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# Комплексный протеомный анализ «легкой» пептидной фракции препарата Церебролизин

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**Резюме**

**Цель исследования.** Проведение комплексного протеомного анализа пептидного состава «легкой» пептидной фракции лекарственного препарата (ЛП) Церебролизин.

**Материал и методы.** Гибридная масс-спектрометрия (МС) с орбитальными ловушками ионов с последующим использованием современных алгоритмов *de novo* МС-секвенирования.

**Результаты.** Установлены аминокислотные последовательности 14 635 пептидов, соответствующих 1643 нейрональным белкам протеома свиньи. Анализ аннотации протеома человека показал, что выявленные пептиды ЛП Церебролизин могут являться пептидами-миметиками соответствующих пептидов человека. В частности, найдено 405 пептидных фрагментов, соответствующих 300 известным биологически активным пептидам, в том числе фрагментам антибактериальных (дефенсины, гистатины), иммуномодуляторных (гранулин, мансерин) и вазоактивных (эндотелин, VIP) пептидов. При этом 8953 из 14 635 пептидов могут являться модуляторами активности 275 сигнальных белков человека, в том числе ингибировать киназы CDK1, CDK2, TGFBR2, GSK3, MTOR, проапоптотические ферменты-каспазы CASP1, CASP3 и CASP6 и др. Результаты исследования подтвердили наличие в составе ЛП Церебролизин Leu- и Мет-энкефалинов, фрагментов нейропептидов орексина, нейропептида VF, галанина и фактора роста нервов, оказывающих нейротрофическое действие.

**Заключение.** Результаты протеомного исследования пептидного состава ЛП Церебролизин указывают на широчайший круг молекулярных механизмов, обусловливающих его фармакологическое действие.

***Ключевые слова:*** *Церебролизин, протеомика, масс-спектрометрия, секвенирование de novo, алгоритмы анализа больших данных.*

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# An analysis of the peptide composition of a «light» peptide fraction of Cerebrolysin

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**Abstract**

**Objective.** To analyze the peptide composition of a light peptide fraction of cerebrolysin.

**Material and methods.** Mass spectrometry (MS) with orbital ion traps and modern de novo MS-sequencing algorithms was performed.

[1]Термин заимствован у J. Giles [1] — «age identity disorder».

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**Results.** The amino acid sequences of 14 635 peptides corresponding to the 1643 porcine proteome neuronal proteins are identified. An analysis of the human proteome annotation shows that these peptides can mimic the corresponding human peptides. In particular, 405 peptide fragments correspond to 300 known biologically active peptides, including fragments of antibacterial peptides (defensins, histatins), immunomodulatory (granulin, manserin) and vasoactive (endothelin, VIP) peptides. At the same time, 8953 of 14 635 peptides can modulate the activity of 275 human signaling proteins, including kinases CDK1, CDK2, TGFBR2, GSK3, MTOR, pro-apoptotic caspases CASP1, CASP3 and CASP6 etc. The results confirm the presence of Leu- and Met-enkephalins, fragments of neuropeptide orexin, neuropeptide VF, galanin and nerve growth factor that have a neurotrophic effect.
**Conclusion.** The results of a proteomic study of the peptide composition of cerebrolysin indicate the widest range of molecular mechanisms responsible for the clinical efficacy of this drug.

***Keywords:*** *Cerebrolysin, proteomics, mass-spectrometry, de novo sequencing, algorithms for big data analysis.*

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Cerebrolysin drug, which is obtained from the peptide extract isolated from the brain of young pigs, exerts a neurotrophic and a neuroprotective effects that are important for the recovery after ischemic stroke (IS) and for the treatment of dementia [1]. Experimental studies demonstrated a neurotrophic [2], neuroprotective [3], nootropic [4, 5], analgesic and anti-inflammatory [6], anticonvulsant [7], and antitumor [8] effects of cerebrolysin. Clinical studies showed the efficacy and safety of using cerebrolysin for the therapy of acute IS [9], after traumatic brain injury (TBI) and subarachnoid hemorrhage (SH) [10], for the treatment of cognitive [11] and autism spectrum disorders [12], as well as confirmed the anti-inflammatory and the antioxidative effects of the drug [13]. Meta-analysis of the clinical studies confirmed the advantages of using cerebrolysin for treating acute IS [14], moderate Alzheimer's disease [15] and TBI [16].

Clinical efficacy of cerebrolysin is due to the features of its peptide structure. A comparative experimental study of a set of neuropeptide extracts of animal origin demonstrated that only cerbrolysin enhanced the results of neurological testing using the ischemic stroke model [17]. Neuroplastic effect of the drug is due to its action on the neuronal signaling system of the nerve growth factor (NGF) [18], which is partially confirmed by neurocytological studies [19]. Previous studies on the peptide structure of the drug using conventional peptide sequencing procedures indicated the presence of the peptide fragments of enkephalins [20], NGF, orexin, galanin, and etc. [21] in the structure of cerbrolysin.

