting to endoplasmic reticulum membrane
F6Q3P6	Misshapen like kinase 1	MINK1	1	149	cytoskeleton reorganization / stress-activated protein kinase signaling
P46196	Mitogen-activated protein kinase	MAPK1	1	42	involved in directing cellular responses to a diverse array of stimuli
F1MBK4	Neurexophilin and PC-esterase domain family 2	NXPE2	1	63	adhesion ?
A0JNN8	Neuropeptide-like protein C4orf48 homolog	N/A	1	10	signaling molecule ? Neuropeptide ?
E1BNN6	NLR family pyrin domain containing 1	NLRP1	1	166	mediator of programmed cell death
Q5NUA6	Nuclear factor erythroid 2-related factor 2	NFE2L2	1	67	transcription activator, binds ARE in the promoter regions of target genes
Q0P569	Nucleobindin-1	NUCB1	1	54	calcium ion binding / DNA binding / protein binding
Q56JW3	Nucleoside-diphosphate kinase NBR-A	LOC574091	2	17	major role in the synthesis of nucleoside triphosphates other than ATP
P31096	Osteopontin	SPP1	2	44	cell migration, adhesion / promotes cell-mediated immune responses
Q5E947	Peroxiredoxin-1	PRDX1	1	24	plays a role in cell protection against oxidative stress
Q9BGI3	Peroxiredoxin-2	PRDX2	4	22	plays a role in cell protection against oxidative stress
Q0VC07	Peroxisomal biogenesis factor 10	PEX10	1	37	biogenesis of peroxisomes / protein import into peroxisome matrix
O02811	Phosphatidylinositol 4-kinase alpha	PI4KA	2	237	signal transduction
A0A3Q1MZ56	Phosphodiesterase	PDE2A	1	105	regulate localization, duration, amplitude of cyclic nucleotide signaling
D3K0R6	Membrane calcium-transporting ATPase	ATP2B4	1	133	ATP binding, metal ion binding
P81265	Polymeric immunoglobulin receptor	PIGR	1	83	transcytosis of soluble polymeric isoforms of immunoglobulin A
P0CH28	Polyubiquitin	UBC	33	77	protein ubiquitination
F1MEC8	Protocadherin 8	PCDH8	1	112	calcium ion binding, cell adhesion
A5D984	Pyruvate kinase	PKM	1	58	catalyzes the final step of glycolysis

Accession number	Name	Gene	Peptides*	MW (kDa)	Function
A2VE99	Septin-11	SEPTIN11	1	49	GTPase activity, cellular protein localization
F1MCD0	Septin-14	SEPTIN14	1	50	GTPase activity, cellular protein localization
Q3SZN0	Septin-6	SEPTIN6	1	49	GTP binding, cellular protein localization, cell differentiation
A2VDZ0	Serine/threonine-protein phosphatase 2A	PPP2R5A	2	56	signal transduction, protein dephosphorylation
A0A0A0MP92	Serpin A3-7	SERPINAA3-7	1	47	negative regulation of endopeptidase activity
A0A0A0MP89	Serpin A3-8	SERPINAA3-8	1	47	negative regulation of endopeptidase activity
A0A140T897	Serum albumin	ALB	14	66	main function is to regulate the oncotic pressure of blood
P02769	Serum albumin	ALB	11	69	negative regulation of apoptotic process, cellular response to starvation
E1BNF9	SLAM family member 9	SLAMF9	1	32	may play a role in the immune response
A0A3Q1NHE6	Small cell adhesion glycoprotein	SMAGP	22	13	may play a role in epithelial cell-cell contacts
Q3T147	Spliceosome RNA helicase DDX39B	DDX39B	4	49	regulation of DNA-templated transcription, mRNA export from nucleus
E1BD13	Synuclein alpha interacting protein	SNCAIP	1	106	ubiquitin protein ligase binding, protein-protein interactions
E1BJT1	T-box transcription factor 18	TBOX18	1	64	transcription factor that plays a crucial role in embryonic development
O46375	Transthyretin	TTR	2	15	carrier protein, transports thyroid hormones in plasma
Q5KR49	Tropomyosin alpha-1 chain	TPM1	3	33	actin-binding proteins involved in the function of cytoskeleton
E1BJB1	Tubulin beta chain	TUBB2A	2	50	GTP binding, microtubule-based process
G1K218	Tyrosine-protein kinase receptor	IGF1R	1	13	signal transduction; key roles in growth, differentiation, and metabolism
P62992	Ubiquitin-40S ribosomal protein S27a	RPS27A	2	18	targeting cellular proteins for degradation by the 26S proteosome
O97681	V-type proton ATPase 116 kDa subunit	ATP6V0A2	1	116	essential component of the endosomal pH-sensing machinery
P80457	Xanthine dehydrogenase/oxidase	XDH	1	146	oxidation of hypoxanthine / generation of reactive oxygen species
F6PS38	Y-box binding protein 3	YBX3	5	39	nucleic acid binding, regulation of gene expression
A6QR64	ZNF131 protein	ZNF131	2	67	DNA-binding transcription activator, regulation of gene expression

*Number of identified peptides corresponding to sequence of the listed bovine protein.

