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Protein Profiling of the Medicinal Leech Salivary Gland Secretion by Proteomic Analytical Methods

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Abstract—Protein diversity of the high molecular weight fraction (molecular mass > 500 daltons) of salivary grand secretion of the medicinal leech *Hirudo medicinalis* has been demonstrated using methods of proteomic analysis. One-dimensional (1D) electrophoresis revealed the presence of more than 60 bands corresponding to molecular masses ranging from 11 to 483 kD. 2D-electrophoresis revealed more than 100 specific protein spots differing in molecular masses and p*I* values. SELDI-mass spectrometry analysis using the ProteinChip™ System based on chromatography surfaces of strong anion or weak cation exchanger detected 45 individual compounds of molecular masses ranged from 1.964 to 66.5 kD. Comparison of SELDI-MS data with protein databases revealed eight known proteins from the medicinal leech. Other masses detected by proteomic analytical methods may be related to both modifications of known proteins and unknown biologically active components of leech saliva secretion.

Key words: medicinal leech, salivary gland secretion, proteomics, 1D-electrophoresis, 2D-electrophoresis, protein chip, SELDI-MS

Salivary gland secretion of the medicinal leech *Hirudo medicinalis* exhibits several important functions required for exclusive feeding of these bloodsuckers. Secretion components are responsible for suppression of protecting mechanisms of host organism at the wound; their functioning allows long-term bloodsucking. Secretion components are also responsible for inhibition of blood coagulation by blocking host vascular-platelet and plasma hemostasis at the wound [1-3]. In the gut of bloodsuckers, the secretion components maintain anticoagulant and fibrinolytic potential of sucked blood; this regulates blood digestion and provides antimicrobial protection [4]. Combined action of various components of the salivary secretion determines the positive effect of hirudotherapy during treatment of various diseases in man by skin application of leeches. Salivary gland secretion "injected" into the wound acts on various systems of the human body directly linked to the hemostatic system. However, only a few protein components have been identified in the leech secretion [5-13]. So identification of proteins secreted by the salivary glands and encoded by the leech genome is still an important problem. Such information may be obtained using proteomics, which allows identification, characterization, and quantification of various proteins in cells based on highly sensitive methods of separation and analysis [14]. However, lack of information on medicinal leech genome seriously complicated proteomic analysis of salivary gland secretion of *Hirudo medicinalis* and so it is impossible to carry out total proteomic analysis of salivary secretion proteins. Nevertheless, in the present study we have tried to map proteins of high molecular mass fraction (>500 daltons) of salivary secretion.

MATERIALS AND METHODS

Preparation of high molecular weight leech salivary gland secretion. Salivary gland secretion was obtained as described previously [15]. Medicinal leeches *Hirudo medicinalis* grown at the Girud I.N. biofactory (Balakovo, Saratov Region, Russia) were starved for at

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least six months. Salivary gland secretion was fractionated using Minitan (Millipore, USA) and 500-dalton membrane (Spectra Medical Industries Inc., USA) and high molecular weight (>500 dalton) fraction (protein concentration 0.5 mg/ml) was used in subsequent analyses. Proteins were precipitated by methanol in the presence of chloroform. Methanol (60 μl) was added to 150 μl of solution (150-500 μg protein) of salivary high molecular weight fraction. After centrifugation, 150 μl of chloroform was added under vigorous shaking. After centrifugation, 450 μl of water was added and the mixture was shaken again. The aqueous phase was separated by centrifugation and interface proteins were precipitated by adding 450 μl of methanol. The precipitate was dried down and dissolved in solution A containing 9 M urea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) (w/v), 0.2% Triton X-100 (v/v), and 100 mM dithiothreitol. Sample in buffer A was subjected to ultrasonic treatment at 50 kHz and amplitude of 30 μm (5 impulses for 1 sec at 4°C) using a Soniprep 150 disintegrator (UK). After the ultrasonic treatment, the sample was centrifuged at 12,000*g* for 15 min, and the resultant supernatant was used in subsequent analysis.

1D-Electrophoresis was carried out by the method of Laemmli [16] using 9-16% gradient gel with applied salivary secretion proteins (25-50 μg) and a mixture of marker proteins of 97-14 kD (Amersham, USA). Gels (1.5 mm thick) of 20 × 20 cm were used for all types of staining. Silver staining in the presence of sodium thiosulfate was carried out according to standard protocol [17]. Gels were scanned using an Epson 1600 (Japan) densitometer. Analysis of gels and densitometry of electrophoretic lanes was carried out using LabWorks 4.0 software (UVP, USA).