Thus, obtaining the most comprehensive information on the peptide structure presents one of the most important tasks of the basic research on cerebrolysin. The information on the peptides comprising cerebrolysin published to date is not complete. For instance, a mass spectrum of cerebrolysin presented in [20] indicated the presence of tens of thousands of peptides in the range of 200 to 6,000 Da in the relatively low-molecular-weight (LMW) peptide fraction (up to 1,500 kDa) comprising the drug. For this reason, high-precision mass spectrometry (MS) with microsequencing of the isolated peptides should be used for comprehensive analysis of the peptide composition of the drug cerebrolysin.

An attempt to conduct such an analysis of cerebrolysin has been made earlier and published in [22]. However, this work has some features that we cannot help but mention. First, the authors purchased cerebrolysin preparations at an internet shop but not from a licensed drug distributor and not from the manufacturer. Hence, we cannot exclude the possibility that it was not the drug cerebolysin but a counterfeit product studied in the work [22].

Secondly, the paper claims that the sequence of 638 peptides was established using the method of *de novo* sequencing (PEAKS software package). Such a very small number of peptides is a quite ambiguous result for a large-scale proteomic study involving tens of thousands of peptide fragments. The extremely low number of peptides indicates a very low efficiency of the MS data analysis procedures used in the study [22]. For comparison, the sequence of 2,221 peptides has been determined in the current study using a standard software for protein mass spectrometry analysis (MASCOT package) only, which is more than three times higher than the number of peptides identified in [22]. Using the *de novo* MS sequencing procedures developed by us earlier, we established the sequence of 14,635 proteins comprising cerebrolysin.

Thirdly, the examples of the amino acid sequences presented in [22] confirm our assumption about the possible counterfeiting of the samples analyzed by the authors of [2]. The authors claim that they did not find any peptide fragments of growth factors but observed mainly fragments of tubulin, actin and myelin basic protein (MBP) in the studied samples. However, we have found numerous peptide fragments of various neurotrophic factors in cerebrolysin samples both earlier [20, 21] and in the current study. On the contrary, only three peptide fragments of actin, namely TEAPLNPK, DFEQEM (as part of a longer LCYVALDFEQEMATAASSSSLEK fragment) and VAPEEHP (comprising VAPEEHPVLLTEAPLNPK), were found by us among those presented in [22], while the other peptides presented by the authors in [22] have not been identified by us. Therefore, as the authors stated themselves in [22], the results obtained by them indicate the problems related to the purchasing of the drugs online rather than the peptide composition of cerebrolysin.

The current paper presents the results of an MS study of the peptide composition of cerebrolysin carried out using one of the

advanced technologies of modern proteomics, the hybrid mass spectrometer, which combines the capabilities of ion chromatography and multivariate analysis of the MS data. MS orbital ion traps in these devices operate in high resolution mode and allow identification of small peptides and proteins. The fast scanning speed and parallel execution of the processes of chromatography and mass spectrometry of peptides allow identification of a vast number of peptide fragments, which is important when analyzing drugs with a complex composition.

## Material and methods

Experimental study of the peptide composition of cerebrolysin included three main stages: purification of the drug, chromatographic separation of peptides and mass spectrometry of the peptide fraction with parallel identification of the amino acid sequences.

**Drug purification** included separation of the lipid fraction and desalting to obtain a purified peptide fraction. The modified method of Brockerhoff—Dawson—Hübscher [23] was used for purification of the drug from the lipid fraction. First, mild alkaline deacylation of phospholipids was performed. The approach was worked out using a mixture of 1 mL of proteoliposomes isolated from 1—20 mg of phosphatidylcholine and 0.05—0.2 g of bacitracin or gramicidin A. Hexane and methanol mixture (1:1, v/v) was added to 1 mL of lyophilized cerebrolysin sample, the resulting solution was diluted 2 times with 0.25M NaOH in methanol. The mixture was incubated at room temperature with shaking for 45 min, then at 75 °C for 15 min. Next, methanol, hexane and water (1:4:4, v/v) were sequentially added to the solution, mixed and centrifuged at 1,000 g for 1 min. The hexane fraction was separated; water-methanol fraction was neutralized with HCl to a pH of 4—6. Hexane was added to the neutralized water-methanol fraction, the resulting solution was mixed and centrifuged at 1,000 g for 1 min; the hexane fraction was carefully aspirated in order to avoid contamination with the interphase precipitate. The procedure was repeated 4 times. Water-methanol fractions were combined and lyophilized; the precipitate was resuspended first in methanol/water (1:1), the supernatant was discharged, then the second resuspension in chloroform/methanol (1:1) solution in 0.2% TFA was conducted, the supernatants were combined, dried from chloroform and desalted.

The peptide fraction was desalted on a mini-column by centrifugation [24]. Two mL of Sephadex G-10 («Pharmacia», Sweden) decanted in methanol/water (85:15, v/v) were added to a 0.75 x 4.5 cm mini-column («Raining Instrument», USA), then 160 μL of the same solution was added drop by drop. The solution was centrifuged at 1,000 g for 1 min. The procedure was repeated until the solution volume reached 150 μL. A total of 150 μL of the sample was applied dropwise to the gel and centrifuged as described above. The gel was replaced after a single use. After the desalination procedure, protein loss did not exceed 35%.