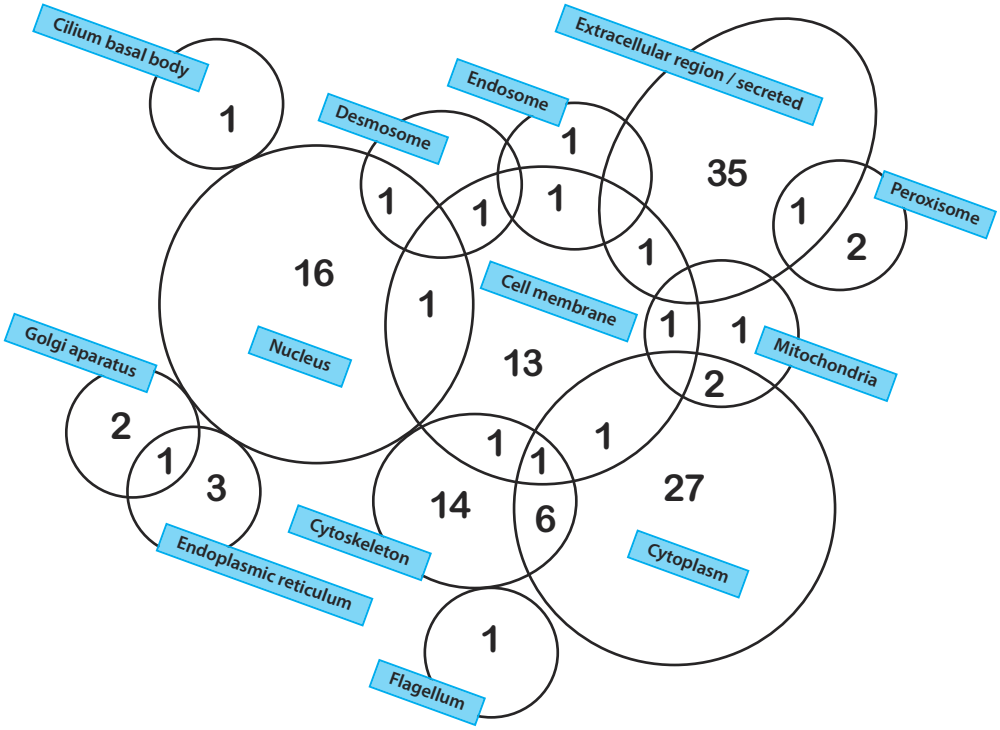


Figure 2. Venn diagram representing the eukaryotic cell compartments of bovine proteins from which the peptides of the extract were identified. Taking into account the proteins' functions, the two dominant subcellular localizations were then used as the basis for constructing the diagram.

