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Morphological Changes of Proteolipid Giant Unilamellar Vesicles Affected by *Macrovipera lebetina obtusa* Venom Visualized with Fluorescence Microscope

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Abstract As a rule, zootoxins are complex and biologically active, and therefore the greater part of zootoxins is subjected to biotransformation and interacts with biological membranes. In this case, the interaction of different venom components with the membranes is not always the same. The present study shows how the giant unilamellar vesicles (GUV) from bovine brain proteolipids interact with *Macrovipera lebetina obtusa* venom. GUV (mean diameter 30 μm) were formed by the electroformation method. We used 8-anilino-1-naphthalenesulfonic acid and pyrene as fluorescence probes, which allowed us to quantify the fluidity changes in the membrane by measuring the fluorescence intensity.

Keywords Proteolipids · GUV · *Macrovipera lebetina obtusa* · ANS · Pyrene

Introduction

Venom as medicine has interested scientists since ancient times, but only in recent years have important achievements been described. Snake venom has a unique composition of biologically active proteins, with specific features for which they are widely used for scientific and medical purposes; for example, lebetox can stop bleeding. Although viperid venoms may contain well over 100 protein components, venom proteins belong to only a few major protein families, including enzymes (serine proteinases, Zn^{2+}-metalloproteinases, L–amino acid oxidase, group II PLA2) and proteins without enzymatic activity (disintegrins, C-type lectins, natriuretic peptides, myotoxins, CRISP toxins, nerve and vascular endothelium growth factors, cystein, and Kunitz-type proteinase inhibitors) (Zakharyan and Ayvazian 2005; Sanz et al. 2008).

Because of their unique biological effects, many types of snake venom have been utilized as valuable pharmacological reagents for studies on the interaction of their content and organized lipid interfaces, including bilayer lipid membranes (BLM), large unilamellar vesicles (LUV), small unilamellar vesicles (SUV), and multilamellar vesicles (MLV) (Burack et al. 1997; Eble et al. 2003). However, usually because of their particular characteristics (size and lamellarity), these model membrane systems are not necessarily accurate descriptions of cell membranes. The binding of proteins to lipid interfaces depends on the physicochemical and structural properties of the membrane surface. Giant unilamellar vesicles (GUV) are a fascinating model system that were first described 40 years ago; they are ideal for studying lipid–lipid and lipid–protein interactions.

The present study was undertaken to elucidate how the proteolipid GUV from bovine brain interacts with *Macrovipera lebetina obtusa* (MLO) venom.

Materials and Methods

Preparation of Pure Proteolipids

The proteolipid fraction was separated from the bovine brain according to the Folch method (Zobelinsky et al. 1984). White matter was dissected from fresh adult bovine brain and scraped free of gray matter. The white matter was homogenized with 1:1 chloroform–methanol mixture (1 g of tissue/20 ml). The suspension was washed with distilled water to remove nonlipid substances.

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The water that remained between the two phases went into the solution, and 1–2 ml of methanol was added. Next was added half the chloroform volume (compared to the extract), and it was dried in a vacuum pump at room temperature (20–22 °C). Distilled water was then added in proportion to 33 times as much as the weight of the sediment; the latter was centrifuged for 1 h at 4,600×*g*. This procedure was repeated 4 times. The last step lasted 10 min at 200×*g* (2 °C). The resulting sediment was a crude proteolipid, which was dried in a vacuum pump, after which a mixture (1:1) of sulfur ether and ethanol (150 times as much as the sediment weight) was added and centrifuged for 10 min at 4,600×*g* (−10 °C). The resulting sediment was pure proteolipid.

The crude proteolipids contained 40–50 % of protein and 60–50 % of lipids. The content of the phospholipid was about 40 %. Purified proteolipid contained 70 % protein and 30 % lipid. The crude and purified proteolipid (compared with the original lipid extracts from which they were isolated) were enriched in acidic phospholipids: phosphatidylserine, phosphatidylinositol, and diphosphatidylglycerol. Acidic phospholipids in total lipid extract made up 15–20 % of the total phospholipids, in crude proteolipids 25–30 %, and in purified proteolipids 70–75 %, where the greater part is phosphatidylserine (35–40 %).

