Original article

Fractionation of *Vipera berus berus* Snake Venom and Detection of Bioactive Compounds Targeted to Blood Coagulation System

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Abstract

Introduction. The performed research focused on a search for new biologically active compounds acting on blood coagulation system proteins and cells. To achieve this goal, we fractionated Vipera berus berus snake venom and studied the action of the separated fractions on human blood plasma, fibrinogen, platelets or red cells.

Methods. Crude venom was fractionated using ion-exchange chromatography. Protein composition of fractions was studied using SDS-PAGE. The ability of fractions to prolong or initiate blood plasma clotting was studied using the prothrombin time test with thromboplastin. Fibrinogen-specific proteases were detected using enzyme-electrophoresis. Action on red cells was estimated using the hemolysis test. Aggregometry was used for the detection of action on platelets. All experiments in this study were performed in vitro.

Results. We obtained fractions containing phospholipase and a protease that is able to hydrolyze fibrinogen, leading to the loss of its ability to polymerize and to maintain platelet aggregation.
Conclusion. Further purification and study of these components can be a promising research direction for biotechnological as well as for biomedical use.

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Introduction

Snake venoms are complex mixtures of bioactive components, particularly proteins, nucleic acids, and organic compounds. The venom's main purpose is to immobilize or kill prey, usually small mammals, reptiles, birds or amphibians. The venom's components get into the prey's blood during the bite, and are instantly distributed through its body via blood flow.

Evolutionary, venomous reptiles have developed different routes to hit the prey, one or a few of which appeared to be more successful and thus started to prevail. Most snake venoms have neurotoxic action [1]; however, some snakes kill their prey by inducing generalized inflammatory tissue response, or by using blood coagulation activators, which cause massive coagulation or conversely cause hemolysis, inability of the blood to clot, destroy blood vessels and lead to internal hemorrhages [2].

In any case, if one way of immobilizing a preferable type of prey prevails, other venom components do not vanish from the venom instantly. Instead, their effects are masked by the more active components. This is why venoms of some snakes simultaneously contain, for instance, enzymes hydrolyzing fibrinogen, which decrease blood coagulation ability, and fibrinogen activators, which stimulate thrombus production, in different proportions.

Since venom comes into contact primarily with the prey's blood, the action of many of its components is aimed at the circulatory system. In particular, the proteases destroying fibrinogen, platelet aggregation inhibitors, activators of blood coagulation factors, phospholipases, etc. were found in snake venoms [2]. Obtaining and describing these components led to the discovery of unique enzymes, used nowadays in medicine and laboratory diagnostics. That is why the study of snake venom composition is a current issue of modern biotechnology [3].

Functionally active components of snake venoms may also be used in fundamental research on the features of certain elements of the hemostasis system. In particular, fibrinogenases (enzymes capable of selectively hydrolyzing fibrinogen) appear to be useful tools for studying the role of certain molecule sites [4].

In our research, we focused on the venom of a snake from Ukrainian fauna, which made our object available for analysis, as well as for potential further biotechnological production. The purpose of the study was to search for Vipera berus berus venom proteins, which can be used as effectors of the blood coagulation system.

Materials and methods

Materials

a) Vipera berus berus *venom*

Crystalline venom of Vipera berus berus was provided by the Laboratory of Experimental Herpetology of the Trypillia Biochemical Factory (Trypillia, Ukraine).

b) Human blood plasma

Blood was taken from the vein using a sharp dry needle with a large diameter without a syringe (to prevent hemolysis), discarding the first 5–6 blood drops. During blood taking, neither a tourniquet nor a massage was applied in order to prevent blood coagulation activation and fibrinolysis. Blood collection was performed under fasting conditions.

A 3.8% sodium citrate solution was introduced into the polyethylene tube using a dispenser and further admixed with blood in a 1:9 ratio. Next, the tube was tightly closed and gently mixed using slow hand movements, without shaking.