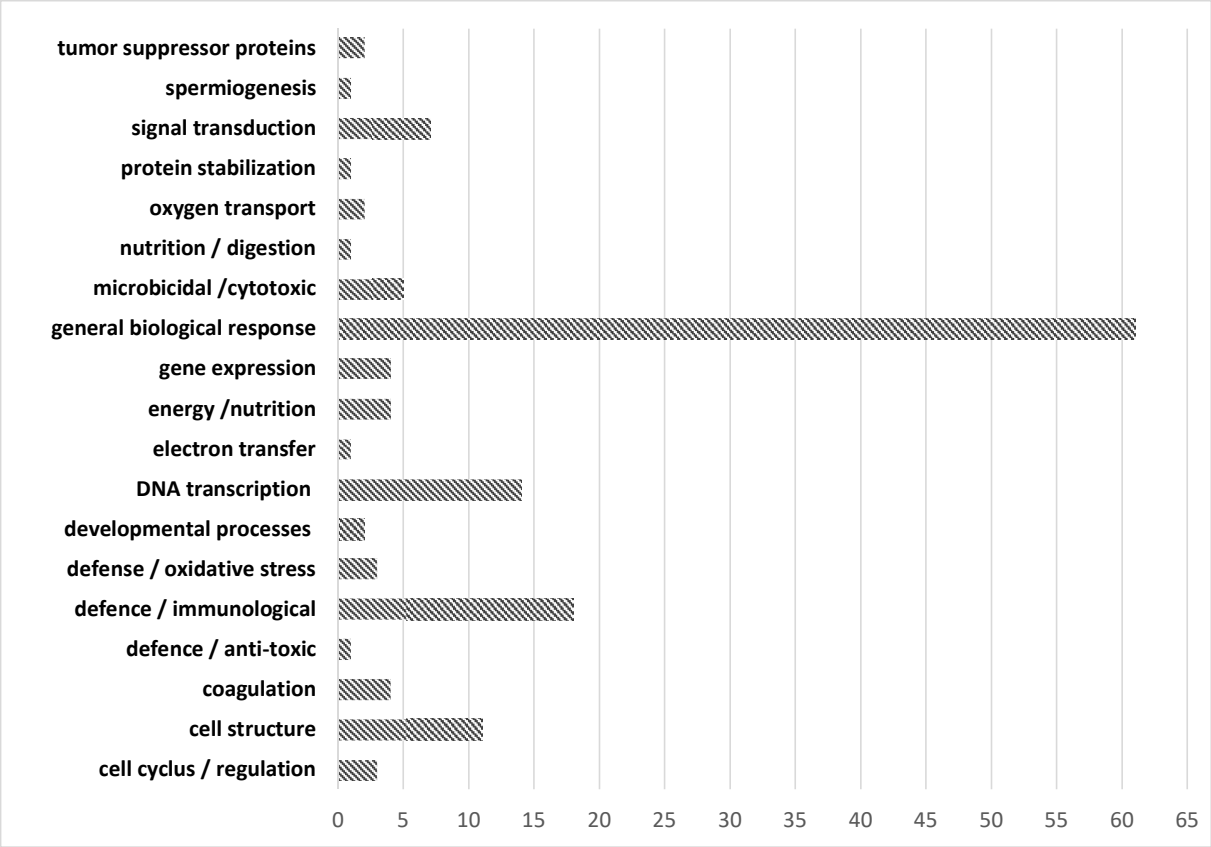


Figure 3. Functional classification of proteins from which the extract's peptides were derived.

Regarding functional classifications, proteins from which the peptides were identified perform dominantly general biological reactions or immunological defense and were the components of cell structures (Figure 3). Both subcellular localization and functional classification categories indicate that peptides from plasma proteins and proteins of cellular organelles and cytosol, of both erythrocytes and leukocytes, are present in the extract. Despite the long list of proteins from which the extract's peptides were derived, there nevertheless remains some uncertainty about a component's identification or explanation of the list of protein variants. Peptides derived from Ig-like domain-containing proteins might in reality be parts of immunoglobulin molecules. Thus, the protein with accession number A0A3Q1MI29 and identified by three peptide sequences is functionally characterized as antigen binding molecule associated with B cell receptor signaling pathway. Similarly, unidentified G5E604 protein corresponds to human immunoglobulin lambda chain of the same accession number. For some other unspecified proteins, a category of involvement in a biological process such as chemotaxis (protein F1MGE0) or immunological defense response (Ig-like domain-containing protein) has been identified. Other proteins are characterized by genes encoding a member of the cell surface signaling molecule (such as SLAM family member 9 or E1BH15 Ig-like domain-containing protein).

In accordance with the molecular weight limit for protein components of the extract of approximately 10 kDa, proteins with higher weight have been cleaved and will not be able to exhibit their assigned biological functions. Nevertheless, the peptides that were formed may have new specific functions. For example, bovine lactoferricin derived from proteolytic cleavage of bovine lactoferrin is a multi-functional peptide with anti-inflammatory and anti-catabolic function (18). Buforins are histone H2A-derived peptides that enter into bacteria and fungi, bind to microbial nucleic acids, and thereby kill them (19). Hemocidins are antimicrobial peptides derived from mammalian alpha and beta hemoglobin chains and might function similarly as do porins by disrupting plasma membranes (20-22). We made a bioinformatics analysis of the MS/MS data obtained from testing the extract to find sequences corresponding to hemocidins or hemorphins among the peptides derived from hemoglobin chains. Seven peptide

sequences corresponded to seven hemocidins, but we failed to identify the peptide sequences that correspond to bovine VV- or LVV-hemorphins and spinorphin (Table 3).

Table 3. Result of identifying peptides derived from bovine hemoglobin subunit alpha and beta with the amino acid sequence corresponding to bovine hemocidins or endorphins. A sequence corresponding to seven hemocidins was found in the extract, but we failed to identify the peptide sequences for bovine VV- or LVV-hemorphins and spinorphin.