**Chromatographic separation of peptides.** Peptides comprising the isolated peptide fraction were separated using an Ultimate 3000 RSLCnano system for parallel chromatographic separation of peptides (Dionex). The peptide fraction samples were applied to an Acclaim PepMap chromatographic trap column (2 cm, inner diameter 75 μm, $C_{18}$, 3 μm, 100 A, manufactured by Thermo Scientific) at a rate of 2 μL/min in combination 0.1% formic acid (v/v) in water. After 5 min, the trap column was automatically connected to the analytical chromatography column (Zorbax 300SB-C18 column, 15 cm × inner diameter 75 μm, 3.5 μm, manufactured by Agilent). Peptides were eluted using a mixture of solvents «A» (0.1% (vol) formic acid solution in distilled water for HPLC) and «B» (0.1% (vol) formic acid solution in 80% (vol) acetonitrile in water). Chromatographic separation of the peptides was carried out using a linear gradient of solvent B (5% to 40%) at a speed of 300 nL/min for 120 minutes followed by a washing step (with 99% solvent B for 10 min) and a re-equilibration step (with 5% solvent B for 10 min).

**Parallel mass spectrometry and peptide identification.** Mass spectrometry analysis was carried out using a Q-Exactive mass spectrometer (Thermo Scientific, Germany). Nanospray «Flex» ion source with ionization voltage of 1,800 V and a capillary temperature of 200 °C was used. Mass spectrum data were collected in accordance with the amount of precursor peptide in the MS chamber; data on the peptide particles resulting from fragmentation of the original peptides in the MS chamber (collision-induced dissociation, CID) were collected. Preliminary scanning was conducted at a resolution of 70,000, 400—1200 m/z scanning range, $1\times10^6$ target AGC values, and 50 ms maximum injection time. Fragmentation of the peptide particles in the MS chamber was carried out for the 20 most frequently observed ions with a normalized collision energy of 30, and a dynamic exclusion of 10 s. The generated ions were subjected to MS/MS with a resolution of 17.500, the target values were $10^5$, the maximum injection time was 100 ms, and the isolation window was 2.0. Peptide sequences were determined using Mascot software (http://www.matrixscience.com), SwissProt amino acid sequence database and *de novo* sequencing.

***De novo* sequencing** of the peptides was based on the collision-induced dissociation (CID) data. During the CID procedure, ions of the studied peptide are accelerated in an electrostatic field and collide with neutral particles (argon). The kinetic energy is converted into internal energy upon collision thus leading to fragmentation of the peptide ion into smaller fragments. These fragments are analyzed by tandem mass spectrometry [25] resulting in a CID mass spectrum of the *molecular fragments of the peptide*. The CID mass spectrum consists of the mass component (list of the observed fragment masses) and the intensity component (list of the observed fragment intensities) [26].

In order to obtain the amino acid sequences of the cerebrolysin peptides, for which only mass, charge and chromatographic retention time are known from [20, 21], we developed a set of DNVSEQP program tools for *de novo* sequencing of amino acid sequences using CID data. These software packages are based, firstly, on the theory of topological [27], metric [28, 29], and combinatorial [30] approaches to the analysis of big data [31, 32] and, secondly, on the theory of chemograph analysis [ 33, 34] applied to chemoinformatic tasks [35] and, in particular, to the identification of amino acid sequences using CID mass spectra.

Chemograph is *a labeled finite graph without loops with a clique number of at most 3*. The set of vertices of the χ graph is isomorphic to the set of atoms in the molecule, the set of edges is isomorphic to the set of chemical bonds in the molecule, and the adjacency matrix contains information on the chemical bond orders. Let Y be the *set of labels* (element names «C», «N», «O», and etc. are used as labels), the μ be the *vertex labeling function*: V→Y; and the *label estimation function* is defined as w: Y→R. A variation of the label estimation function is the *weighting function* m, which calculates the atomic mass of the corresponding label of the chemograph's vertex.

Let X=X(V, E) be a chemograph with the adjacency matrix $M(X)=\{m_{ij}\}$ corresponding to the studied peptide. The set $\Gamma=\{(V, E)|V\subset N, E\subset N^2\}$, which is the set of all subgraphs of the

infinite complete graph $G=(N, N^2)$, will be called the *set of all possible graphs*, $\forall X \in \Gamma$, N is the natural series. An arbitrary structural fragment of a molecule corresponds to a certain *subgraph* of the corresponding chemograph X, i.e. a graph containing some subset of vertices of the given graph and the subset of edges incident to these vertices. In other words, the set of all structural fragments of the molecule is isomorphic to the *set of all closed subgraphs* $\Pi(X)$ of the chemograph X(V,E),

$$\mathbf{\Pi}(X) = \left\{ (\mathbf{v},\mathbf{e}) \mid \mathbf{v} \subseteq V, \mathbf{e} \subseteq E, \overset{e}{\forall}(v_1, v_2) : v_1 \in \mathbf{v}, v_2 \in \mathbf{v} \right\}.$$

Let us introduce the *chemograph modification operator* $s^{n_x}: \Gamma^{n_x} \to \Gamma$, where $n_x$ is a small natural number. Practically, $n_x$ rarely exceeds 2, since the probability of a trimolecular and, especially, a tetramolecular reaction is close to zero. Let

$$S = \{ s_1^{n_1}, s_2^{n_2}, \ldots, s_{|S|}^{n_{|S|}} \},$$

be the *set of chemograph modifications*, the elements of which correspond to *post-translational modifications of proteins*, *addition/removal of OH groups*, and etc. The set of possible MS fragments is defined as $S \times \Gamma$. Thus, a certain element of the set $S \times \Pi(X)$ corresponds to an arbitrary fragment of the molecule X observed during MS with CID.

