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Protein–Lipid Particles of Medicinal Leech Salivary Gland Secretion; Their Size and Morphology

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Abstract—The relative location of proteins and lipids in particles of medicinal leech salivary gland secretion (SGS) is revealed for the first time. Their sizes and morphology are described. Using scanning electron microscopy and transmission electron microscopy, it was determined that SGS consists of particles of different sizes and form. This picture is supported by confocal laser scanning microscopy of SGS preparations treated with fluorescein isothiocyanate. After incubation with nonionic detergents (Brij 35 and Tween 20), transmission electron microscopy revealed the dissociation of fragments composing protein–lipid particles (PLP), and in this case an increase in free protein concentration determined by a modification of the Lowry method was observed. Perylene probing of lipids in SGS preparations showed that they are concentrated mainly inside PLP and are almost absent on the surface. Cholesterol was detected during SGS probing using the cholesteryl-Bodipy (hydrophobic fluorescent analog of cholesterol) on surface sections during confocal analysis of electron microphotographs of SGS. This analysis detected PLP structures in SGS resembling caveoles full of cholesterol. SGS, preliminary frozen at –70°C, transformed into a multitude of similar small particles visualized by transmission electron microscopy, whose fixed distribution resembled water crystal structure.

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Key words: medicinal leech salivary gland secretion, scanning electron microscopy, transmission electron microscopy, confocal laser scanning microscopy, nonionic detergents, lipids, cholesterol

The composition and structure of salivary gland secretion (SGS) of the medicinal leech are of high interest because they determine the humoral effect of hirudotherapy – a broadly used method of treatment of many diseases [1, 2]. A large spectrum of biological activities of the leech secretion (antithrombotic, thrombolytic, anti-atherosclerotic, anti-inflammatory [1]) has been shown during hirudotherapy, and the secretion can activate mast cells [3] and influence lymphatic and blood vessels of the microcirculation system (unpublished data). Using proteomic analysis, it has been shown that SGS contains multitude of proteins and peptides of both high and low molecular weight [4, 5], as well as low molecular weight non-protein biologically active substances [6], similar to the proteome of human saliva, which has micellar structure [7, 8].

Not much is known about lipids of SGS. The results of their analysis published in work by Rabinovich [9] show that SGS contains neutral lipids, phospholipids, and cholesterol. However, this data does not reflect the real composition of SGS because the author was analyzing not native SGS but "saliva fluid" referring to the preparation method described in [10]. Using this method SGS is secreted by a leech to fluid that simulates blood, and then it is ingested by the animal together with this solution. The secretion that is squeezed out through the mouth from the leech's stomach naturally contains not only traces of blood that leech was fed with, but also

Abbreviations: CLSM, confocal laser scanning microscopy; FITC, fluorescein isothiocyanate; PLP, protein–lipid particles; SEM, scanning electron microscopy; SGS, salivary gland secretion; TEM, transmission electron microscopy.

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products secreted by the wall of the digestive channel. This kind of "SGS" was analyzed by the authors of the method described above [10]. In fractionation products they found a phospholipid fraction that inhibited thrombocytic aggregation stimulated by the factor activating blood platelets [11]. The work [12] merits attention, where the authors separated steroids from extracts of intact medicinal leeches and studied their structure. They suspect that the presence of cholesterol and its derivatives as part of sanguivorous leeches is a result of their adaptation to feed on blood of homoiothermic animals, which is connected with the evolution of leeches and reflects the properties of their food.

Our study of the lipids in native SGS were limited to comparative analysis of weight correspondence of lyophilized SGS and its dried lipid-soluble fraction. It revealed that the latter constitutes about 20%. In the lipid-soluble SGS fraction we could not observe phospholipids either qualitatively or quantitatively. However, it was shown using chemiluminescent immunoassay and microchromatography–mass spectrometry that steroid hormones were present in native SGS of the medicinal leech [6].

Present work is a first step to the understanding of SGS structure characterized by the interaction of proteins and lipids. Here we report the distribution of proteins and lipids in SGS particles revealed by electron and laser microscopy using specific probes and the influence of detergents on solubilization of proteins in SGS of the medicinal leech.

