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Proteomic Analysis Methods for Characterization of Proteins from the Salivary Gland Secretions of the Medicinal Leech during Different Seasons

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Abstract—Salivary gland secretion (SGS) of the medicinal leech *Hirudo medicinalis* in summer and winter was studied by proteomic analysis methods, and season-associated difference was found in the distribution of fractionated proteins with the same pattern of their positions. Differences were detected for proteins with molecular weights from 15 to 250 kD fractionated by two-dimensional SDS-PAGE and for 2-10- and 10-60-kD proteins analyzed by SELDI-MS. Thirty-two and 20 proteins were detected by MALDI-TOF-MS in the high-molecular-weight fraction of the summer and winter SGS, respectively, isolated from the corresponding two-dimensional electrophoregrams, and this was less than 20% of the total SGS protein. The N-terminal amino acid sequences were determined for 12 proteins. The peptide maps and N-terminal amino acid sequences of the proteins studied were identified, and no known proteins were revealed. These findings suggest a high content of newly revealed proteins in SGS of medicinal leech, and this correlates with multiple positive clinical effects of hirudotherapy realized through SGS, but the mechanisms of these effects remain unclear.

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Key words: proteomic analysis, salivary gland secretion, medicinal leech (*Hirudo medicinalis*), seasonal prevalence, two-dimensional electrophoresis, SELDI mass spectrometry, N-terminal protein sequencing

The humoral effect of hirudotherapy, which is widely used for treatment of various diseases [1-5], is associated with the action of the salivary gland secretion (SGS) of the medicinal leech (*Hirudo medicinalis*). However, the composition of SGS can be nonhomogenous, and this can significantly influence results of the treatment. Thus, we showed that the SGS portions secreted by the leech salivary glands during blood suction had different absorption at the wavelength of 210-320 nm depending on the moment of suction [6]. We also observed that the maximum antithrombin activity of the leech secretion occurred in winter (I. P. Baskova and G. I. Nikonov, unpublished data). Thus, it is important to compare the protein composition of SGS in summer and winter.

The determination of profile of the high-molecular-weight fraction of SGS by methods of proteomic analysis revealed the multiplicity (more than 100) of proteins different in molecular weights and isoelectric points [7]. These methods, two-dimensional SDS-PAGE and SELDI-MS, which combine chromatography of proteins by microarray technique and their detection by mass

Abbreviations: 2D-electrophoresis) two-dimensional electrophoresis; HMWF) high-molecular-weight fraction; IEF) isoelectric focusing; MALDI-TOF-MS) mass spectrometry of laser desorption-ionization; MS) mass spectrometry; SELDI-MS) mass spectrometry of laser desorption-ionization with a preliminary choice of the fraction by microarray technique; SGS) salivary gland secretion of the medicinal leech *Hirudo medicinalis*.

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spectrometry (MS) [8], have been used in the present work for comparison of SGS preparations obtained from medicinal leech in summer and winter. We have also attempted (in the absence of data on the medicinal leech genome) to obtain peptide maps for some proteins from the summer and winter secretions and compare them with the databases of known proteins.

MATERIALS AND METHODS

Preparations analyzed. SGS were obtained from the medicinal leech (*Hirudo medicinalis*) as described in [6]. Each preparation was a pool of SGS from at least 50 leeches. The high-molecular-weight fraction (HMWF) of SGS was prepared as described in [7]. The summer and winter secretion was taken in June-August and November-February, respectively. One preparation of the native summer secretion, one preparation of HMWF from the summer secretion, and two preparations of the winter secretion were used in the present work. Both the native secretion and its HMWF were concentrated as described in [7].