Giant Unilamellar Vesicles

Giant unilamellar vesicles were prepared according to the electroformation method, developed by Angelova et al. (1992). GUV were formed in a temperature-controlled chamber that allowed a working temperature range of 20–50 °C. GUV were prepared using the following steps: ~2 µl of the proteolipid stock solution was spread on two sample chamber platinum wires. The chamber was then dried for ~1 h to remove any remaining trace of organic solvent. The chamber and the buffer (Tris–HCl 0.5 mM, pH 7.4) were separately equilibrated to temperatures above the lipid mixture phase transition (~10 °C over the corresponding transition temperature), and then 2 ml of buffer was added to cover the wires. Immediately after addition of the buffer, the platinum wires were connected to a function generator, and a low-frequency AC field (sinusoidal wave function with a frequency of 10 Hz and amplitude of 2 V) was applied for 90 min (Bagatolli and Gratton 1999). The diameter of the GUV was up to 300 µm.

After formation of GUV in the electroformation chamber, we added 1 µM 8-anilino-1-naphthalenesulfonic acid (ANS), then incubated it for 5 min at 25 °C (Verstraeten et al. 2005). We next prepared an observation chamber for the microscope study. Images were collected by an epi-fluorescent microscope (FM320-5M; AmScope, USA).

Macrovipera lebetina obtusa venom was added to the electroformation chamber before the vesicles were formed for spectrophotometric study. For the microscope study, we added venom both before and after formation of GUV.

Planar Bilayer Lipid Membranes

The lipid bilayer membranes were formed from the total lipid fractions of the bovine brain on a Teflon aperture by means of the Muller method (Mueller et al. 1962). A Teflon cylindrical cup with a 0.8-mm hole was coupled to a glass chamber so that the cup separated two compartments, each filled with 5 ml electrolyte. Electrical access to the baths was through a pair of Ag/AgCl electrodes.

Optical reflectance, electrical resistance, and capacitance indicated the formation of planar lipid bilayers. The electrical parameters of the planar BLM were determined on a device equipped with a Keithley 301 differential feedback amplifier (Keithley, USA) in voltage-fixation mode, which let us keep up the membrane potential on any level, independent of ionic streams. The potential setting on the exit of the generator completely fell on the membrane, the resistance of which was much higher than that of the resistance of electrodes and electrolytes, as well as the effective resistance of the current's gauge. Electrometric devices can measure a current through membrane under a fixed value of transmembrane difference of potentials. The amount of membrane resistance was calculated by the following formula:

$$R_{\mathrm{m}} = R_{\mathrm{f}} U_{\mathrm{m}} / U_{\mathrm{f}} (\Omega),$$

where U_{f} potential from source of direct current, U_{m} difference of potentials on membrane, R_{m} membrane resistance, and R_{f} resistance of chain.

Conductivity of membrane is expressed by

$$\mathrm{g_m} = 1/R_{\mathrm{m}} (\Omega^{-1}),$$

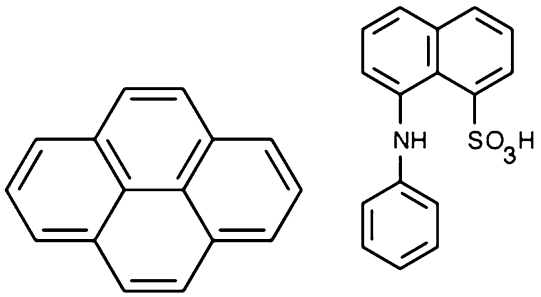
in which specific electrical conductance (g) is expressed in $\Omega^{-1}/\mathrm{mm}^2$, and 0.1 M KCl, NaCl, LiCl, and $CaCl_2$ serve as ionic media.

The breaking potential of membrane recorded in the experiments by a shielded camera was taken as the threshold value of the voltage applied. The potential of membrane rapture was a criterion for valuation of the natural defects of model membranes. Under electrical potential, the radius of these holes increased. There was a critical value of radius (r_{o}), and when $r_{\varphi} > r_{\mathrm{o}}$, membrane was destroyed.

Fluorescence Labeling and Measurements

The fluorescence emission was observed from 350 to 600 nm. The membrane fluorescence probes, ANS and

pyrene, were used to assess the state of the membrane and specifically mark the phospholipid domains. ANS and pyrene allow us to quantify the fluidity changes in the membrane by measuring the fluorescence intensity. These probes were added to the sample chamber after vesicle formation. A total of 1 μM ANS was added to the sample, then incubated for 5 min at 25 °C (Verstraeten et al. 2005). The fluorescence intensity of ANS was inversely proportional to the value of the membrane potential. A total of 3 μM pyrene was added to the samples and then incubated by stirring constantly at 25 °C for 2 min (Galla and Sackman 1974). The fluorescent spectra were acquired on a Varian Eclipse spectrofluorometer instrument; the excitation wavelength for the pyrene monomers and dimers were 286 and 334 nm ($L_{emission}$ were 395 and 470 nm, respectively); the excitation and emission wavelengths for ANS were 360 and 490 nm, respectively (Bordushkov et al. 1993).