✓ >sp| P01966X1 | HBA_BOVIN Hemoglobin subunit alpha [2—24]; Hemocidin Hba 2-24 (1-23) OS=Bos taurus OX=9913 GN=HBA PE=1 SV=2

VLSAADKGNVKAAWGKVGGHAAE

✓ >sp| P01966X2 | HBA_BOVIN Hemoglobin subunit alpha [34-62]; Hemocidin Hba 34-62 (33-61) OS=Bos taurus OX=9913 GN=HBA PE=1 SV=2

FLSFPTTKTYFPHDFLSHGSAQVKGHGAK

✓ >sp| P01966X3 | HBA_BOVIN Hemoglobin subunit alpha [99-115]; Hemocidin Hba 99-115 (98-114) OS=Bos taurus OX=9913 GN=HBA PE=1 SV=2

FKLLSHSLLVTLASHLP

✓ >sp| P01966X4 | HBA_BOVIN Hemoglobin subunit alpha [108-137]; Hemocidin Hba 108-137 (107-136) OS=Bos taurus OX=9913 GN=HBA PE=1 SV=2

VTLASHLPSDFTPAVHASLDKFLANVSTVL

✓ >sp| P01966X5 | HBA_BOVIN Hemoglobin subunit alpha [134-142]; Hemocidin Hba 134-142 (133-141) OS=Bos taurus OX=9913 GN=HBA PE=1 SV=2

STVLTSKYR

✓ >sp| P01966X6 | HBA_BOVIN Hemoglobin subunit alpha [138-142]; Hemocidin Hba 138-142 (137-141) OS=Bos taurus OX=9913 GN=HBA PE=1 SV=2

TSKYR

✓ >sp| P02070X1 | HBB_BOVIN Hemoglobin subunit beta [32-40]; VV-hemorphin-7 OS=Bos taurus OX=9913 GN=HBB PE=1 SV=1

VVYPWTQRF

✓ >sp| P02070X2 | HBB_BOVIN Hemoglobin subunit beta [31-40]; LVV-hemorphin-7 OS=Bos taurus OX=9913 GN=HBB PE=1 SV=1

LVVYPWTQRF

✓ >sp| P02070X3 | HBB_BOVIN Hemoglobin subunit beta [31-37]; Spinorphin OS=Bos taurus OX=9913 GN=HBB PE=1 SV=1

LVVYPWT

✓ >sp| P02070X4 | HBB_BOVIN Hemoglobin subunit beta [126-145]; Hemocidin Hbb 126-145 OS=Bos taurus OX=9913 GN=HBB PE=1 SV=1

QADFQKVVAGVANALAHRYH

The composition of nucleotides in the extract lacks the pyrimidine deoxynucleoside thymosin

The dominant nucleotide identified in the extract was adenosine monophosphate (Table 4). The second most abundant nucleotide was inosine monophosphate, which is typically present in animal tissues and meat industry waste. The cytidine diphosphate and a group of other unidentified diphosphates were present in smaller amounts, but we were unable to demonstrate a presence of thymidine monophosphate that is used as a monomer in DNA. Even repeated analysis did not show its presence.

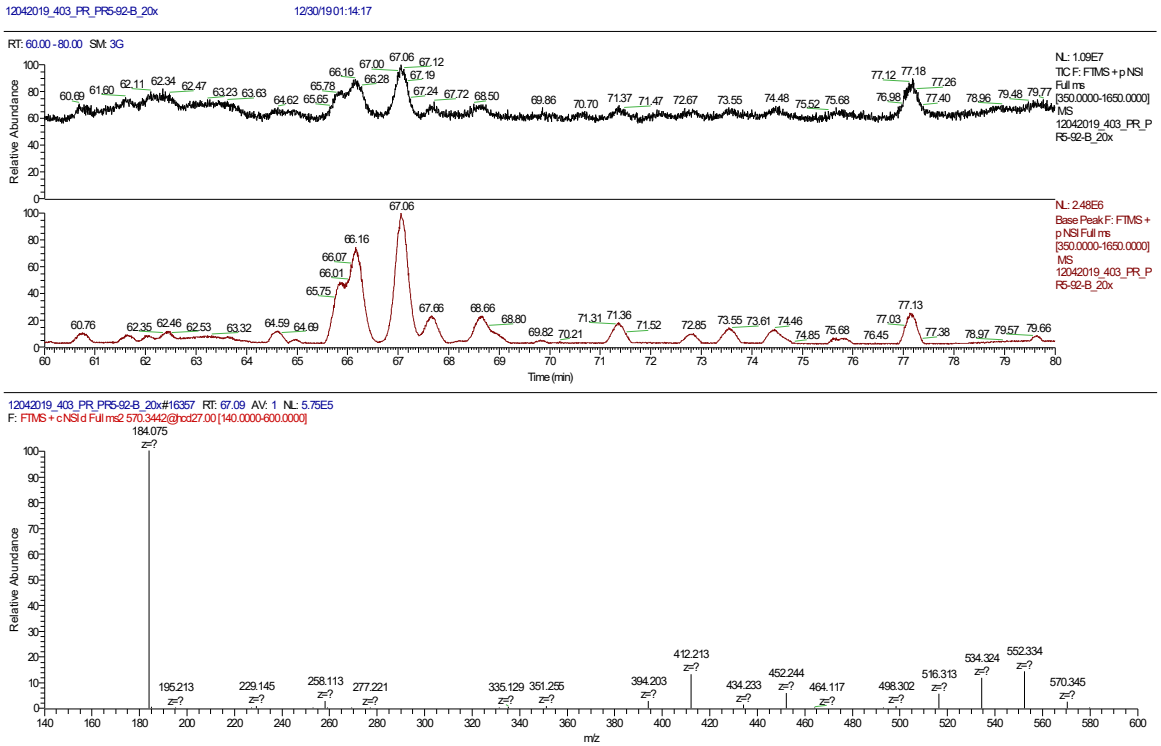
Table 4. Nucleotide content of bovine tissue extract. Two independent samples of the extract were subjected to nucleotide analysis using technical triplicates.