Separation of proteins by 2D-electrophoresis. Two-dimensional electrophoresis was performed using a Protean IEF Cell apparatus (Bio-Rad, USA) for immunoelectrophoresis (IEF) of proteins and a Protean II xi Cell (Bio-Rad) setup for 2D-SDS-PAGE. IEF was performed in glass tubes (internal diameter 1.5 mm, length 170 mm). To separate the proteins in the first direction, the capillary tubes were filled with a solution containing 3.5% (w/v) acrylamide, 8.5 M urea, 1.5% (w/v) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 0.5% (w/v) NP-40 (nonaethylene glycol octylphenyl ether) or PEG (octylphenyl ether), 0.3% (v/v) ampholytes (pH 3.5-10), 0.3% (v/v) ampholytes (pH 5.0-8.0), 0.05% (v/v) TEMED, 0.05% (w/v) ammonia persulfate. A specimen containing 240 μg protein was mixed with two volumes of buffer containing 7 M urea, 2 M thiourea, 60 mM DTT, 65 mM CHAPS, 5% ampholytes (pH 3.5-10.0). After incubation for 15 min at room temperature, 15 μl of the specimen was placed onto the basic end of the gel in the tube. As cathode and anode buffer, 50 mM NaOH in water and 6 mM H_3PO_4 in water, respectively, were used. The proteins were separated using the following electric scheme: every step of 100, 200, 300, 400, and 500 V for 45 min and 700 V overnight. In the second direction, the proteins were separated in a standard 9-16% polyacrylamide gel at 10°C, 30 mA onto the gel, as described in [8]. The proteins were stained with silver in the presence of sodium thiosulfate [9].

Detection of proteins. Corresponding gel fragments were excised from the stained spots and treated successively with sodium bicarbonate and acetonitrile, then the protein of the gel was hydrolyzed with trypsin, and the produced peptides were extracted according to protocols [10]. The resulting specimens were analyzed by MALDI-TOF-MS using a Reflex III MALDI-TOF mass spectrometer (Bruker, USA) with a UV laser (336 nm) in the regimen of positive ions in the mass range of 0.5-8 kD. The proteins were identified using sets of peptide products of trypsinolysis with the Peptide Fingerprint option of the Mascot program (Matrix Science, USA).

Analysis by SELDI-MS. The method included microarray technique with detection of proteins by SELDI-MS. SGS was diluted with deionized water to protein concentration of 0.1 mg/ml. The diluted secretion (2 μl) was applied onto an NP20 microarray with the normal phase based on silica gel and washed according to the producer's instruction (Ciphergen Biosystems Inc., USA). Mass spectra of the extracts were obtained by SELDI-MS using a ProteinBioSystem™ II instrument (Ciphergen Biosystems Inc.). The proteins were analyzed in the range of 2-10 and 10-60 kD, using as a matrix α-cyanohydroxycinnamic acid (Sigma, USA) as 50%-saturated solution in 0.1% trifluoroacetic acid in water containing 50% (v/v) acetonitrile. Mass spectra of compounds with molecular weight of 2-10 kD were obtained at the laser intensity of 200 and sensitivity 8; for compounds with molecular weight >10 kD the laser intensity was 230 and sensitivity 9 (internal conventional characteristics of the system).

Further calibration of the mass spectra was performed using equations of the external calibration (Peptide and Protein Calibration Kits; Ciphergen Biosystems Inc.). The accuracy of the weight determination was better than 1%.

Determination of N-terminal amino acid sequence of proteins. The proteins present in the polyacrylamide fragments were hydrolyzed in 6 M HCl and analyzed routinely by the Edman method using a Protein Sequencer model 477 (Applied Biosystems, USA).

Isolation of total DNA from medicinal leech. Leech tissue (10-20 mg) was frozen in liquid nitrogen, homogenized, resuspended in 800 μl of buffer TE (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)), supplemented with SDS to the final concentration of 1%, and heated at 37°C for 30 min, then supplemented with proteinase K to the final concentration of 400 μg/ml, and incubated for 18 h at 56°C. Then DNA was successively extracted with phenol and chloroform and precipitated by addition of two volumes of 96% ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2). After centrifugation at 14,000 rpm, the DNA was washed in 70% ethanol, dried, and resuspended in water.

Synthesis of oligonucleotides. Based on the N-terminal amino acid sequence of the initial peptide QGTELEL with possible substitutions (A in position 1 and N in position 3) the variants of synthesis included the initial sequence, the sequencing variants by the Edman method, and degenerated nucleotides.