MATERIALS AND METHODS

SGS of medicinal leech was obtained using the method we described before [13] from individuals raised at the Biofactory of Scientific-Production Company Girud I. N. (Balakovo, Saratov Region), 1.3-1.5 g in weight, starving for not less than four months. We used pools of SGS collected from 10-15 animals kept not longer than 2-3 days at 4°C.

Protein concentration was determined by a modification of the Bradford method [14] using BSA as a standard. The amount of protein in SGS preparation after interaction with detergents was determined by a modification of the Lowry method [15]. Unlike the original method, in this case the D solution did not contain detergents, but it was added at specific concentrations to construct the calibration curve.

Scanning electron microscopy (SEM). SGS preparations were fixed in physiological solution containing 3% of glutaraldehyde overnight at 4°C. After centrifugation and washing in physiological solution, preparations were dehydrated with ethanol solutions of increasing concentrations by a well-known method with subsequent processing with acetone [16]. Preparations were then dried to critical point, shadowed with gold in vacuum, and studied using SEM (Amray 18301, USA; Hitachi S-405A, Japan).

Transmission electron microscopy (TEM) contrast study. SGS preparations were put on copper grids (0.5 cm diameter) and left for 30-60 sec, and the remaining liquid was removed with absorbent paper. For contrast study a drop of 2% phosphotungstic acid was applied on the surface of preparations, the excess being removed with absorbent paper after 30-60 sec [16]. Preparations were visualized using TEM (JEM100B; JEOL, Japan).

Introduction of fluorescent labels into SGS preparations. SGS with fluorescent label for protein was obtained by processing with fluorescein isothiocyanate (FITC) according to the method described in [17]. To 1 ml of SGS solution in 50 mM carbonate buffer, pH 9.0, during gentle stirring (50*g*), we added FITC solution in the same buffer. The molar protein/FITC ratio was 1 : 1. After 1.5-2.0 h incubation, the solution was dialyzed against 30 liters of water for 12 h in Sigma (USA) tubing (molecular weight cut-off, 12,000 Da). The dialyzate was lyophilized, and the residue for dissolved in ethanol, applied onto a glass plate, and analyzed using confocal laser scanning microscopy (CLSM). SGS preparations after incubation with detergents were processed similarly.

Lipid-labeled SGS was prepared using perylene as a fluorescent lipophilic probe (kindly provided by U. G. Molotkovsky, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences). SGS was treated with perylene according to the method described in [18] with a few modifications recommended by U. G. Molotkovsky. Perylene was dissolved in acetone (1 ml per mg perylene) and during intensive stirring was added to the SGS solution (5 μl of perylene solution per 0.5 ml of secretion) using a syringe. Final acetone concentration was 0.01%. After incubation at 37°C for 30 min with gentle stirring, the preparation was applied onto a microscope slide and analyzed using CLSM.

Cholesterol-labeled SGS was prepared using cholesteryl-Bodipy C-3927 (Molecular Probes, USA) according to the method described in [19]. Preparations were applied onto glass slides and analyzed using CLSM.

Confocal laser scanning microscopy. The morphology of SGS labeled with fluorescent probes was studied using a Zeiss LSM 510 confocal laser microscope (Germany) with fluorescence attachment (for FITC-labeled SGS, at λ_{ex} = 488 nm, λ_{em} = 543 nm; for cholesteryl-Bodipy-labeled SGS, at λ_{ex} = 458 nm, λ_{em} = 514 nm).

Perylene-labeled SGS was analyzed using a Nikon Eclipse TE-2000 Configuration (Japan) electron microscope with an objective Nikon 60 × 1.4 and fluorescence attachment (λ_{ex} = 488 nm, λ_{em} = 543 nm). Exposure was performed using the Image-Pro Express 4.5 program. Fluorescence signals from laser-excited fluorophores were detected and registered as pictures with 12-bit color rendering.