Nucleotides		Total content mg/100 g
Cytidine monophosphate	CMP	136 ± 16
Inosine monophosphate	IMP	390 ± 38
Guanosine monophosphate	GMP	211 ± 10
Uridine monophosphate	UMP	389 ± 56
Adenosine monophosphate	AMP	1 694 ± 94

Phospholipids

The lipids extracted from extract samples were subjected to LC-MS analysis. Similarly as in the case of proteins, only lipids with lower molecular weight were detected and subsequently their identification was based on m/z

values of lipid species using the ALEX[123] lipid calculator accessible via the LipidMaps database homepage. The quantity of (phospho)lipids in the extract suggests that their content constitutes only a residuum of phospholipids that failed to be removed by the technology used for extract production. For this reason, we identified only the dominant lipid, which is probably sphingolipid LIPC 20:1,2 (Figure 4).



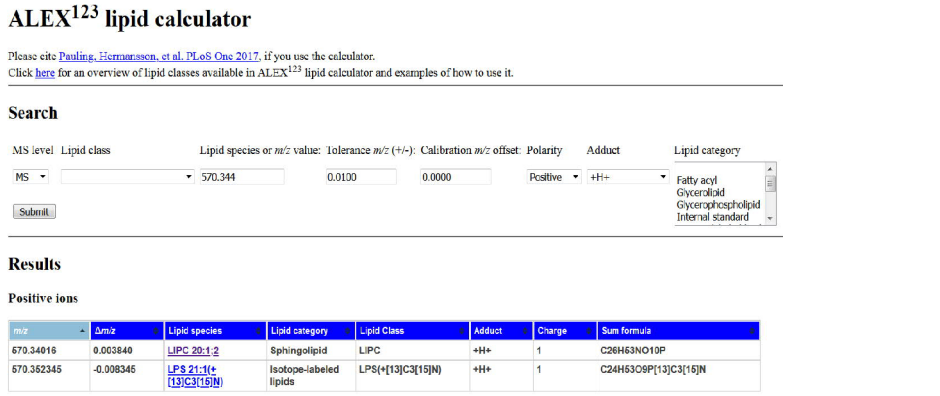


Figure 4. Identification of the extract's lipid content. One of the detected lipids was identified as sphingolipid LIPC 20:1;2.

Discussion

Many experimental or clinical studies have been performed with focus on the harmlessness and biological effects of bovine tissue extract. Little attention has been given, however, to composition of the tested preparation. Previous analyses were mainly focused on general definition as to the content of amino acids, the proteins as a whole, and nucleotides. Such analyses cannot, however, elucidate the modulatory effect of the extracts demonstrated

by changes in biological responses within *in vitro* and *in vivo* systems. Our analyses have focused on more detailed analysis of the molecular composition of bovine alcohol–ether extract to approach the possibility of understanding the relationship between the components of the extract and their potential modulatory effect.