The following oligonucleotides were synthesized.

Forward:

1_18nf, ATGTTGACCGAGCTGGAG;

1_23nf, ATGTTGACCGAGCTGGAGAAAGC;

Deg1f, ATG(T,C,G)(T,C)(G,A)(A,T)C(C,T)GA(G,A)-CTGGAGAA;

Deg2f, ATG(T,C,G)(T,C)(G,A)(A,T)C(C,T)GA(G,A)-CTGGAG;

Deg3f, ATG(T,C,G)(T,C)(G,A)(A,T)C(C,T)GA(G,A)-CTGGAGAA(A,G)GCCTTG;

Edm0, CAGGGCACCGAACTGGAACTG;

Edm40, CARGGBACBGARCTGGARCTG;

Edm100, CARGGNACNGARYTNGARYTN.

Reverse:

141_121nr, CCTGATATACTGAGGACACTC;

132_111nr, CTGAGGACACTCGGTCTCTAGC;

90_70nr, GTAGACGGCATGGAAATTCCC;

Deg1r, ACTGAGG(G,A)CACTC(G,A)GT;

Deg2r, ACTGAGG(G,A)CACTC(G,A)GT(A,C)(G,T)-(T,C)(G,T)A(G,C)C.

PCR amplification. PCR amplification was performed using DNA polymerases Taq and Pfu (Fermentas MBI, Lithuania). Temperature conditions of the amplification were as follows: 95°C for 3 min; 95°C for 30 sec, 50-65°C for 30 sec, 72°C for 30 sec (30-40 cycles); 72°C for 5 min.

Results of amplification are presented in Fig. 1. The amplicons were excised from the gel and purified using a Wizard PCR Preps kit (Promega, USA). The ligation was performed into the plasmid vector pGEM-Teasy (Promega), and the sequencing was performed using an ABI Prism 3100 automated sequencer (Applied Biosystems). The sequencing was performed from the primers complimentary to the plasmid sequences:

M13 for GTAAAACGACGGCCAGT,

M13 rev CAGGAAACAGCTATGAC.

The sequencing was performed of the amplicons resulting from amplification with the following pairs of primers: clones 10, 11 – Deg3f, Deg1r; clones 15, 16 – Deg3f, Deg2r; clones 17, 23, 28, 29 – Edm0, Deg1r; clones 38, 39 – Edm40, Deg1r.

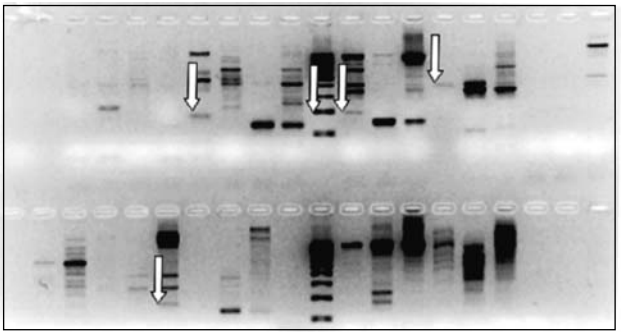

Fig. 1. Electrophoregram of PCR products. The arrows indicate the amplicons that were later subjected to cloning and sequencing.

The nucleotide sequences were compared using the BLAST program (www.ncbi.nlm.nih.gov).

RESULTS

Seasonal differences of SGS preparations. Comparison of 2D-electrophoregrams. Quantitative and qualitative differences between the winter and summer SGS of medicinal leech were studied by 2D-electrophoresis using IEF in tubes at pH values from 3.5 to 10. Gel staining with silver revealed, on average, 156 protein spots in the range of molecular weight from 15 to 250 kD on the standard (18 × 20 cm) 2D-gel. Figure 2a presenting averaged data of the SGS preparations (each obtained from at least 50 leeches) shows that at pH values from 5 to 8 the proteins are mainly located in the range of 30-50 kD. Notwithstanding the common pattern of protein spot location in the winter and summer leech secretion, significant differences have been revealed in the protein composition of the two secretions and in the expression level of individual proteins manifested by the staining intensity of the spots. Quantitative evaluation of the staining intensity (in relative units) of only the spots present in both gels is shown in Fig. 2b as a histogram after processing the gel using the PDQuest Advanced 8.0 program (Bio-Rad Laboratories).