2D-Electrophoresis. Supernatant was analyzed after addition of 0.2% Ampholine™ pH 3-10 (Amersham). 2D-Electrophoresis was carried out using a Protean IEF Cell for protein isoelectrofocusing and Protean II xi Cell (Bio-Rad, USA).

For protein separation in the first dimension by isoelectrofocusing glass tubes (Bio-Rad) with inner and outer diameters of 1.5 and 4 mm, respectively, were used. The length of gel was 17 cm and the amount of the applied sample did not exceed 100 μg of protein per tube. Before sample application the tubes for isoelectrofocusing were filled with standard 4.5% polyacrylamide gel (30% T, 3% C) (for definitions % T and % C, see [18]) containing 8 M urea, 4% CHAPS (w/v), 0.5% NP-40 (w/v), 3.6% (v/v) Ampholine™ pH 5-8, 1.2% (w/v) Ampholine™ pH 3-10. The isoelectrofocusing was initially carried out at 250 V for 1 h, then at 450 V for 2 h, and finally at 950 V up to total 14,000 kV·h. After isoelectrofocusing, the tubes were incubated for 30 min in 0.125 M Tris-buffer, pH 6.8, containing 6 M urea, glycerol (300 g/liter), and SDS (20 g/liter). Separation of proteins in the second dimension was carried out in sodium-Tricine-SDS-polyacrylamide gel prepared by using a standard protocol [18].

ProteinChip technology. For separation and characterization of high molecular weight fraction proteins, we used the ProteinChip System with SELDI-MS detection (Ciphergen Biosystems Inc., USA). Two types of chips were employed: SAX2 with a chromatographic surface on the basis of strong anion exchanger and WCX2 with the chromatographic surface on the basis of weak cation exchanger. In the first case (SAX2) we used binding 20 mM Hepes buffer, pH 7.0, containing 0.1% Triton X-100 (v/v); in the case of WCX2 binding buffer was 0.1 M ammonium acetate, pH 4.5, containing 0.1% Triton X-100 (v/v). Chips were prepared as follows: using a 96-well bioprocessor (Ciphergen Biosystems Inc.) each well was treated twice (5 min each) with 200 μl of the corresponding binding buffer. After buffer removal 50 μl of high molecular weight fraction diluted by the binding buffer (1 : 3) was added to each well; the system was incubated during 30 min at room temperature with shaking. Unbound sample was removed and the wells were washed twice (5 min each) with 200 μl of the corresponding binding buffer. Chips taken from the bioprocessor were soaked (5 times) with water for HPLC (Merck, Germany) and then dried under air.

For MS-detection, matrix solution (2 × 0.5 μl) was added. α-Cyano-4-hydroxycinnamic acid (Sigma, USA) (20% saturation) in 50-% acetonitrile (v/v) with 0.5% trifluoroacetic acid (v/v) was used as matrix for detection of compounds ranging from 1 to 10 kD. For compounds of higher molecular mass (>10 kD) saturated solution of sinapic acid in 50% acetonitrile (v/v) with 0.5% trifluoroacetic acid (v/v) was used.

The chips were placed into a PBS II mass-spectrometer (Ciphergen Biosystems Inc.). Mass spectra for compounds of 1-10 kD were obtained at laser intensity of 200 and sensitivity mode 8, whereas for compounds >10 kD laser intensity and sensitivity mode were 230 and 9, respectively (these are intrinsic arbitrary characteristics of this system). Spectra were calibrated using external standards (Peptide and Protein Calibration Kits, Ciphergen Biosystems Inc.). Accuracy of molecular mass determination was 1%.

RESULTS AND DISCUSSION

1D-Electrophoresis. Figure 1 shows a typical 1D-electrophoregram of separation of high molecular weight fraction isolated from leech salivary secretion (25 and 50 μg). 1D-Electrophoresis revealed the presence of many proteins ranging from 6 to >200 kD. Densitometric analysis of these electrophoregrams revealed precise molecular masses of each identified protein (Table 1). This suggests the presence of more than 60 proteins (from

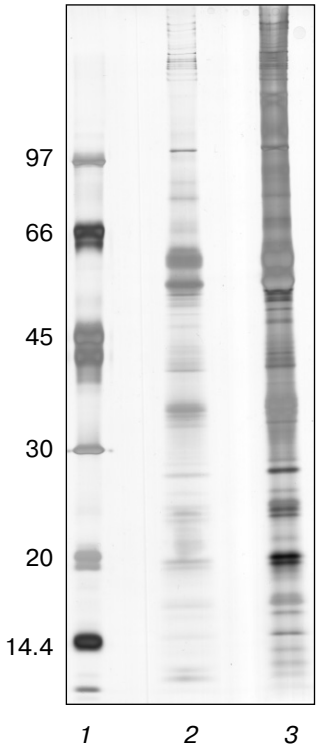

Fig. 1. Separation of high molecular weight components of the medicinal leech salivary gland by SDS-PAGE. Lanes: *1*) protein molecular mass markers (on the left, their molecular masses in kD); *2*, *3*) high molecular mass fraction (25 and 50 μg, respectively).