Platelet-rich plasma (PRP) was obtained by centrifugation at 200 g for 30 min [5]. During centrifugation, the tubes were open so as not to prevent oxygen access to the platelets.

Platelet-poor plasma was obtained by centrifugation at 450 g for 20 min at a temperature lower than 20 ˚C.

This study was approved by the ethical committee of the Palladin Institute of Biochemistry of NAS of Ukraine, on 9 December 2021, N12. Volunteers signed informed consent forms prior to blood sampling, in accordance with the Declaration of Helsinki.

c) Human fibrinogen

Fibrinogen was obtained from human blood plasma by salt extraction using 16% Na2SO4. The content of protein coagulated by thrombin was 96–98%. To remove vitamin K dependent proteins – prothrombin, factors IX and X of the blood coagulation system, and protein C – plasma was cooled to +4 ˚C and, under constant mixing, admixed with BaSO4 (30 g/L of blood), then centrifuged at 1300 g for 10 min. The BaSO4 addition procedure was repeated twice.

In the next step, blood plasma was heated to room temperature, and 1 M glycine buffer (glycine and 1 M NaOH, pH 9) was added at a 1:10 ratio to the entire volume. After that, one fraction was precipitated using 16% Na2SO4 (slowly adding a small portion thereof with constant mixing). The resulted precipitate was spun-down at 1300 g for 30 min at ambient temperature. Supernatant was carefully discarded, and then fibrinogen precipitation was performed using 16% Na2SO4. After that, fibrinogen was spun-down for 30 min at 1300 g at a temperature of 10–15 ˚C. The precipitate was then diluted in 0.2 M NaCl – 250 mL of solvent was used for solving the precipitate from 1 L blood plasma.

The obtained fibrinogen was re-precipitated by an equal volume of 16% Na2SO4, and then the precipitate was diluted in 0.2 M NaCl (150–200 mL for 1 L blood plasma). After that, the fibrinogen solution was added with 0.5 M phosphate buffer (KH2PO4 and NaOH, pH 6.5) at a 1:5 ratio to the entire fibrinogen solution volume, and then again re-precipitated by equal volume of 16% Na2SO4. The order of steps is analogous with the first precipitation, but re precipitated fibrinogen was diluted with 0.15 M NaCl (70–150 mL of solvent for 1 L blood plasma).

Re-precipitated fibrinogen was placed on ice with a temperature of +4 ˚C for the night in order to separate the cryoforms of fibrinogen. The expected concentration of fibrinogen while cryoforms were separating was 10–12.5 mg/mL. Then, the fibrinogen solution was centrifuged for 30 min at 1300 g, the supernatant liquid was carefully discarded, and another re precipitation by 16% Na2SO4 was performed. The precipitated fibrinogen was diluted in 0.15 M NaCl, after which the solution was frozen and stored at -20 ˚C [6].

d) Reagents

The following materials and reagents were used in the present experiments: acrylamide, bis-acrylamide, molecular mass markers for electrophoresis (Fermentas, EU), tris (Merck, USA), SDS (Bio-Rad Laboratories), Q Sepharose (Pharmacia, Sweden), PD-10 columns. The thromboplastin (Thromborel) was bought from Siemens, Germany. Molecular weight markers SM0671 (250; 130; 100; 70; 55; 35; 25 та 10 kDa) were purchased from Fermentas (EU). BluEye molecular weight markers (245; 180; 100; 75; 63; 48; 35; 25; 20; 17 та 11 kDa) were purchased from Sigma-Aldrich (USA).

Methods

a) Ion-exchange chromatography

Q Sepharose is the anion-exchange agent, which allows efficient fractioning of the protein mixture under alkaline pH [7]. To fraction the venom, Q Sepharose was balanced with 0.05 M tris-HCl buffer, pH 8.9. The column volume was 12.5 mL, and flow speed was 1 mL/min. The adsorbed proteins were eluted with the same buffer in staged gradient: 0.1, 0.2, 0.5 and 2 M NaCl, pH 8.9. For fractioning, the Acta Prime (Acta, USA) chromatographic system was used.