Pyrene **ANS**

Statistical Analysis

For quantitative analysis of electrical parameters of BLM, a Student's t test was used to compare differences at each time point, with $P < 0.05$ considered to be significant. All data are presented as mean ± standard error of the mean.

Results

In the first stage of our experiments, we studied the ANS fluorescence intensity with the proteolipid GUV, which were modified by venom. It is interesting to follow the above interactions when we got GUV from pure proteolipids instead of the usual vesicles: according to the division method, after chemical purification, the proteolipids mainly consist of acidic phospholipids (mainly of phosphatidylserine) and membrane proteins (Zobelinsky et al. 1984). Because they are negatively charged, the phospholipid GUV interaction with ANS, which is also negatively charged, is very superficial (Fig. 1a; Table 1).

The study of venom influence on GUV fluidity with the help of pyrene made it possible to discover certain features of the interaction between the fluorescence probe and GUV modified by venom. As a hydrophobic probe, pyrene was not dissolved in buffer solution, and its fluorescence intensity was very weak. However, after incubation in GUV solution, the pyrene fluorescence intensity sharply increased (Fig. 1b). Unlike ANS, pyrene interacts with proteolipid GUV; this hydrophobic interaction is an interesting information source for lipid–protein interaction. The fluorescence of pyrene was not so intense, but as a result of proteolipid GUV, we got two peaks with high intensity that corresponded to monomer and dimer fluorescence peaks in the lipid–lipid and lipid–protein contact points.

The pyrene monomer fluorescence was more intense, which was due to the greater number of lipid–protein contacts. This makes sense, because according to the chemical structure of the proteolipids, there are fewer free lipid bilayer areas in these vesicles, as these phospholipids are hydrophobic. When we compared pyrene interaction in the GUV modified by venom and in proteolipid GUV, we noted that by having the properties of protein, the components of the venom could play the role of membrane protein. However, in case of negatively charged proteolipids, the venom binding mechanisms were superficial.

For the next stage of our research, we combined the GUV formation technique and fluorescence microscopy for the visualization of the above-mentioned interaction. Figure 2 shows the obvious decrease of GUV diameter in the course of modification with venom (low concentration) at 21 °C in the liquid phase. During 47 s, the GUV shrank until virtually nothing remained of the vesicle, instead of the usual vesicles formed from phospholipids (Ayvazian and Ghazaryan 2012). It is worth noting that GUV with a high concentration of proteins are more stable and there is little increase in size, unlike the GUV with a low concentration of proteins (Fig. 3).

The other series of experiments was performed by visualization of GUV modified with MLO venom over the course of GUV formation. This was done to ensure the involvement of venom components in the membrane and to investigate the properties and morphological characteristics of GUV with a damaged lipid bilayer. The GUV with a high concentration of proteins were again stable instead of vesicles with a low concentration of proteins, whereas there were no structured GUV of media with a low venom concentration (Fig. 4a). We also tried to form GUV in media with a high venom concentration. Our preliminary results demonstrated that some GUV formed in this media result in some shape deformation and a very short life span, so we posit that this is a reason for lipid package violation (Fig. 5).