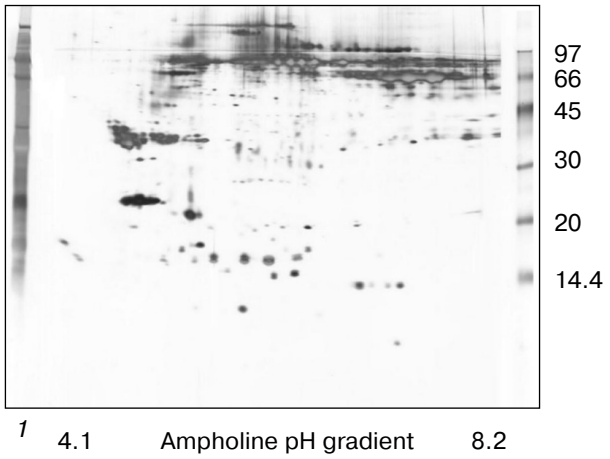

Fig. 2. 2D-PAGE of high molecular weight components of medicinal leech salivary gland secretion. Lane *1* shows the 1D electrophoregram of the same proteins. On the right, molecular masses of protein markers in kD.

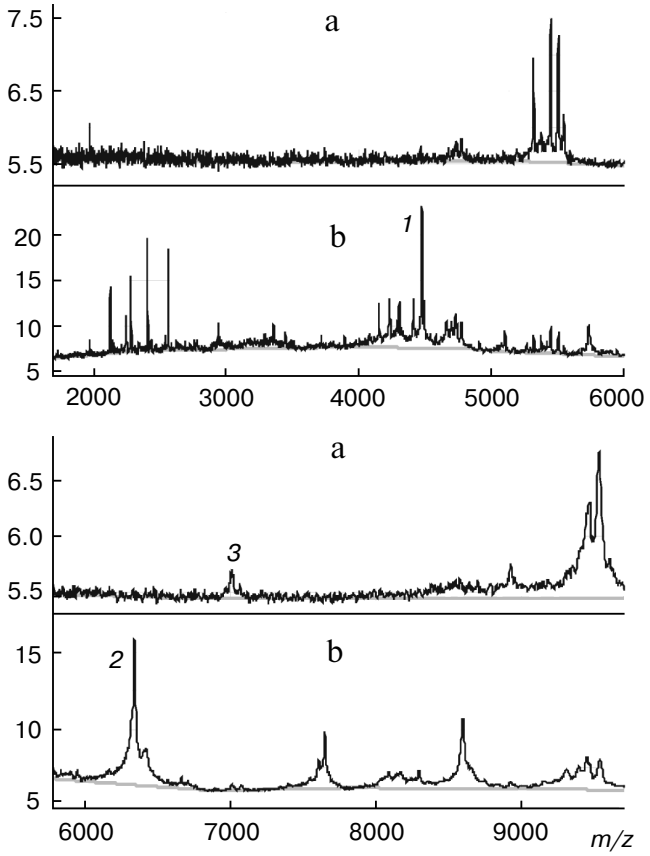

Fig. 3. SELDI-mass spectrum of high molecular weight components (1-10 kD) of the medicinal leech salivary gland secretion. a) Separation using strong anion-exchanger ProteinChip (SAX2); b) separation using weak cation-exchanger ProteinChip (WCX2). Vertical axis shows relative signal intensity, horizontal axis represents mass/charge ratio (*m*/*z*). *1-3*) Peaks corresponding to known proteins produced by the medicinal leech (see Table 3).

11 to 483 kD) in the high molecular weight fraction of salivary gland secretion.

2D-Electrophoresis. Combination of isoelectrofocusing with electrophoresis provides further separation of protein fractions obtained during 1D-electrophoresis, and this allows the detection of additional proteins with different isoelectric points. Figure 2 shows the separation pattern of high molecular weight fraction proteins of salivary gland secretion. (1D-Electrophoregram is shown for comparison.)