The obtained material was described using SDS-PAGE.

b) Protein concentration identification

Protein concentration in obtained fractions was approximately identified through spectrophotometry, by optic absorbency under 280 nm subtracting the absorbency under 320 nm using the Optizen POP (Optizen, Korea) spectrophotometer. The Bradford method was used for quantitative identification of protein concentrations [8].

c) Electrophoresis in polyacrylamide gel

Electrophoresis in polyacrylamide gel (PAGE) with SDS was performed according to the Laemmli method, where the tris-glycine system is used in the device for vertical gel electrophoresis in the plates [9].

Samples for electrophoresis were prepared in accordance with the following algorithm: the 1 mg/mL protein solution was added with a sample buffer that contained 5% sucrose or glycerin, 2% SDS and bromophenol (bromophenol is added until coloration appearance).

To identify the sites containing proteins, gel-plates were developed by staining it in a solution (0.125% Coomassie G-250 in 25% isopropanol and 10% acetic acid) for 10 minutes. To discard the staining remains, we used a 3–9% acetic acid solution. The method resolution was 1 μg of protein.

To establish the molecular mass of studied proteins, molecular markers were used (Fermentas, EU): SM0671 (250; 130; 100; 70; 55; 35; 25 та 10 kDa).

d) Enzyme-electrophoresis with fibrinogen as substrate

Enzyme-electrophoresis was performed using the Laemmli method in 15% PAAG [9]. The 0.5 mg/mL fibrinogen was put into the separating gel before polymerization. After electrophoresis, SDS was removed from the gel through trice washing with 2.5% Triton X-100 for 30 min. The gel was incubated in 0.1 M glycine buffer, pH = 8.3, for 12 hours. After that, the gel was stained with Coomassie G-250, and the sites of proteolytic activity were identified by uncolored spots on the gel [9].

To establish the molecular mass of studied proteins, molecular markers were used (Sigma-Aldrich, USA): BluEye (245; 180; 100; 75; 63; 48; 35; 25; 20; 17 та 11 kDa).

e) Prothrombin time (PT)

This method consists of measuring the coagulation time of citrate plasma after thromboplastin and Ca^{2+} addition. Normal prothrombin time is 12–17 s. Elongation of prothrombin time may be linked to deterioration of vitamin K-dependent factor synthesis, which are involved in the external pathway of blood coagulation – VII, X, II – and also to indirect anticoagulant therapy. Shortening of prothrombin time is linked to anticoagulant content decrease in the blood, or to pathological activation of the procoagulant chain of the hemostasis system [10]. To perform the test, 80 μL of blood plasma was added with 20 μL of examined fraction (or equal volume of 0.05 M tris-HCl buffer, pH 8.9, in control), and with 100 μL of 0.025 M $CaCl_2$ solution, and then polymerization was initiated by adding 100 μL of thromboplastin (Thromborel, Siemens, Germany). The time of clot formation was controlled.

f) Establishing hemolytic activity

Red blood cells were obtained from citrate blood via centrifugation for 20 min at 125 g and a temperature of 20 ˚C. The precipitated erythrocytes were washed by trice re-suspending in 10 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl, 5 mM KCl, 1 mM $MgSO_4$, and 10 mM sucrose, with following precipitation at 125 g and a temperature of 20 ˚C.

The suspension of washed human red blood cells obtained using this method [11] was diluted in a 1:8 ratio with 10 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl, 5 mM KCl, 1 mM $MgSO_4$, and 10 mM sucrose. The amount of 50 μ L of the suspension was added with the studied sample (total volume – 1 mL), mixed, and incubated at 37 ˚C for 30 min.