Apart from the data on the amino acid composition of the preparation, we did not have many possibilities to compare our findings with data in the literature. Comparisons of the data from amino acid analyses performed over several decades show near-conformity of the results, thus demonstrating the constancy of the preparation's production. Current, modern amino acid analyses, however, have also revealed the presence of taurine in the extract. Taurine (2-aminoethanesulfonic acid) is an organic acid that can be found in lower concentrations in most mammalian plasma and tissues. Taurine is a derivative of the amino acid cysteine and is therefore sometimes classified as an amino acid, although it lacks a carboxyl group. This organic acid is responsible for a vast array of neurochemical changes in the brain and, subsequently, the modulation of one's organ system. Here, the neuroendocrine system is thus reflected in the function of other organ systems (23). The L-amino acids alanine, serine or aspartic acid might be converted to D- form by the process known as intrinsic racemization. Both D-Asp and D-Ser participate in processes underlying neurotransmission and in neuroendocrine regulation and signaling (24). The aromatic amino acids (tryptophan, phenylalanine, and tyrosine) serve as substrates for generating amino acid metabolites by the gut symbiont *Clostridium*, which affects intestinal permeability and systemic immunity (25). Tryptophan can be converted by gut microbiota into indole and indole derivatives that have important cellular functions, for example as intracellular signal molecule (indole), neurotransmitters (serotonin), or a highly potent neuroprotective antioxidant that scavenges hydroxyl radicals (3-indolepropionic acid) (26). Among the amino acids of the extract that are building blocks for polypeptides and ultimately protein construction are essential (phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine) as well as conditionally essential (arginine, cysteine, glycine, glutamine, proline, and tyrosine) amino acids. Supply of amino acids, and especially of essential amino acids, in balanced proportion is necessary for a high degree of net protein utilization to ensure optimal exploitation of resources for protein synthesis and metabolic energy.

The results obtained from 1-D electrophoresis demonstrated the presence of proteins and peptides with maximum molecular weight of 10 kDa. This means that, due to the technological procedures used, proteins of the original bovine tissue were chopped into peptide fragments. This is evidenced by the presence of peptides from a number of significantly higher molecular weight source proteins (see Table 2). Only proteins with molecular weights not exceeding 10 kDa could be present in the extract in their entirety. Meeting such molecular weight limitation were only apolipoprotein C-III, basal body orientation factor 1, and the group of four defensins, which are moreover defined as cationic peptides.

Destruction of proteins to peptide sequences by the technology used can be demonstrated on the hemoglobin chains, which have molecular weights only slightly higher than the mass limit of 10 kDa (15, resp. 16 kDa). The peptide fingerprint of hemoglobin alpha and beta chains disclose the presence of seven peptide sequences that correspond almost completely to the sequences of hemocidins. Hemocidins comprise a group of microbicidal peptides that arise from fragmentation of heme-binding proteins, and especially hemoglobin chains (27). The identification of hemocidins and defensins among the components of the extract can clarify the insignificant bactericidal effect of the extract, especially evident on Gram-negative bacteria (12). On the contrary, we failed to demonstrate the presence of a second group of biologically active peptides derived from hemoglobin chains, hemorphins, which could elucidate other biological effects of such extracts (28, 29). We specifically tried to identify these because they have significant therapeutic potential (30).

The analysis of the nucleotide content of the extract failed to identify the thymidine nucleotides, but other nucleotide monophosphates, cytidine diphosphate, and a group of other unidentified diphosphates were clearly identified. The failure to identify the thymidine nucleotide(s) may indicate that the nucleotides contained in the extract are either derived from the cytosol of the cells or originate from the free nucleotides of the plasma and not from nuclear DNA. Extracellular nucleotides and nucleosides participate in a number of biological processes, including regulation of cell proliferation, cell migration, and production of various growth and immune mediators. Moreover, the signaling via extracellular nucleotides is a fundamental system for intercellular communication (31, 32). Thus, it can be said that even the nucleotide content in the extract can have very important biological functions that could potentially influence the overall health status of the recipients of such animal tissue extracts.

The last group of molecular structures that were expected as a component of the extracts of animal tissues was phospholipids, which are an essential structural unit of cell membranes. We were aware that the conditions for producing the extract would remove most of the membrane phospholipids from the extract, and we wanted to see the effectiveness of the technological process of animal tissue selective extraction. The comparison of the phospholipid content of the extract with the phospholipid standard of animal tissues actually confirmed that only the low molecular phospholipids or fragments of phospholipids with higher molecular are present in the extract. In terms of possible modulatory activities of such alcohol–ether extracts of animal tissues, the presence of unspecified sphingolipids in the extract may be significant. The sphingolipids are important components of eukaryotic cells, many of which are bioactive signaling molecules. Of these, for example, ceramide is a central metabolite regulating a variety of such basic cellular characteristics as cell growth, viability, differentiation, and signaling (33).