These results suggest that high molecular proteins (>70 kD) are major components of salivary gland secretion. Proteins of 30-45 kD are preferentially concentrated within the pH range 4.5-5.0 and 5.5-6.5. At pH > 6.5 proteins of 35-45 kD are separated. Proteins of below 20 kD are equally distributed over the whole pH range.

Protein characterization by means of ProteinChip/SELDI-MS. Figures 3 and 4 show results of protein pro-

Table 1. Molecular masses (kD) corresponding to protein bands on lanes *2* and *3* (Fig. 1) obtained during densitogram analysis of SDS-PAGE electrophoregrams of high molecular weight components of the medicinal leech salivary gland secretion

Lane					
2	3	2	3	2	3
	483.04	115.53	115.77	34.424	34.544
455.87	452.61	90.538		33.74	34.142
430.87	435.22	77.385	78.769	33.056	33.016
406.96	405.87	67.846	70.615	32.453	31.769
386.3	386.3	62.172	64.411	30	30.04
372.17		60.222	60.656		28.65
366.74		53.794	55.167	27.383	27.741
336.3		53.217	53.072	25.868	26.033
327.61	326.52		52.66		25.096
308.04	309.13	51.525	51.865	24.656	24.463
292.83		50.73	51.411	24.077	23.967
288.48	289.57	49.482	49.652	23.416	22.011
283.04	281.96	47.667	47.44	19.98	20.468
273.26	271.09	45.511	44.718	19.616	19.98
266.74	265.65	42.869	43.794		17.534
259.13	255.87		42.346	16.826	17.069
240.65	35.22	41.582	41.501		16.159
218.91	218.91	40.777	40.617	14.461	14.501
	171.09	40.214			13.349
	161.56	39.692	12.358	12.439	
	132.54			11.812	11.752

filing of high molecular weight fraction of salivary gland secretion on ion-exchange chips. Use of two ion-exchange surfaces resulted in detection of 29 compounds with molecular masses of 1-10 kD (Fig. 3). Four of them coincided for both ion-exchange surfaces. Among compounds of molecular masses >10 kD (Fig. 4), there were 24 proteins, and four of them also coincided for strong anion exchanger and weak cation exchanger. Thus, use of both ProteinChips revealed 45 individual compounds from 1.964 to 66.5 kD (Table 2).

Table 3 shows the molecular masses of known proteins isolated from medicinal leech. Comparison of these data with previous results of mass-spectrometric determination revealed coincidence of eight peaks with known leech proteins (with accuracy of molecular mass determination of 1%). Other detected masses might represent modification of known proteins and also unknown biologically active components of salivary secretion.

Table 2. Analysis of high molecular weight components of the medicinal leech salivary gland secretion by means of ProteinChip/SELDI-MS

Parameter	Strong anion exchanger (SAX2)	Weak cation exchanger (WCX2)
Number of individual compounds (1-10 kD)	11	18
Coincidence for both surfaces	4	
Number of individual compounds (>10 kD)	10	14
Coincidence for both surfaces	4	
Total number of individual compounds	45	