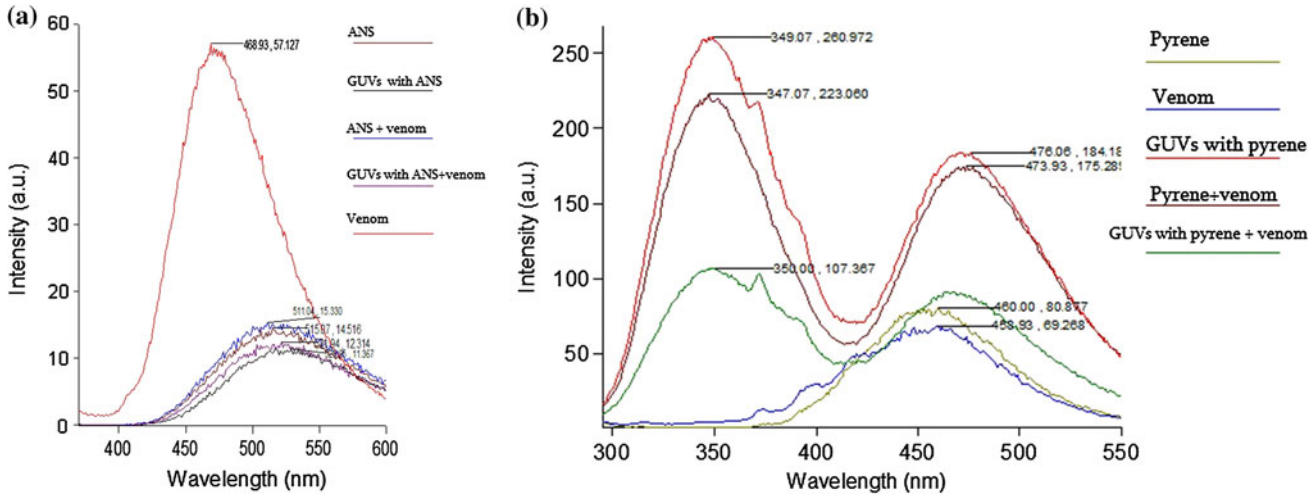


Fig. 1 ANS (**a**) and pyrene (**b**) fluorescence intensity in proteolipid GUV membrane modified with snake venom

Table 1 Pyrene and ANS fluorescence intensity in GUV membrane modified with viper venom

Probe	Pyrene fluorescence intensity (membrane fluidity)		ANS fluorescence intensity
	Protein–lipid contact	Lipid bilayer	
Control proteolipid GUV	260.9	184.2	11.2
Proteolipid GUV containing venom	107.4	85.5	12.3

$P > 0.01$ by Student's t test relative to the corresponding control

The average data were obtained from three independent experiments; the standard errors were calculated as described by Lakin (1992) and did not exceed 5 % if not indicated

Taking into consideration the supposed importance of a membrane surface curvature for its interaction with enzymatic components of venom, we worked out the following scheme, relying on the fact that some principal components of the venom (such as PLA_2) could not easily go into the flat bilayer. That is, under the influence of electric current, these vesicles approach the bilayer flat membrane of the Teflon aperture and become part of the BLM. The introduction of the modified GUV into the BLM is easier, as a lipid–lipid interaction takes place, and as a result BLM is modified by venom components. The addition of modified GUV leads to the channel-like activity, which is periodic in the case of high voltage (200 mV). In fact, the penetration of the GUV bilayer is larger and results in the described effect. It is worth mentioning that the insertion of clean proteolipids with the BLM leads to a different kind of interaction. The BLM modified with such proteolipids have low resistance (10^7–10^8 Ω, $U_m = 50$ mV) and channel-like activity that is not periodic and that shows the work of different membrane proteins (Table 2).

Discussion

We conclude that MLO viper venom, which has a complicated chemical composition of biologically active substances, interacts with proteolipid GUV and leads to a tendency to decrease the GUV membrane fluidity formed by pure proteolipids, both in the lipid–lipid contact area and in lipid bilayer. The GUV with a high concentration of proteins are more stable, unlike the GUV with a low concentration of proteins. The proteolipid GUV interacting with the BLM go into the lipid bilayer, and the bilayer has low resistance and channel-like activity that is not periodic,

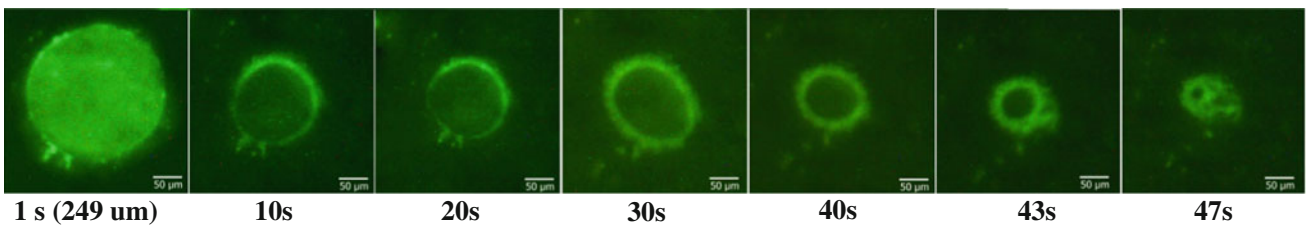


Fig. 2 Rapid changes of the size of the ANS-containing GUV in the course of MLO venom (47 s). Dried lyophilized MLO toxin was dissolved in Tris–HCl buffer (pH 7.4), final concentration 3×10^{-5} M, and 1.1 µl of this solution were added to the fluorescent microscope sample. The initial size of the GUV was 249 µm