Comparison of SELDI-MS analysis results. Seasonal differences of the SGS preparations manifested themselves by changes in the protein composition in the range of 2-10 kD and of 10-60 kD (Table 1). Thus, there are distinct differences in the distribution of low-molecular-weight proteins and peptides. The proteins with molecular weights of 2812.3, 3069.8, 5151.0, 5151.6, and 7713.5 daltons detected in the summer SGS were not found in the winter SGS, whereas proteins with molecular weights of 3403.2, 4309.6, 5454.8, 5513.1, and 7026.5 daltons

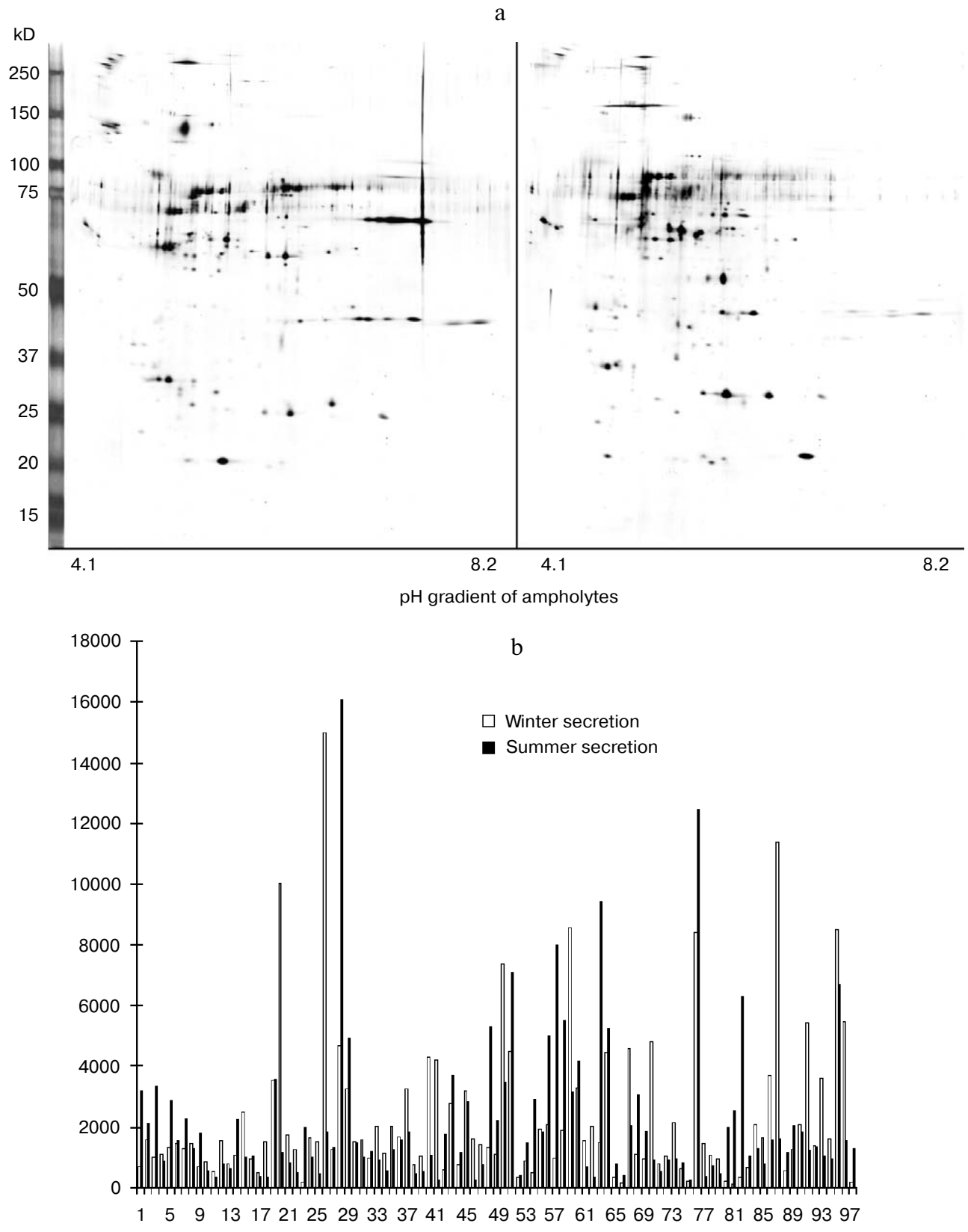

Fig. 2. a) 2D-PAGE of the winter (to the left) and summer (to the right) salivary gland secretion of medicinal leech. Along the abscissa axis, the ampholyte gradient in the pH range 4.1-8.2. Along the ordinate axis, molecular weights from 10 to 200 kD. b) Histogram of the intensity of 97 spots in the winter and summer SGS expressed in relative units (along the ordinate axis).

Table 1. Characteristics of the main (significant) peaks from SELDI-MS for summer and winter SGS

Summer secretion		Winter secretion	
molecular weight/ charge	relative intensity of the signal	molecular weight/ charge	relative intensity of the signal
Range of molecular weights from 2 to 9 kD			
2384.3 + H	17.7	2383.9 + H	9.47
2812.3 + H	21.6	2615.0 + H	7.36
3069.8 + H	24.6	3024.1 + H	7.90
3260.5 + H	56.2	3260.6 + H	41.05
		3403.2 + H	5.79
3559.2 + H	19.4	3558.9 + H	11.3
4052.1 + H	16.5	4052.8 + H	10.5
		4309.6 + H	10.0
4734.4 + H	12.5	4744.5 + H	11.8
4840.8 + H	12.9	4826.0 + H	10.3
5151.0 + H	10.8		
5151.6 + H	10.3		
		5454.8 + H	11.4
		5513.1 + H	10.5
		7026.5 + H	10.3
7713.5 + H	9.5		
8099.4 + H	12.3	8101.1 + H	8.3
Range of molecular weights from 10 to 60 kD			
		13102.9	9.5
15374.8 + H	10.3		
17438.7 + H	9.2	17492.8	10.8
20548.9 + H	7.7		