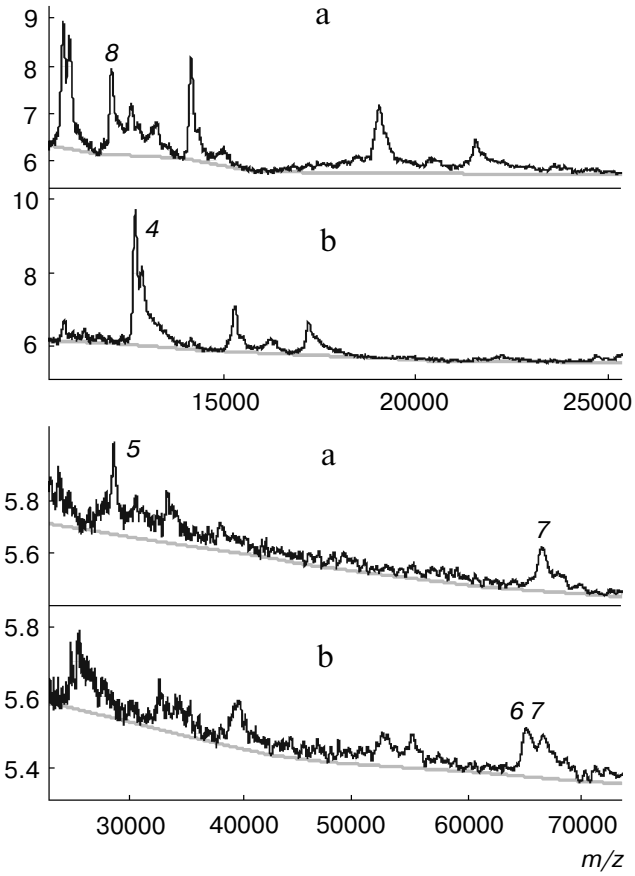

Fig. 4. SELDI-mass spectrum high molecular weight components (>10 kD) of the medicinal leech salivary gland secretion. a) Separation using strong anion-exchanger ProteinChip (SAX2); b) separation using weak cation-exchanger ProteinChip (WCX2). Vertical axis shows relative signal intensity, horizontal axis represents mass/charge ratio (*m/z*). *4-8*) Peaks corresponding to known proteins produced by the medicinal leech (see Table 3).

In the present study we have demonstrated that the high molecular mass fraction of medicinal leech salivary secretion contains more than 100 proteins and only eight of them exhibiting particular functions are known to date. For example, leech secretion components tryptase inhibitor and bdellastasin (trypsin inhibitor) block corresponding enzymes produced by mast cells of host subcutaneous adipose tissue [17, 19] during their activation by salivary gland secretion. This reduces response of host protective mechanisms to skin damage induced by leech bite. Hyaluronidase provides penetration of salivary gland secretion through tissue barriers by decomposing hyaluronic acid, a major component of the intercellular matrix [20]. Salivary gland secretion proteins are effective inhibitors of vascular-platelet and plasma hemostasis. For example, saratin and calin inhibit platelet adhesion to damaged vascular wall [11, 13, 21]. Factor Xa inhibitor and hirudin, a highly specific thrombin inhibitor, block plasma hemostasis [9, 22]. Destabilase-lysozyme is responsible for antibacterial activity of the leech secretion [4]. γ-Glutamyl transpeptidase [23] together with leech secretion destabilase-monomerase [15] (its molecular mass still requires precise elucidation) are involved in maintenance of liquid state of host blood by cleaving endo-ε-(γ-Glu)-Lys isopeptide bonds in stabilized fibrin and D-dimer. However, these proteins cannot account for the wide functional spectrum of medicinal leech salivary secretion. The present study clearly demonstrates that the protein consortium described in this study is obviously responsible for manifestation of

Table 3. Comparison of molecular masses of high molecular weight components of the medicinal leech salivary gland secretion separated by means of ProteinChip/SELDI-MS with literature data on calculated and experimentally detected molecular masses of proteins produced by the medicinal leech (error in mass determination by SELDI spectrum did not exceed 1%)

Protein	Molecular mass, kD	Peak number on SELDI spectrum and molecular mass (kD) (Figs. 3 and 4)
Tryptase inhibitor [5]	4.6093 (native) 4.4808 (native) 4.7369/4,7194 (native) 4.60935 (calculated) 4.48118 (calculated) 4.73745 (calculated)	1 4.4740
Bdellastasin (bdellin A) [6]	6.3326 (determined) 6.3342 (calculated)	2 6.3394
Hirudin [7]	7.045 7.030 7.087	3 7.0103
Saratin [13]	12.000	8 12.0528
Destabilase-lysozyme [8]	12.6776 12.9382 12.8397 ± 0.2 dalton 12.8034 12.7844 12.7241 12.7497	4 12.6774
Hyaluronidase [10]	28.500	5 28.5220
Calin [11]	65.000	6 65.1635
γ-Glutamyl transpeptidase [12]	65.520	7 66.6541

multiple functions of medicinal leech salivary gland secretion.

In the present study, we were able to identify proteins that had previously been isolated from leech extracts and related to salivary gland secretion. These include tryptase inhibitor, bdellastasin, hirudin, destabilase-lysozyme, hyaluronidase, calin, and γ-glutamyl transpeptidase. Factor Xa inhibitor previously isolated from the leech secretion [9] represents the only exception. Its molecular mass [9] was determined for recombinant (but not native) protein obtained during mutation of the corresponding gene. This might explain why it has not been detected among proteins of salivary gland secretion. It should also be noted that some other proteins previously isolated from the medicinal leech (e.g., bdellin B [24], hirustasin [25], carboxypeptidase inhibitor [26], eglin *b* and eglin *c* [27]) were not found in the salivary gland secretion, possibly due to their low concentration.

Thus, use of the method of proteomic analysis demonstrated diversity of protein components in high molecular weight fraction of medicinal leech salivary gland secretion. Identification of their structures and functions require further investigation.

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