Thus, the set of chemographs $S \times \Pi(X)$ describing the *possible fragments of the molecule* X corresponds to the chemograph X. Graph *invariant*, which is a numerical characteristic or an ordered list of such characteristics (tuple), the value of which is the same for each element of an arbitrary class of isomorphic graphs, can be determined for each of these fragments. Let us call an invariant $\iota: \Gamma \to R$ the *elementary* invariant and an invariant $\iota: \Gamma \to R^n$, $n \geqslant 2$ the *tuple invariant*. The additive numerical invariant of the chemograph X for each element a=(v(a), e(a)) of the set $S \times \Pi(X)$, $v(a)=\{v_1(a), v_2(a), \ldots, v_{|v(a)|}(a)\}$, is defined as

$$\iota(a) = \sum_{j=1}^{|v(a)|} w(\mu(v_j(a))),$$

so that the tuple invariant $\iota(X)=(\iota(a)|a \in S \times \Pi(X))$ corresponds to the set $S \times \Pi(X)$. The molecular mass of an element a=(v(a), e(a)) is calculated as a special case of the elementary invariant i(a) provided that the function «w» is the weighting function «m».

A tuple invariant i(X) corresponds to the mass component of the CID mass spectrum of a molecule X and includes all the MS peaks possible for X. The experimentally observed mass component of a CID mass spectrum of the molecule, $\iota_в(X)$, is a subset of the tuple $\iota(X)$, $\iota_в(X) \subseteq i(X)$. Analysis of the solvability/regularity of the task of isomorphism assessment using tuple invariants allows us to select the most informative elements of the invariant tuples $\iota(X)$. Then, by using machine learning methods on the sets of $((\iota(X), \iota_в(X)))$ use cases and obtaining the function $f: R^n \to R^n$, $\iota_в(X)=f(\iota(X))$, one is able to calculate the mass component of the CID mass spectrum from a tuple $\iota(X)$. Calculation of the $f(\iota(X))$ function values is a key component of the *de novo* sequencing algorithm used in this study.

## Results and discussion

In this study, the composition of the peptide fraction of three series of cerebrolysin drug has been studied: B4RF1A series, manufactured on 06.2018; PB2298 series, manufactured on 06.2014; A4AB1A series, manufactured on 02.2015. All drug samples have been purchased from the governmental drug stores SBHI «Moscow Health Department of the Drug Supply Center». All samples were within their shelf life, they were released