RESULTS AND DISCUSSION

Electron microscopy (SEM and TEM) of SGS preparations of medicinal leech. *Native SGS.* Photographs of different SGS preparations obtained by SEM are presented in Figs. 1 and 2. Together with protein–lipid particles (PLP) about 1 μm in diameter (Fig. 1), we also note larger multiglobular formations about 4 μm in diameter. PLP about 5.6 × 4.0 μm containing small inclusions about 0.04 × 0.4 μm on the surface are presented in Fig. 2.

SEM data of SGS particles (Figs. 1 and 2) showed that PLP about 1 μm in diameter and less can be present not only in small closely located particles (Fig. 1), but also as larger formations (Fig. 2) where particles interact more close making a quite homogenous conglomerate. Separate particles settle on its surface.

SGS after storage at −70°C. A TEM image of SGS that was subjected to prolonged storage at −70°C is shown in Fig. 3. It reveals unusual transformation of the particles (Fig. 3). Their degradation is obvious, revealing multiple particles of less than 0.05 μm size. The reason for such degradation might be a mechanical destruction of PLP by ice crystals that appear during freezing of SGS, determined by the dynamic properties of water [20, 21].

These results are consistent with our repeated observations and with experimental data on the loss of biological properties of leech secretion after freezing and lyophilization [10]. They demonstrate how important for biological activity is the "packaging" of the SGS components incorporated in particles shown in Figs. 1 and 2. In Fig. 3 we note unusual distribution of SGS PLP remnants on the surface. It appeared that SGS preparation held at 4°C for some time after freezing kept the form of organization of small SGS particles, determined not only by physical properties of water at −20°C [22], but probably by the properties of the degraded SGS particles themselves, particularly by the properties of incorporated proteins [23, 24]. This phenomenon is quite remarkable and further studies are needed for its explanation.

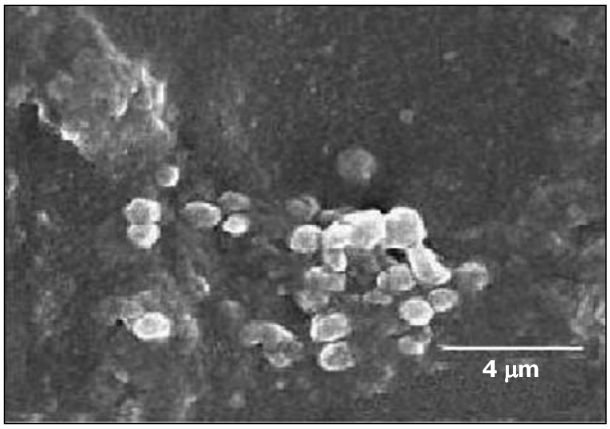

Fig. 1. SEM of small particles of leech secretion (Hitachi S-405A).

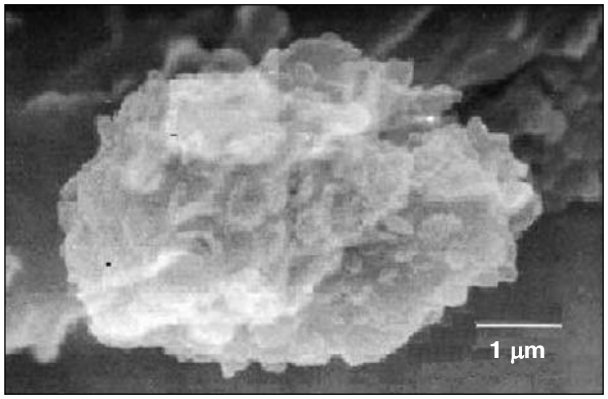

Fig. 2. SEM of a large homogenous conglomerate of leech secretion with small particles settled on its surface (Amray 18301).

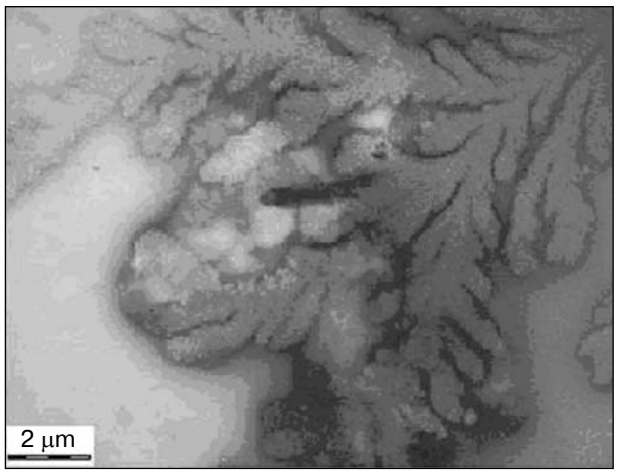

Fig. 3. TEM of leech secretion after deep freezing (JEM100B, JEOL).