		26235.6	9.2
29932.6 + H	7.1	29926.1	7.7
		52374.6	7.2

were detected only in the winter SGS. Judging by the intensity of the corresponding peaks, the concentration of the proteins with molecular weights in the ranges of 2383.9-2384.3, 3260.5-3260.6, 3558.9-3559.2, 4052.1-4052.88, and 8099.4-8101.1 daltons was higher in the summer SGS than in the winter secretion. Distinct differences were also found in the distribution of high-molecular-weight proteins. In particular, protein peaks of the winter SGS were more diverse than those of the summer SGS.

Analysis by 2D-PAGE of the winter salivary gland secretion of medicinal leech. Twenty proteins of the winter secretion were revealed by 2D-PAGE of the more concentrated preparation, and this allowed us to exclude the stage of protein concentration by precipitation with methanol in the presence of chloroform (Fig. 3). To obtain peptide maps, each protein obtained upon trypsinolysis was analyzed by MALDI-TOF-MS. The resulting peptide maps of 20 proteins were analyzed using the MasCot Search program, and no reliable homology was found with any of known proteins.

Analysis by 2D-PAGE of the high-molecular-weight fraction (HMWF) of summer salivary gland secretion of medicinal leech. The HMWF of the medicinal leech summer secretion was analyzed similarly; concentrating its protein and 2D-PAGE were described earlier [7]. In Fig. 4, 32 proteins are indicated for which peptide maps were obtained and compared with known databases. The data were processed using the MasCot Search program, and the results indicated a high homology of protein No. 22 (the homology index was 82) with calgranulin, a human calcium-binding protein. Lower values of the homology index were obtained for proteins Nos. 5 (76), 6 (61), 10 (60), 11 (48), 17 (64), and 18 (61).