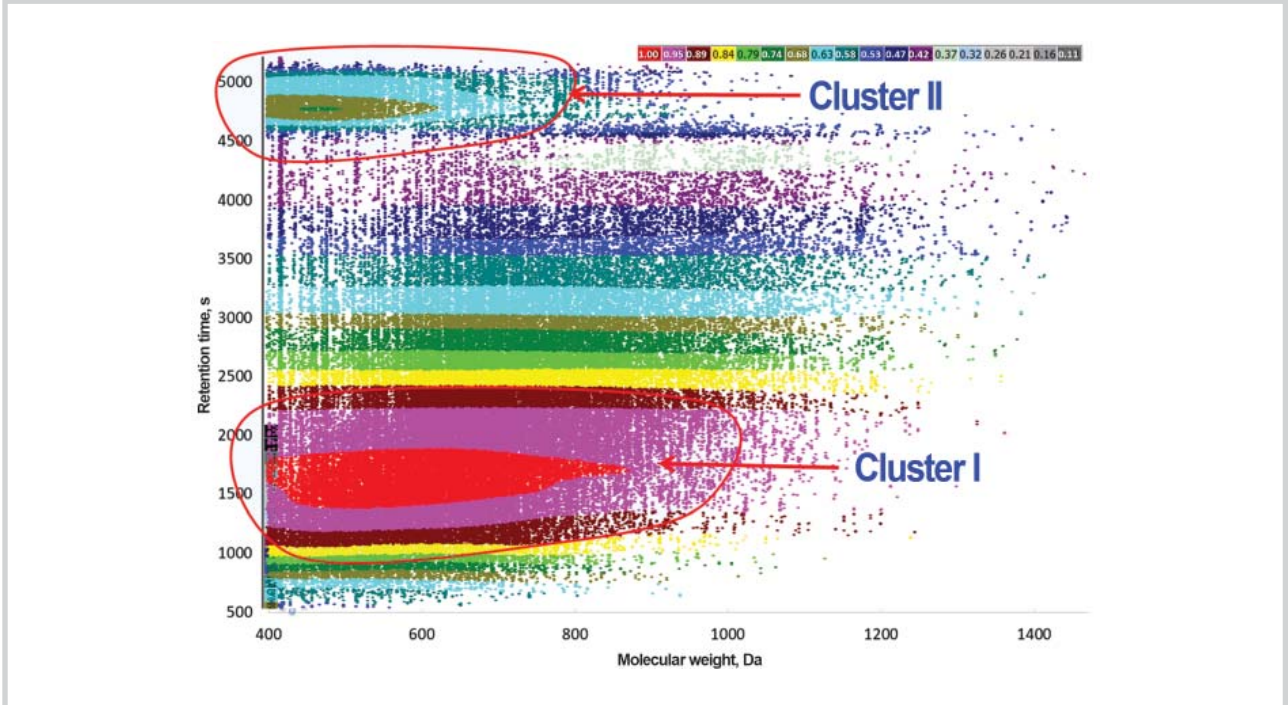


***Fig. 1.*** **Cluster analysis of the density peaks in the combined 2D mass spectrogram of the peptide fraction of cerebrolysin. Points with higher density values (red, purple, dark red, etc.) correspond to a higher abundance of peptides. Clusters (i.e., encircled density peaks) correspond to the most common values of the molecular masses of the representatives of the peptide fraction.**

within the past 4 years and studied under various conditions of the proteomic experiment. In total, 10 independent proteomic MS experiments were conducted.

The results of MS experiments are presented graphically as two-dimensional (2D) mass spectrograms. Each point on the 2D spectrogram corresponds to a peptide; the X axis represents the molecular weight of this peptide, the Y axis represents the chromatographic retention time of the peptide (which characterizes the rate of the peptide passage through the chromatographic column).

A combined 2D mass spectrogram of the peptide fraction of cerebrolysin **(Fig. 1)**, which includes the results of all proteomic experiments (171,039 peptides in 10 proteomic experiments), indicates the key features of the peptide composition of cerebrolysin. In particular, analysis of the density of point arrangement in the combined 2D mass spectrogram of the peptide fraction of cerebrolysin indicates the predominance of peptides with molecular masses in the range of 400 to 800 Da (which approximately corresponds to an average peptide length of 5 to 9 amino acids) in the low-molecular-weight (LMW) peptide fraction of cerebrolysin.

Cluster analysis of the density peaks in the combined 2D mass spectrogram of the LMW peptide fraction of cerebrolysin confirmed that almost 90% of all registered cerebrolysin peptides are of sufficiently low molecular weight (400 to 600 Da, cluster II; 400 to 800 Da, cluster I). Cluster II peptides, which have high retention time (i.e., the time of passage through a chromatographic column is less than 5,000 s), turned out to be predominantly hydrophobic peptides with no established biological function.

Determination of the exact amounts of individual peptides using mass spectrometry data is a complex experimental task. The solution to this problem becomes even more complicated in the case of studies of natural peptide-containing drugs (in which the amounts of individual peptides can vary significantly). Therefore, in this work, we used a 10-point scale to estimate the number of peptides. This scale is based on the hypothesis that the higher the concentration of the peptide in the studied sample is, the more proteomic experiments reveal the presence of this peptide. For a given number of proteomic experiments (10 experiments in the present study), the score for evaluating the content of a given peptide is the number of proteomic experiments which revealed a peptide with a given amino acid sequence.

As a result, using this scale, we identified 14,635 peptides corresponding to 1,643 proteins of the porcine proteome involved in neuronal function **(Fig. 2)**. Using *de novo* MS sequencing, we determined the amino acid sequences of these peptides, and each of the peptides was observed in at least 2 out of 10 proteomic samples (i.e., a score not lower than 2).

In order to evaluate the biological role of the identified peptides, we compared them with the proteins of the human proteome. The identified 14,635 peptides corresponded to 23,334 peptide annotations in the human proteome. Human proteome annotations were obtained using UNIPROT data analysis [36]. An expert analysis of the obtained annotations demonstrated that the sequenced cerebrolysin peptides can be mimetics of the corresponding peptides in the human proteome. A total of 13,997 annotations corresponded to mimetic peptides that can inhibit 275 human kinases, including CDK1, CDK2, TGFBR2, ABL1, GSK3-beta, and MTOR. Inhibition of GSK3-beta and MTOR kinases corresponds to the neurotrophic and neuroprotective effects of cerebrolysin, while inhibition of kinases CDK1, CDK2, TGFBR2, and ABL1 corresponds to the antitumor effect of the drug [8]. Moreover, 5,657 of the identified annotations corresponded to 405 peptide fragments from more than 300 biologically active peptides (including neurotrophic neuropeptides). Another 1,967 annotations corresponded to mimetic peptides that can potentially inhibit the pro-apoptotic caspase enzymes CASP1, CASP3, and CASP6.