Confocal laser scanning microscopy of SGS. *Protein probing with FITC.* Ten optical sections of SGS PLP (thickness 0.65 μm) are presented in Fig. 4 (see color insert). This image presents not only dimensional parameters of 85 × 55 μm particle but also reveals the distribution of protein in different layers because of different staining intensity. It indicates that on the surface of SGS particle there are only limited regions (light colored) where proteins are incorporated. The main protein mass is in layers distant from the surface.

These results indicate the presence of hydrophobic components in PLP of SGS. So we tried to identify lipids in the particles of leech secretion.

Lipids in SGS particles. Perylene was chosen as a fluorescent lipid probe to estimate lipid incorporations in SGS particles. It is used for visualization of hydrophobic

components, lipids in particular [25, 26]. After incubation of SGS with perylene, hydrophobic components and lipids were detected using CLSM.

A picture of perylene-treated SGS particles is presented in Fig. 5a (see color insert). Comparing fluorescence and reflected light images reveals that lipids (colored regions) occupy only part of the inner volume of the particle seen in reflected light (right upper image). The same thing is revealed during the superimposition of fluorescence and reflected light images (left lower in Fig. 5a). Presenting fluorescence images as a collection of sections shows that lipids are not present on the surface of the particle and on the sections close to it. The main mass is incorporated inside the particle (Fig. 5b).

Cholesterol in SGS particles. As cholesterol and its derivatives were detected in the extract of medicinal leech [12], in further experiments to visualize specific hydrophobic component as a part of the conglomerate of leech secretion we used cholesteryl-Bodipy, a fluorescent analog of cholesterol that has high affinity to this steroid [27]. Two PLPs of SGS differing in form, size, and structure were chosen to demonstrate our results.

Fluorescence images and images made in reflected light are presented in Figs. 6a and 7a (see color insert); fluorescence images of the sections of analyzed PLP of SGS are presented in Figs. 6b and 7b. These images show the distribution of cholesterol incorporated in individual fragments of SGS particles. It appears (Fig. 6b) that cholesterol is mostly concentrated inside the particles incorporated distant from the surface of PLP. This is less true for the SGS preparation presented in Fig. 7b. The comparison of these results with optical sections presented in Fig. 4 labeled with FITC shows that proteins and peptides occupy larger volume inside SGS particles than cholesterol and lipids (Fig. 6b), though we should not ignore morphological and structural differences (Figs. 1 and 2) of the compared particles. Figure 6 with a cluster of fragments of almost similar form attracts attention. These fragments filled with cholesterol look very similar to caveoles [28, 29], specialized subdomains of cell structures [30]. It is remarkable that each of these fragments is surrounded by "envelope" that resembles protein covers of caveoles incorporated into the membrane of endothelial and smooth muscle cells [31]. However, Fig. 7 demonstrates cholesterol fluorescence that does not concentrate in single SGS microfragments but is distributed along analyzed particle though mostly it is concentrated inside it.

Change in content of detectable protein of medicinal leech SGS after incubation with detergents

Detergent	Detergent content, %	Content of detectable protein of SGS, %
DOC	7	105
–"–	10	113
SDS	0.25	113
–"–	0.5	100
Tween 20	0.5	269
–"–	1	285
–"–	2	300
Triton X-100/protein	1 : 1	155
–"–	2 : 1	141
–"–	5 : 1	105
–"–	10 : 1	115
Brij 35	4*	203
–"–	10*	94

* In μM.