Summary and Perspectives

The data from testing of animal ethanol–ether tissue extracts (such as Retisin, Juvenil, and Imuregen) on human volunteers have collectively demonstrated the extracts' health safety for human use (3, 34-36). Such products are regarded as regenerators of weakened or impaired biological functions. For this reason, the functional positive effects of the extract on a totally healthy human population will not be seen as dramatic. Nevertheless, some data have documented that ethanol–ether animal tissue extracts and analogous preparations will improve preparedness for adverse conditions such as psychological stresses or infections. According to these data, the extract does not act directly on metabolic processes but rather through the microbiota–gut–brain axis to modulate energy balance, production of neurohormones, or other molecular signals. Sufficient evidence has been accumulated over the long period of the ethanol–ether animal tissue extracts' availability to document their harmlessness, biological efficacy, and benefit to the human body, specifically, if the body is weakened by stress, infection, or a need for convalescence. If we summarize all available data concerning alcohol–ether extracts of animal tissues, their effect can be most accurately characterized as influencing psychobiotic processes arising from modulation of the microbiota–gut–brain axis. Accordingly, such extracts can be termed psychobiotics (37). To produce an accurate understanding of the relationship between the molecular composition of the extracts and their biological effects will require conducting further experimental and clinical studies.

Author contributions

Klara Kubelkova participated in conceptualization, decisions on methodology, and writing of the original draft.
Martin Hubalek participated in amino acids identification and MS/MS analysis of proteins.
Pavel Rehulka participated in MS/MS analysis of proteins and identification of peptides'
Helena Rehulkova participated in phospholipid identification.
David Friedecky participated in amino acid and nucleotides identification.
Jitka Zakova participated in preparation of electrophoretic procedures.
Ales Macela participated in conceptualization, investigation, decisions on methodology, data curation, and writing of the original draft.

Acknowledgements

The authors wish to thank Juvenil Products, a.s. Prezletice, Czech Republic for providing extract from bovine tissue for our experiments.

Funding

This study was supported by the Ministry of Defence of the Czech Republic - long-term organization development plan Medical Aspects of Weapons of Mass Destruction of the Faculty of Military Health Sciences, University of Defence.

Declaration of conflict of interest

The authors state that there are no conflicts of interest regarding the publication of this article.

Adherence to Ethical Standards

This article does not contain any studies involving animals performed by any of the authors. This article does not contain any studies involving human participants performed by any of the authors.

References

1. https://www.urmc.rochester.edu/profiles/display/124207
2. Kubelkova K, Macela A. A short history of Imuregen – an original tissue extract. Mil Med Sci Lett (Voj Zdr Listy). 2019;88(3):1–6.
3. Dolezal B, Rakusan B, Urbanek G, et al. Existing experiences with tissue preparations RTN. Military Medical Letters. 1956; Suppl. 1, Part II.: 3-14.
4. Richter J, Stiborova I, Kral V, et al. Glucan supplementation regulates secretory immunity andstress. Am. J. Immunol.. 2017;13(1):81-85.
5. Svozil V, Richter J, Vetvicka V. High exposure to pollution requires nutritional improvements in children. Arch Nutr Food Sci. 2020;1(2):30-34.
6. Nam SM, Maeng YS. Wound Healing and Mucin Gene Expression of Human Corneal Epithelial Cells Treated with Deproteinized Extract of Calf Blood. Curr Eye Res. 2019;Nov;44(11):1181-1188.
7. Konturek SJ, Drozdowicz D, Pytko-Polonczyk J, et al. Solcoseryl in prevention of stress-induced gastric lesions and healing of chronic ulcers. J Physiol Pharmacol. 1991;Mar;42(1):73-84.
8. Guekht A, Skoog I, Edmundson S, et al. ARTEMIDA Trial (A Randomized Trial of Efficacy, 12 Months International Double-Blind Actovegin): A Randomized Controlled Trial to Assess the Efficacy of Actovegin in Poststroke Cognitive Impairment. Stroke. 2017;May;48(5):1262-1270.
9. Søndergard SD, Dela F, Helge JW, et al. Actovegin, a non-prohibited drug increases oxidative capacity in human skeletal muscle. Eur J Sport Sci. 2016;Oct;16(7):801-7.
10. Machicao F, Muresanu DF, Hundsberger H, et al. Pleiotropic neuroprotective and metabolic effects of Actovegin's mode of action. J Neurol Sci. 2012;Nov 15;322(1-2):222-7.
11. Kubelkova K, Macela A. Microbiota-gut-brain signaling: a minireview. Mil. Med. Sci. Lett. (Voj. Zdrav. Listy). 2020;89,1-11.
12. Kubelkova K, Rychlik I, Crhanova M, et al. Gut microbiota alteration by nutritional supplement Imuregen, Mil. Med. Sci. Lett. (Voj. Zdrav. Listy). 2020;89(3):114-125.
13. Rehulka P, Zahradnikova M, Rehulkova H, et al. Microgradient separation technique for purification and fractionation of permethylated N-glycans before mass spectrometric analyses. J Sep Sci. 2018;May;41(9):1973-1982.
14. Matyash V, Liebisch G, Kurzchalia TV, et al. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res. 2008; May;49(5):1137-46.
15. Stübiger G, Pittenauer E, Belgacem O, et al. Analysis of human plasma lipids and soybean lecithin by means of high-performance thin-layer chromatography and matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom. 2009;Sep;23(17):2711-23.
16. Friedecky D, Bednar P, Prochazka M, et al. Analysis of intracellular nucleotides by capillary electrophoresis-mass spectrometry. Nucleosides Nucleotides Nucleic Acids. 2006;25(9-11):1233-6.
17. Friedecky D, Tomkova J, Maier V, et al. Capillary electrophoretic method for nucleotide analysis in cells: application on inherited metabolic disorders. Electrophoresis. 2007;Feb;28(3):373-80.
18. Yan D, Chen D, Shen J, et al. Bovine lactoferricin is in human articular cartilage and synovium. J Cell Physiol. 2013;228(2):447-456.
19. Cho JH, Sung BH, Kim SC. Buforins: histone H2A-derived antimicrobial peptides from toad stomach. Biochim Biophys Acta. 2009;Aug;1788(8):1564-9.
20. Nedjar-Arroume N, Dubois-Delval V, Miloudi K, et al. Isolation and characterization of four antibacterial peptides from bovine hemoglobin. Peptides. 2006;Sep;27(9):2082-9.
21. Machado A, Sforça ML, Miranda A, et al. Truncation of amidated fragment 33-61 of bovine alpha-hemoglobin: effects on the structure and anticandidal activity. Biopolymers. 2007;88(3):413-26.
22. Nedjar-Arroume N, Dubois-Delval V, Adje EY, et al. Bovine hemoglobin: an attractive source of antibacterial peptides. Peptides. 2008;Jun;29(6):969-77.
23. El Idrissi A. Taurine Regulation of Neuroendocrine Function. Adv Exp Med Biol. 2019;1155:977-985.