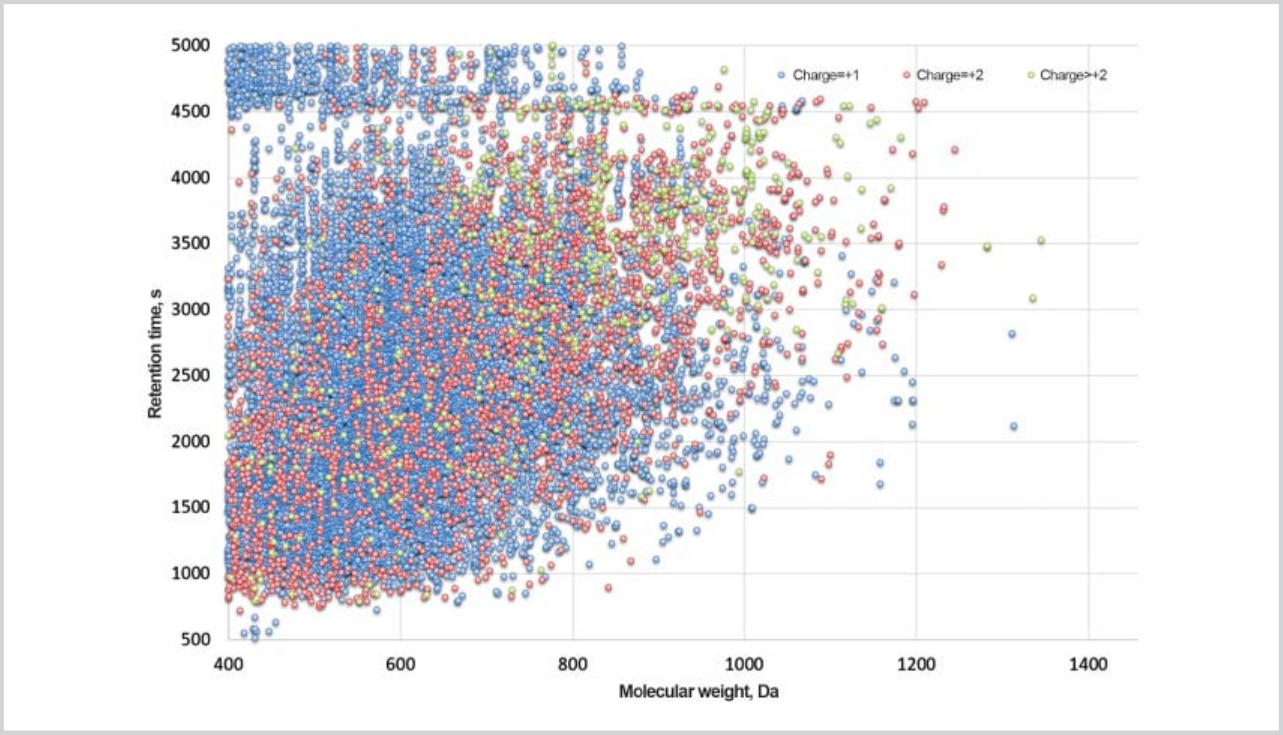


***Fig. 2.*** **2D mass spectrometry of 14,635 peptides, each of which was found in at least 2 out of 10 proteomic samples. The points corresponding to the selected peptides are colored according to the peptide charge.**

**Table 1. Confirmation of previously obtained data on the peptide composition of cerebrolysin using *de novo* mass spectrometric sequencing. "Est. No" – estimated number of peptides according to the 10-point scale (see article text).**

| Studied peptides [21] | NCBI/SWISSPROT protein annotation | Current study | Est. No |
|---|---|---|---|
| GEFSV, NSYCTTT | Q29074 Nerve growth factor (NGF) | 54 peptides, including.: | |
| | | DLEASG | 10 |
| | | PDLEAS | 7 |
| | | VLFSTQ | 5 |
| | | PPVAAD | 5 |
| | | NVPAGH | 5 |
| | | GEFSV | 5 |
| | | NSYCTTT | 4 |
| YGGFL, GGFLR | P01214 Beta-neoendorphin-dynorphin | 13 peptides, including.: | |
| | | YGGFLRR | 10 |
| | | YGGFL | 8 |
| | | GGFLR | 8 |
| | | YGGFLR | 6 |
| | | GEGDGD | 6 |
| | | GGFLRR | 5 |
| | | DAQQND | 3 |
| YGGFM | P01192 Enkephalins, opiomelanocortin | 85 peptides, including.: | |
| | | DLSAET | 10 |
| | | LLLTLLL | 10 |
| | | YGLVAE | 10 |
| | | PLVTLF | 9 |
| | | YGGFM | 8 |
| | | QPLTEN | 6 |
| | | LVAEAE | 5 |
| | | LTENPR | 5 |
| | | DLSTES | 5 |
| PQRF | ACQ82801 Neuropeptide VF | 45 peptides, including.: | |
| | | LPLRFG | 10 |
| | | LNFEEL | 10 |
| | | FANLPL | 10 |
| | | SFANLP | 8 |
| | | VPNLPQ | 4 |
| | | PLRFGR | 4 |
| | | NLHSK | 4 |
| | | NFEELKD | 4 |
| | | MEVSLV | 4 |
| | | EVSLVR | 4 |
| | | PQRF | 3 |
| CCRQK | O77668 Orexin | 17 peptides, including.: | |
| | | LYELLH | 7 |
| | | YELLHG | 3 |
| | | QASGNH | 3 |
| | | CCRQK | 3 |
| WWLNSAGY | P07480 Galanin | 21 peptides, including.: | |
| | | LNSAGY | 10 |
| | | GLGSPV | 5 |
| | | YLLGPH | 4 |
| | | LQSEDK | 4 |
| | | LGLGSPV | 3 |
| | | FLAFLH | 3 |
| | | LGSPVK | 2 |