Influence of detergents on SGS of medicinal leech. To estimate the level of interaction between protein and lipid components in secretion particles, we tried to disintegrate PLP with detergents and follow the changes of their size

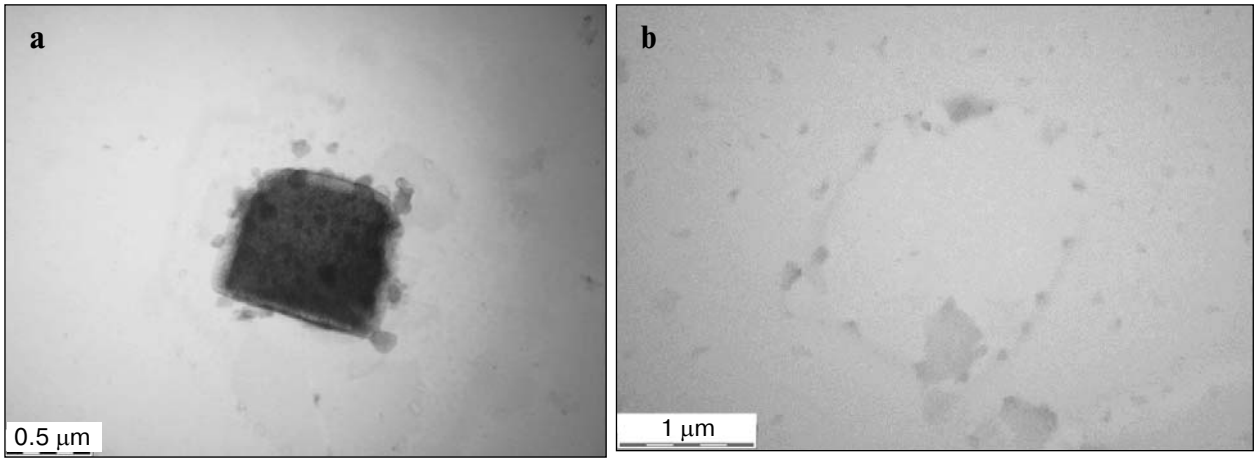

Fig. 8. Photographs of SGS at early (a) and late (b) stages of incubation with Brij 35 detergent (4 μM) obtained using TEM.

and form. SGS was incubated with anionic (sodium deoxycholate (DOC), sodium dodecyl sulfate (SDS)) and nonionic (Triton X-100, Brij 35, Tween 20) detergents for 2 h at 37°C, and then protein concentration was determined using the modified Lowry method. All detergents were taken in concentrations corresponding to 2, 5, and 10 critical concentrations of micellar formation. These are usually sufficient to disintegrate and fragment PLP. Comparative results of determining protein concentration before and after the detergent treatment are presented in the table (each value is the mean of three experiments).

Anionic detergent has practically no influence on SGS protein content (table). However, nonionic detergents, Tween 20 and Brij 35 (concentration 4 μM), increase the detectable protein 2-3-fold, e.g. they destroy the primary PLP organization. We have chosen Brij 35 to visualize structural changes of SGS under the action of detergents that cause maximum increase in concentration of detectable protein (2% Tween 20 and 4 μM Brij 35). Preparations were analyzed using TEM and CLSM.

Images obtained using TEM (Fig. 8) show the result of protein solubilization (vast areas of somewhat higher electron density around the particle) and separation of smaller fragments from the surface of the particle (Fig. 8a). In Fig. 8b the end of the process is shown: solubilization of the protein and degradation of the particle; we can see only faint contours of smaller fragments which are SGS particles and/or its residues lacking protein. Such effects were presented using CLSM of SGS preparations where protein was labeled with fluorescent dye FITC (picture not shown).

So, SGS contains a set of particles of different sizes and forms containing compounds of protein and lipid nature. The latter are concentrated mostly inside PLP of SGS. The effectiveness of nonionic detergents that are known to destroy lipid–lipid and lipid–protein interactions [32] shows that protein–protein interactions are the strongest among SGS components. The detection of lipids and cholesterol in SGS particles suggests that hydrophobic interactions define the structure of leech secretion particles, holding together their elements or fragments. To reveal the role of proteins and lipids in the organization and structure of SGS particles is a next step of our work.

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