Proteins and Peptides of the Salivary Gland Secretion of Medicinal Leeches Hirudo verbana, H. medicinalis, and H. orientalis

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Abstract—The protein and peptide composition of medicinal leech salivary gland secretion (SGS) was analyzed in preparations obtained in July from three species—Hirudo verbana, H. medicinalis, and H. orientalis. Two-dimensional electrophoresis (molecular mass 10-150 kD and pH 3-10) revealed no distinctions in the distribution of over 100 silver-stained proteins. Differences were noted only in intensity of 10 protein spots at 30-90 kD and pH 4.7-7.5. Mass spectrometric profiling of SGS of the three leech species using the Zip-Tip/golden chip scheme and cation-exchanging chips CM-10 revealed over 50 components in SGS of each of the three leech species. It was noted that 30-40% of the individual masses of the SGS of each leech species fall within the masses present in SGS of at least one of the two other species. This rather small part of the total mass may be indicative of a high polymorphism of amino acid sequences or a high frequency of posttranslational modifications of the SGS proteins and peptides. Calculation of Jacquier’s coefficient showed that H. medicinalis and H. orientalis are closest to each other in SGS composition, which is consistent with data in the literature on the phylogenetic relationship between these two species of medicinal leech. Comparison of detected molecular masses with those of six known biologically active compounds produced by medicinal leeches revealed their uneven distribution in SGS of each of the three medicinal leech species. This opens prospects for using certain species of medicinal leech for targeted therapy of various pathologies.

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At the present time therapy using medicinal leeches of the genus Hirudo (hirudotherapy) is actively used in clinical practice in Russia and countries of former Soviet Union as well in USA, Canada, France, Germany, Holland, etc. The salivary gland secretion (SGS) of medicinal leeches is a humoral agent of hirudotherapy and its composition defines the therapeutic effect of this method in different diseases. Analysis of composition of the medicinal leech SGS allowed us to identify in it eight biologically active compounds [1] out of 13 described as being produced by medicinal leeches [2]. No doubt, these do not cover the whole arsenal of biologically active compounds of SGS, which provide for positive effects of hirudo-therapy in treatment of a broad spectrum of diseases in different fields of medicine (cardiology, gynecology, urology, surgery, stomatology, ophthalmology, etc.) [2].
Proteomic analysis detected over 100 proteins with molecular mass from 10 to 97 kD in SGS [1]. It was shown that their distribution on electrophoregrams varies depending on the season [3]. Heterogeneous also are doses of secretion which are injected into the wound caused by the animal’s bite during the whole time of blood suction by the leech [4]. Owing to the absence of information concerning the genome of medicinal leeches, it is impossible now to identify proteins and peptides revealed by proteomic analysis. The problem is even more complicated because doctors use different medicinal leech species (Hirudo verban, H. medicinalis, and H. orientalis). We have used methods of proteomic analysis such as two-dimensional electrophoresis and SELDI-MS and tried to determine how much species peculiarities of medicinal leech, determined by analyses at the molecular level [5], affect the SGS composition in general and the distribution in it of individual biologically active compounds in particular.

**MATERIALS AND METHODS**

SGS was obtained as described in [4] from medicinal leeches of species H. verban, H. medicinalis, and H. orientalis supplied by the factory for biological preparations “Girud I. N.” (Balakovo, Saratov Region) after starvation for no less than four months. To level temporal changes in the leech secretion composition, SGS of all three leech species were taken for three days in July. Each pool corresponded to a single leech species and contained SGS of no less than 60 individual leeches.

**Two-dimensional electrophoresis.** Each SGS preparation was dissolved in a buffer containing 8 M urea, 2 M thiourea, 5% ampholines (pH 3.0-10.0), 80 mM dithiothreitol (DTT), 30 mM Chaps (all from Amersham, USA), and 10% NP-40 and sonicated in an ultrasound bath for 5 min. Samples were centrifuged at 13,000 rpm for 15 min. Protein concentration in the samples was determined according to Bradford using the Quick Start Bradford dye reagent (BioRad, USA). Isoelectrofocusing was carried out in 18 cm glass tubes in 4% polyacrylamide gel (water for chromatography Merck, Germany), 8 M urea, 4% acrylamide/methylene-bis-acrylamide, 1.75% ampholines (pH 3.0-10.0), 3.5% ampholines (pH 5.0-8.0), 65 mM Chaps, 10% NP-40, 0.1% TEMED, 0.02% ammonium persulfate (all from Amersham). Focusing was carried out under the following conditions: 20, 40, and 35 mA per gel for 20 min, 2 h, and 2.5 h, respectively. When electrophoresis was complete, gels were marked and stained by silver with thiosulfate [6]. The data were analyzed using the PDQuest 8.0 program (BioRad). Two-dimensional electrophoregrams of SGS from each species were obtained six times.

**SELDI-MS. Obtaining mass spectra using the Zip-Tip/golden chip scheme.** A 12-µl sample of leech SGS was desalted using Zip-Tip C18 tips (Millipore, USA) according to the manufacturer’s protocol (www.millipore.com). A 1-µl sample of desalted sample was applied onto the protein chip, air-dried, then the matrix solution was applied twice, 0.5 µl each. Solution of α-cyano-4-hydroxycinnamic acid (Bruker, Germany) (2.5 g/liter) in 50% (v/v) acetonitrile containing 0.5% (v/v) trifluoroacetic acid was used as matrix.

Samples were profiled using a SELDI-TOF Protein Biology System II (PBS II) mass spectrometer (Ciphergen, USA). Spectra were registered automatically. In this case two protocols were used: one optimized for peptides and low-molecular proteins (up to 20 kD) and the other for proteins with molecular mass of 6-60 kD. In the first protocol laser intensity was 190, in the second one it was 195. Detector sensitivity was 8, and 90 laser pulses fell on each spot. Calibration was carried out with external calibration standards using the Protein Calibration Standard I (Bruker Daltonics, Germany) that included insulin (5734 daltons), ubiquitin (8566 daltons), horse cytochrome c (12,361 daltons), and the sperm whale myoglobin (16,952 daltons). Three spectra of each SGS preparation were taken.

**Obtaining mass spectra using CM-10 cation-exchange chips.** Aliquots of the medicinal leech SGS were diluted twice with 50 mM ammonium acetate, pH 4.5, containing 0.02% Triton X-100, then 3-µl aliquots were applied onto a mild cation-exchange chip CM-10 (activated in advance with 10 mM HCl and equilibrated with the above-described buffer) and incubated for 30 min at room temperature in a humid chamber. Then spots on the chip were washed five times with buffer and twice with deionized water and then air-dried. Matrix was applied and spectra were taken as described for the Zip-Tip/golden chip scheme. Spectra of each SGS preparation were taken three times.

**Data processing.** Peaks of mass spectra from 2300 to 12,500 daltons on peptide mass spectra and from 4000 to 34,200 daltons on protein mass spectra were analyzed. Peak were detected automatically using the Ciphergen ProteinChip Software program. Minimal signal/noise ratio for detected peaks was 5. Peak masses and their intensities were exported into MS Excel tables. Data processing using the program options of Ciphergen ProteinChip software 3.1 revealed multiple-charge ions. When the number of peaks corresponding to individual