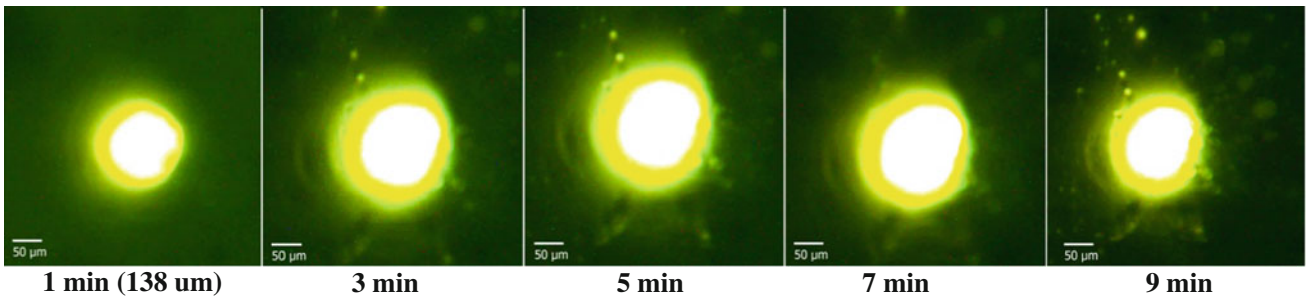


Fig. 3 The increase in size of the high-concentration protein GUV depended on MLO venom. Dried lyophilized toxin of MLO was dissolved in Tris–HCl buffer (pH 7.4), final concentration 3×10^{-5} M; 2.2 μl of this solution was added to the fluorescent microscope sample

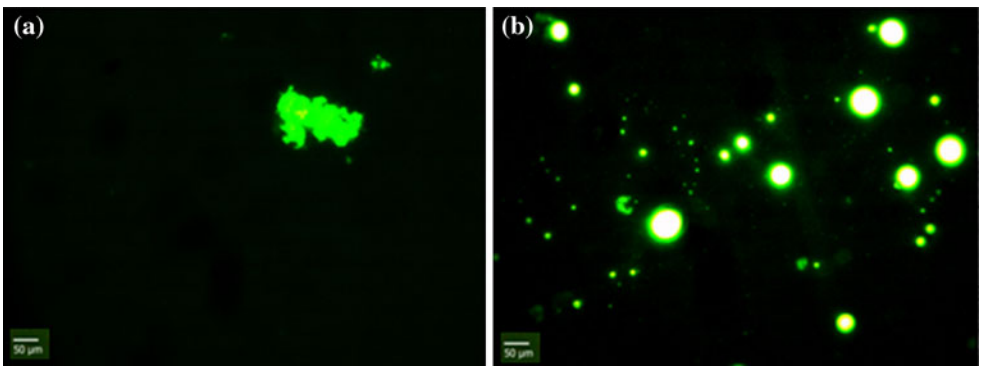


Fig. 4 **a** The fluorescent image of nonstructured ANS-containing lipids damaged with MLO venom in the course of GUV formation. **b** Stable GUV with a high concentration of proteins. Dried lyophilized MLO toxin was dissolved in Tris–HCl buffer (pH 7.4); final MLO concentration, 10^{-5} M

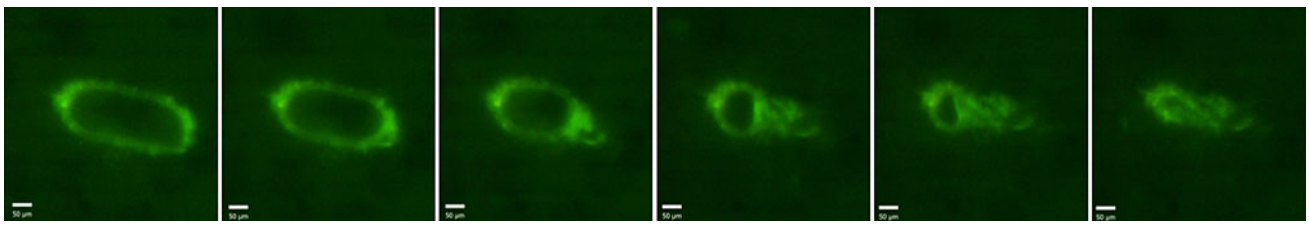


Fig. 5 Rapid changes of the size of the ANS-containing GUV formed in MLO venom solution (58 s). We tried to prepare GUV according to the electroformation method in a temperature-controlled chamber containing Tris–HCl buffer (pH 7.4); final MLO concentration, 10^{-3} M