Attempt to identify protein No. 22. The results of data processing using the MasCot Search program suggested that protein No. 22 could be similar in the structure to the Ca-binding human protein A8, calgranulin A. The N-terminal analysis of the protein No. 22 extracted from polyacrylamide gel showed the following N-terminal amino acid sequence: <Q G **T E L E** L>**(A) (N)**.

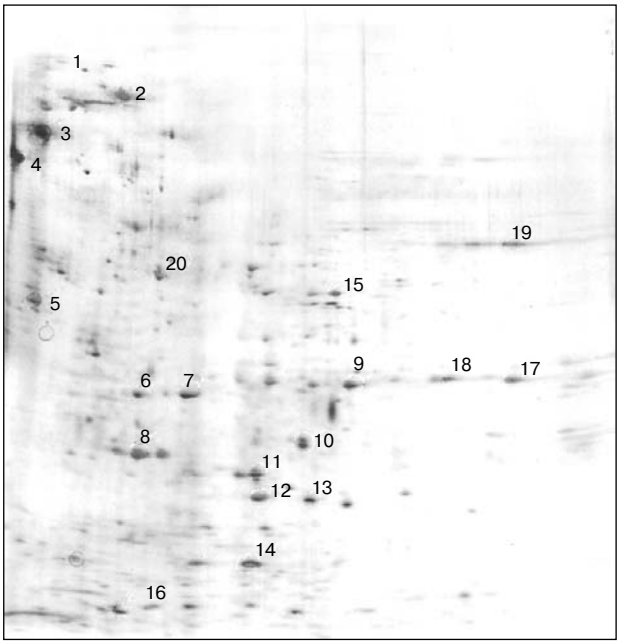

Fig. 3. 2D-PAGE of winter SGS. The protein spots, marked by figures, are those for which peptide maps were obtained and compared with known databases.

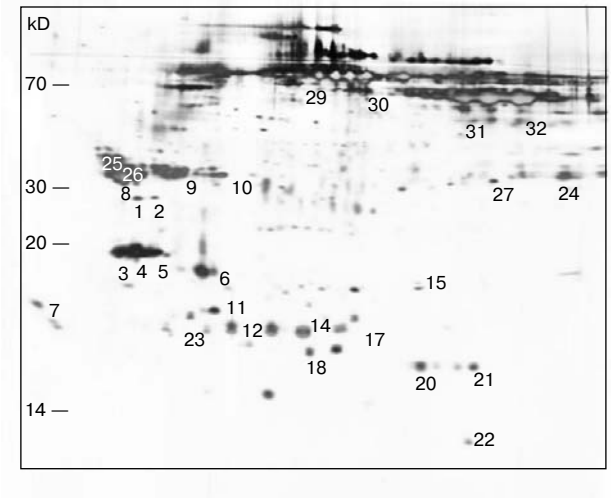

Fig. 4. 2D-PAGE of high-molecular-weight fraction of summer SGS. The protein spots, marked by figures, are those for which peptide maps were obtained and compared with known databases.

This sequence corresponded to residues 3-6 of the N-terminal sequence of calgranulin A: M L **T E L E** K A L N ... [11]. Based on this, we have attempted to detect the corresponding cDNA of leech and compare it with the human cDNA encoding the synthesis of calgranulin A. Because only the sequence consisting of four amino acid residues was homologous for these proteins, the probability of the coincidence of nucleotide sequences encoding them was very low, and we failed to reveal it. The experimental part of this work, which included the isolation of total DNA from leech, synthesis of oligonucleotides, performing of polymerase chain reaction, purification and cloning of amplicons, and determination of the nucleotide sequence of the resulting amplicons, was presented in "Materials and Methods".