One of the most valuable results of the current study is *the confirmation of the previously obtained data on the presence of the fragments of neurotrophic factors among the peptides of cerebrolysin*. Analysis of the peptide composition of the drug, which have been conducted earlier using standard protein sequencing procedures, indicated the presence of enkephalin peptide fragments (YGGFL, GGFLR, YGGFM), NGF (GEFSV, NSYCTTT), orexin (CCRQK), galanin (WWLNSAGY), and etc. in cerebrolysin [21]. The current study confirmed the presence of these peptide fragments as well as the presence of other peptide fragments of the above-mentioned neurotrophic factors. In addition, semi-quantitative estimates of the peptide content in cerebrolysin were obtained **(Table 1)**.

The mechanisms of action of the neurotrophic factors presented in Table 1 were considered in detail in [20, 21]. Briefly, *nerve growth factor* (NGF) activates TrkA and LNGFR receptors and participates in restoration of the damaged neuron networks in IS and TBI. Some model peptides exhibiting NGF-like activity, including GEFSV peptide (119—123), are known [37].

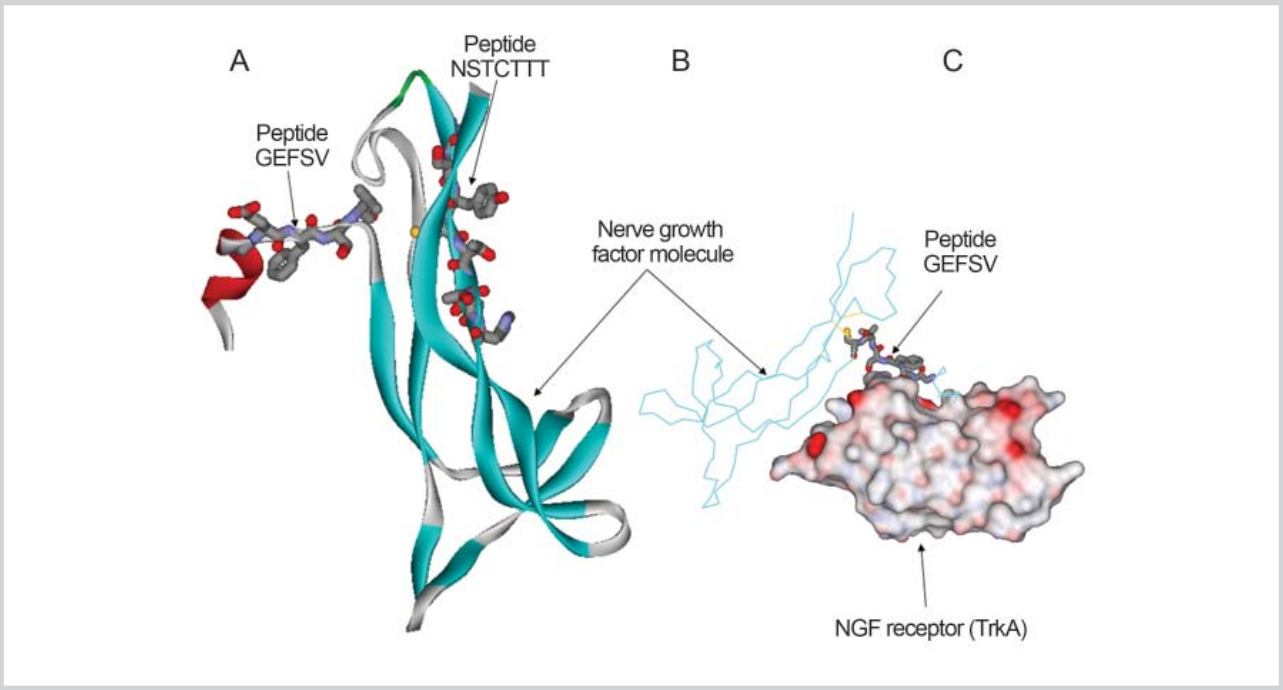


***Fig. 3.*** **The structure of the nerve growth factor molecule. A) Cerebrolysin peptides. B) Interaction of the GEFSV peptide (fragment of the NGF molecule, residues 119-123) with the NGF receptor molecule (PDB-1www-based model). C) Peptides comprising the NGF pro-peptide (PDB-1a6f-based model).**

The NGF peptide fragments found in this study, which result from the non-specific proteolysis of the porcine proteome during the production of cerebrolysin, belong to (1) the *fragmented NGF pro-peptide* (amino acids 19—121, Q29074, UNIPROT database), including peptides VLFSTQ 75—80, PPVAAD 82—87, PDLEAS 91—96, DLEASG 92—97, and etc. **(Fig. 3)**, (2) the *NGF signal peptide* (NVPAGH residues 13—18 and etc.), and (3) the mature NGF molecule (peptides GEFSV 119—123, NSYCTTT 187—192, Fig. 3). As shown earlier [21], cerebrolysin peptides GEFSV and NSYCTTT can interact with the TrkA receptor, which is involved in the neurotrophic action of NGF.