After incubation, the red blood cells were precipitated through centrifugation at 1000 g for 10 min and examined the supernatant's absorbency at 543 nm against the buffer. Hemolysis caused by 50 μL of 1% Triton-X100 was treated as 100%.

Absorbance was measured using the Optizen POP spectrophotometer (Korea).

g) Platelet aggregation study

Platelet aggregation was studied using the SOLAR AP2110 aggregometer (Belarus). The 0.2 mL blood platelet-rich plasma (PRP) was put into the spectrophotometer cuvette and added with 50 μL of studied fraction, or the equal volume of 0.05 M tris-HCl buffer, pH 8.9, in control. The mixture was incubated for 3 min, then 0.025 mL of 0.025 M CaCl2 was added and it was again incubated in the cuvette for 3 min. After that, 0.025 mL of 25 μM ADP was added to the cuvette [12]. The entire aggregation process was recorded, and the aggregation level was estimated.

h) Statistical analysis

Statistical analysis was performed using Microsoft Excel (Microsoft package). All analyses were conducted in the series of three replicates; standard deviation was considered in data analysis. Student's T-test was used. Results are presented as mean ± standard deviation. Data were considered significant when $p < 0.05$.

Results

Fractionation of V. berus berus *venom*

For fractionation, 50 mg of crude Vipera berus berus venom was diluted in 1 mL of 0.05 M tris-HCl buffer, pH 7.4. Before injection, the venom solution was centrifuged at 200 g for 15 min. Using the Akta Prime chromatographic system, the venom was fractionated at a speed of 4 mL/min using the column, filled with chromatographic sorbent Q Sepharose of 15 mL volume.

After we collected the fraction, which did not bond with the sorbent under present conditions, the column was washed with 0.05 M tris-HCl buffer, pH 7.4. Elution was performed using stepping gradient of NaCl: 0.1, 0.2, 0.3, 0.5 M of NaCl in 0.05 M tris-HCl buffer, pH 8.9. Finally, the sorbent was washed with a buffer of 1 M NaCl. The chromatogram is presented in Figure 1.

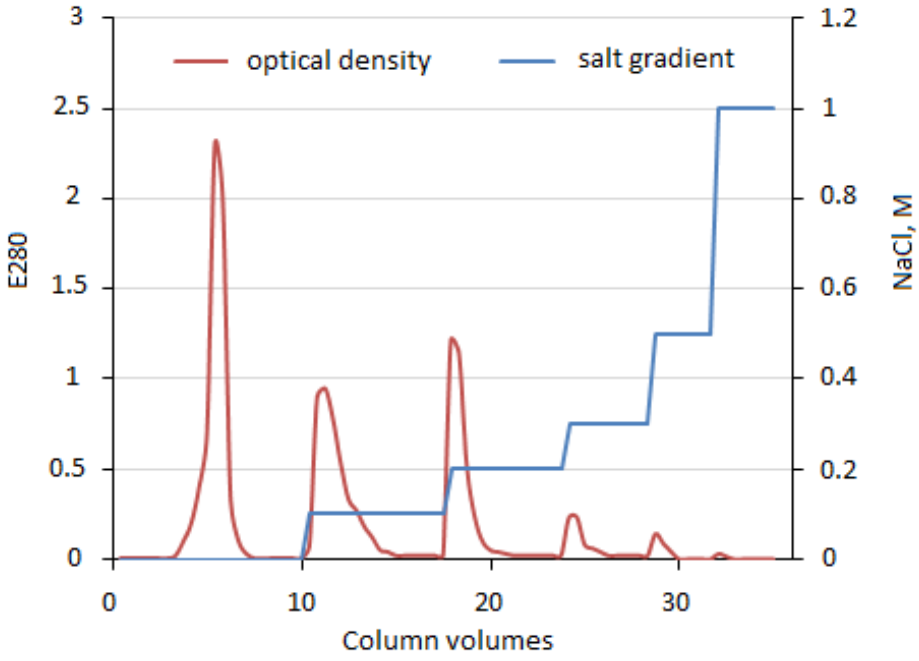

Figure 1. Separation of crude Vipera berus berus venom on Q Sepharose. Speed of elution was 4 mL/min (elution by stepping gradient of NaCl (0.1, 0.2, 0.3, 0.5, 1 M) in 0.05 M tris-HCl buffer, pH = 7.4).