Table 2. The N-terminal amino acid sequence of proteins detected by 2D-electrophoresis presented in Fig. 4

Number on electrophoregram	Amino acid sequence
3	D P N L S E E K D V
5	Y X E Q S L X E M X
6	X L V E D E T
10	A G G N P D X F V Q
11	D L S Q D D E K A G
12	D G G E Q N K
17	D G G G G Q D T N R
18	X M E N S D S A
19	D E D G P L E
20	Q H V R E A E
21	A G K S E D H
22	Q G T E L E L

The N-terminal amino acid sequence of proteins revealed by 2D-electrophoresis (Fig. 4). The N-terminal amino acid sequence was determined for the proteins detected by 2D-electrophoresis. The protein number corresponds to the figure denoting this protein in the electrophoregram (Table 2).

DISCUSSION

We were the first to determine the profiles of SGS proteins [7]. Of 45 proteins with molecular weight from 1.964 to 66.5 kD characterized by us, only eight were known before [7]. These known proteins included tryptase inhibitor [12], bdellastasin [13], hirudin [14], saratin [15], destabilase-lysozyme [16], hyaluronidase [17], calin [18], and γ-glutamyl transpeptidase [19]. We failed in detecting another six known proteins of medicinal leech: bdellin [20], hirustasin [21], carboxypeptidase inhibitor [22], eglins *b* and *c* [23], and inhibitor of factor Xa [24]. Our failure could be caused by the low concentrations of these proteins in the secretion. Proteomic analysis methods are now widely used for identification of protein systems of different origin, in particular, for detection of trypsin inhibitors in extract from whole medicinal leeches [25]. Because we are especially interested in studies on composition and properties of medicinal leech native SGS, in the present work we have realized the next step in the analysis of this remarkable source of biologically active proteins that determine the effect of hirudotherapy in various human diseases [26].

Analysis of the winter SGS by SELDI-MS using microarray with a normal hydrophilic phase on the base of silica gel (NP20 Array) revealed only a tryptase inhibitor with molecular weight of 4744.5 + H and hirudin (molecular weight 7026.5 + H). The chips used provided for the identification of proteins in limited ranges of molecular weights (2-10 and 10-60 kD), and the other SGS proteins revealed by us earlier [7] could not be detected under these experimental conditions.

Nevertheless, this method revealed a difference in the distribution of proteins and their intensity in the SGS preparations obtained from medicinal leech in summer and in winter (Table 1). Just the existence of this difference suggests a seasonal prevalence of the hirudotherapy as it is: the treatment efficiency of various diseases can be different depending on the season. Table 1 shows that the peak corresponding to the molecular weight of hirudin (7026.5 + H) is expressed only in the winter SGS. This findings correlate with our long-standing observations that the maximal antithrombin activity of SGS occurs in winter. Because at present there are no clinical data illus-

trating the seasonal prevalence of hirudotherapy, publication of the SELDI-MS data will be appraised by physicians. Informative data have also been obtained by comparison of 2D-electrophoregrams of the protein distribution in the summer and winter secretions (Figs. 2-4).

Detection by MALDI-TOF-MS of 32 proteins in the high-molecular-weight fraction of the summer SGS and 20 proteins in the winter secretion preparatively isolated from the gel (Figs. 3 and 4) and analysis of the resulting peptide maps did not reveal considerable homology with any known protein. The homology found between protein No. 22 and human calgranulin A8 also cannot be considered as a proof of the presence of the latter in the leech secretion because the data available are insufficient to identify the gene encoding the synthesis of this protein. The presence of numerous unidentified proteins in the SGS is supported by results of the N-terminal amino acid sequence of 12 proteins (Table 2) obtained by 2D-electrophoretic fractionation (Fig. 4); their comparison with the databases also did not reveal known proteins.

Thus, we have analyzed less than 20% of the protein constituents of SGS. It is not surprising that they all seem to be new and undescribed in the literature, because hirudotherapy gives a wide spectrum of effects, and their mechanisms are unclear.

No doubt, to more reliably identify the SGS proteins by the methods used it is necessary to know the medicinal leech genome. There are still no such data, so we are going to create a library of cDNA of the leech secretion to promote further studies on its new protein constituents.

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