*Enkephalins* YGGFL and YGGFM are endogenous opioid peptide neurotransmitters that maintain numerous physiological and psychological functions, including regeneration of neurons. *Orexin* upregulates neurotrophin-3 expression [38] thus contributing to the survival and differentiation of neurons. The CCRQK peptide may be important for the interaction of orexin-A with receptors [20]. *Galanin* modulates the secretion of acetylcholine, serotonin, and norepinephrine [39], it is necessary for the sprouting of axons [40].

In addition to confirmation of the results of the previous studies on the composition of cerebrolysin, the present analysis indicated the existence of 405 peptide fragments in cerebrolysin corresponding to known biologically active peptides, including antibacterial peptides (defensins, histatins, etc.), immunomodulatory peptides (granulin, manserin, etc.), peptides regulating lipid metabolism (spexin, gastrin, obestatin, etc.), and vasoactive peptides (endothelin, VIP, etc., **Table 2**).

Among the peptides listed in Table 2, *proenkephalin* (peptides KELLQL and LLQLSK) is a synergist of enkephalins, the *PACAP peptide* is a vasoactive neurotransmitter and neuromodulator. *Neuroendocrine regulatory peptide-1* inhibits salt-induced or angiotensin 2-induced secretion of vasopressin in the hypothalamus and the pituitary gland [41]. *Galanin-like peptide* maintains the effects of galanin, while *neuropeptide-glycine-glutamic acid* mediates the energy metabolism in neurons. *Neuropeptide K* has a hypotensive and a nootropic effects, it also regulates the energy metabolism of the central nervous system [42]. *Neuromedin—B—32* regulates neuronal growth, glucose levels, arterial blood pressure and exhibits nootropic effects through CREB [43]. *Neuromedin-S* maintains a circadian rhythm, regulates the synthesis of other peptide hormones and exerts a neurotrophic effect [44].

## Conclusion

Despite the evidence and the results of numerous experimental studies, the exact mechanisms of the pharmacological action of the drug cerebrolysin deserve a detailed study, since they remain out of sight for most neurologists. *De novo* sequencing of the peptide fraction of the drug cerebrolysin carried out in the current study using hybrid mass spectrometry data allowed us to establish the amino acid sequences of 14,635 peptides associated with the functioning of neurons. One of the most important results is confirmation of the presence of active neuropeptide fragments of Leu- and Met-enkephalins, orexin, neuropeptide VF, galanin, and nerve growth factor in the composition of cerebrolysin. In addition, for the first time, 8,953 peptides comprising cerebrolysin that can act as modulators of the human kinome (including kinases CDK1, CDK2, TGFBR2, GSK3, MTOR) and inhibitors of pro-apoptotic and pro-inflammatory caspases CASP1, CASP3 and CASP6 have been identified. These results make a significant contribution to the understanding of the wide range of pharmacological effects of the drug cerebrolysin (primarily, neurotrophic, neuroprotective and anti-inflammatory effects). Identification of the peptides that (1) are the most abundant in cerebrolysin samples, (2) modulate human kinome

**Table 2. Examples of other neuropeptide fragments identified in the current study. Peptides are ordered in descending order of estimated number.**

| Peptide | SWISS-PROT | Neuropeptide | Est. No |
|---|---|---|---|
| LELELG | Q5JTD0 | Neurokinin B | 6 |
| EWLSPR | O15130 | Neuropeptide AF | 5 |
| KELLQL | P01210 | Proenkephalin | 5 |
| LAAVLG | P18509 | Pituitary adenylate cyclase-activating polypeptide 27 (PACAP) | 5 |
| YGGFMR | P01210 | Met-enkephalin-Arg-Gly-Leu | 5 |
| DLPEPR | P08949 | Neuromedin-B-32 | 4 |
| FHLLRE | P06850 | Corticoliberin | 4 |
| LLGGSE | O15240 | Neuroendocrine regulatory peptide-1 | 4 |
| LLLMDD | Q8WNQ7 | Galanin-like peptide | 4 |
| LLQLSK | P01210 | Proenkephalin | 4 |
| PYLALK | P20382 | Neuropeptide-glycine-glutamic acid | 4 |
| QVALLK | P20366 | Neuropeptide K | 4 |
| ELELGQ | Q5JTD0 | Neurokinin B | 3 |
| LDLTFH | P06850 | Corticoliberin | 3 |
| LDKFKDK | Q5JTD0 | Neurokinin B | 3 |
| SVAFPA | P20382 | Neuropeptide-glycine-glutamic acid | 3 |
| VAFPAE | P20382 | Neuropeptide-glycine-glutamic acid | 3 |
| VDFTKK | Q5H8A3 | Neuromedin-S | 3 |
| AALLTG | Q8WNQ7 | Galanin-like peptide | 2 |

activity by enhancing the neurotrophic effects of the drug and antitumor protection, (3) are characterized by a multi-target action (one peptide can inhibit several kinases at once and etc., which underlies the pleiotropic effect of cerebrolysin) and (4) have an anti-inflammatory effect as well presents a promising trend for further studies of the peptide composition of cerebrolysin.

## Author's Credentials

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