24. Bastings JJAJ, van Eijk HM, Olde Damink SW, et al. D-amino Acids in Health and Disease: A Focus on Cancer. Nutrients. 2019;Sep 12;11(9):2205.
25. Dodd D, Spitzer MH, Van Treuren W, et al. A gut bacterial pathway metabolizes aromatic amino acids into nine circulating metabolites. Nature. 2017;Nov 30;551(7682):648-652.
26. Wang G, Huang S, Wang Y, et al. Bridging intestinal immunity and gut microbiota by metabolites. Cell Mol Life Sci. 2019;Oct;76(20):3917-3937.
27. P Mak P, Wojcik K, Silberring J, et al. Antimicrobial peptides derived from heme-containing proteins: hemocidins. Antonie Van Leeuwenhoek, 2000; Apr;77(3):197-207.
28. Zhao Q, Garreau I, Sannier F, et al. Opioid peptides derived from hemoglobin: hemorphins. Biopolymers. 1997;43(2):75-98.
29. Nyberg F, Sanderson K, Glämsta EL. The hemorphins: a new class of opioid peptides derived from the blood protein hemoglobin. Biopolymers. 1997;43(2):147-56.
30. Ali A, Alzeyoudi SAR, Almutawa SA, et al. Molecular basis of the therapeutic properties of hemorphins. Pharmacol Res. 2020;Aug;158:104855.
31. Giuliani AL, Sarti AC, Di Virgilio F. Extracellular nucleotides and nucleosides as signalling molecules. Immunol Lett. 2019;Jan;205:16-24.
32. Fliegert R, Heeren J, Koch-Nolte F, et al. Adenine nucleotides as paracrine mediators and intracellular second messengers in immunity and inflammation. Biochem Soc Trans. 2019;Feb 28;47(1):329-337.
33. Hannun YA, Obeid LM. Sphingolipids and their metabolism in physiology and disease. Nat Rev Mol Cell Biol. 2018;Mar;19(3):175-191.
34. Dolezel B, Rakusan B, Urbanek G, et al. Retisin, a new tissue preparation. Cesk Farm. 1954;Sep;3(7):246-7.
35. Richter J, Sima P, Pfeifer I. Protective and imunomodulative influence of supplementing DNA, comparing of clinical testing and experimental model. Final Research Report of the project No.: NJ 6888-3, Usti nad Labem, CZE, 2004;1-61.
36. Richter J, Svozil V, Kral V, et al. Effects of dietary nucleotides on immune mechanisms and physical state in children with chronic respiratory problems. Am J Immunol. 2015;11(2):26-32.
37. Sarkar A, Lehto SM, Harty S, et al. Psychobiotics and the Manipulation of Bacteria-Gut-Brain Signals. Trends Neurosci. 2016;39(11):763-781.