Table 2 Change in BLM electric parameters after insertion with venom-modified GUV

Sample	R_m (Ω)	g_m (Ω^{-1}/mm^2)	$U_{rupture}$ (mV)
Control	1×10^{11}	2×10^{-11}	448
Proteolipid GUV with venom	1×10^{8}	2×10^{-4}	219

R_m Resistance, g_m conductivity, and U_r breaking potential formed from brain lipids in media with K^+

The average data were obtained from three independent experiments; the standard errors were calculated as described by Lakin (1992) and did not exceed 5 % if not indicated

and it demonstrates the work of different membrane proteins.

Our data confirm previously obtained results on PLA_2 mechanisms in GUV through the LAURDAN and PRODAN probes (Bagatolli and Gratton 2000), but it is worth mentioning that unlike the results of this other study, we assessed the complex structure of the venom, and the interaction of its separate particles on GUV may be different. The synergy is especially important for the subfamily Vipera because they have no real toxins in the venom (like the three-finger toxins of Elapidae), but they

form complexes with other nonenzymatic proteins to achieve higher efficiency through synergy. Our results demonstrate that the addition of MLO venom to proteolipid GUV shows gross morphological changes, both distortions in vesicle membrane and shrinking of the vesicle size. These effects will likely disturb the local packing and hydrolysis of the surface phospholipids by venom.

Macrovipera lebetina obtusa venom action with GUV from brain proteolipids and the study of the membrane change dynamics with the methods outlined above allow us to clarify a few essential parts of this interaction, as these mechanisms have not been thoroughly studied.

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Conflict of interest The authors report that they have no conflicts of interest.

References

Angelova MI, Soleau S, Meleard P, Faucon JF, Bothorel P (1992) Preparation of giant vesicles by external fields. Kinetics and application. Progr Colloid Polym Sci 89:127–131

Ayvazian NM, Ghazaryan NA (2012) Lipid bilayer condition abnormalities following Macroviperalebetinaobtusa snake envenomation. Toxicon 60:607–613

Bagatolli LA, Gratton E (1999) Two-photon fluorescence microscopy observations of shape changes at the phase transition in phospholipid giant unilamellar vesicles. Biophys J 77:2090–2101

Bagatolli LA, Gratton EA (2000) Correlation between lipid domain shape and binary phospholipid mixture composition in free standing bilayers: a two-photon fluorescence microscopy study. Biophys J 79:434–447

Bordushkov Yu, Goroshinskaya I, Frantzyantz E (1993) Effects of electromagnetic field exposure on some structural and functional properties of blood lymphocyte and erythrocyte membranes. Biofizika 38:1098–1103

Burack WR, Dibble AR, Allietta MM, Biltonen RL (1997) Changes in vesicle morphology induced by lateral phase separation modulate phospholipase A_2 activity. Biochemistry 36:10551–10557

Eble JA, Bruckner P, Mayer U (2003) Viperalebetina venom contains two disintegrins inhibiting laminin-binding beta1 integrins. J Biol Chem 278:26488–26496

Galla HJ, Sackman E (1974) Lateral diffusion in the hydrophobic region of membranes: use of pyreneexcimers as optical probes. Biochim Biophys Acta 339:103–115

Lakin GF (1992) Biometry. Vishaya Shcola, Moscow [in Russian]

Mueller P, Rudin D, Tien H (1962) Reconstruction of cell membranes structure in vitro and its transformation into an excitable system. Nature 194:979–980

Sanz L, Ayvazyan N, Calvete JJ (2008) Snake venomics of the Armenian mountain vipers Macroviperalebetinaobtusa and Viperaraddei. J Proteomics 71:198–209

Verstraeten SV, Hammerstone JF, Keen CL, Fraga CG, Oteiza PI (2005) Antioxidant and membrane effects of procyanidin dimmers and trimers isolated from peanut and cocoa. J Agric Food Chem 53:5041–5048

Zakharyan AE, Ayvazian NM (2005) Modeling of BLMs in aspect of phylogenetic development of vertebrates. In: Ottova-Leitmannova A (ed) Advances in planar lipid bilayers and liposomes, 2nd edn. Elsevier, Amsterdam, pp 238–259

Zobelinsky CA, Pomazansky LF, Chirkovskaya EB (1984) Brain proteolirids of representatives of different vertebratae classes. J Evol Biochem Physiol 20:240–246