The obtained fractions were de-salted and concentrated using centrifuge microconcentrators Amicon Ultra 3K. For detection of fractions' protein composition, gel-electrophoresis was used.

SDS-PAGE

It was demonstrated that all obtained fractions had different protein compositions and contained proteins of molecular mass from 120 to 10 kDa (Figure 2). This was important for describing each fraction, and for revealing their potential unique physiological effects.

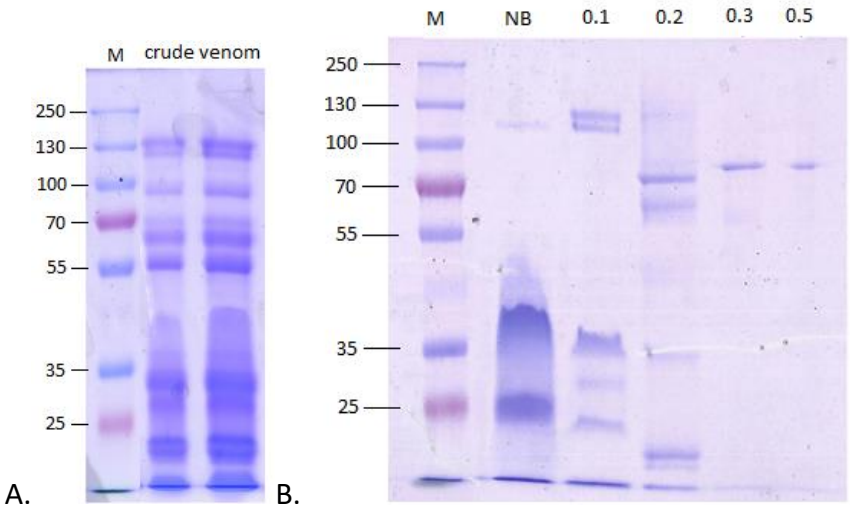

Figure 2. A. – electrophoregram of crude Vipera berus berus venom. B. – electrophoregram of Vipera berus berus venom fractions obtained on Q Sepharose. M – molecular mass markers (250-10 kDa). NB – fraction which did not bind to the sorbent under present conditions. 0.1, 0.2, 0.3, 0.5 – fractions eluted by 0.1, 0.2, 0.3, 0.5 M NaCl, respectively.

Fractions' impact on fibrinogen and fibrin polymerization

To estimate the ability of obtained fractions to inhibit fibrin polymerization, we detected the coagulation time prolongation for blood plasma in the prothrombin time test in the presence of studied fractions. For this purpose, 80 µL of blood plasma was incubated with 20 µL of the fraction at the final concentration of 0.05 o.u./mL (or equal quantity of buffer) for 5 min. The coagulation was initiated by adding 100 µL of 0.025 M CaCl2, and 100 µL of thromboplastin solution. It showed prolongation of the coagulation time for blood plasma of up to 60 s (compared to 40 s in control) in the presence of the fraction eluted by 0.2 M NaCl. Such prolongation may be linked to the presence of the enzyme which can hydrolyze fibrinogen, and which was found in this fraction and in the crude venom. Other fractions did not have such ability (Figure 3).

Identification of proteolytic action toward fibrinogen in the chosen fraction was performed using enzyme-electrophoresis with fibrinogen as the substrate. The enzyme-electrophoresis method combines electrophoretic separation and enzymography.

In particular, it was proved that the fraction eluted by 0.2 M NaCl contained the enzyme capable of hydrolyzing fibrinogen. Its approximate molecular mass was 50 kDa (Figure 4). We concluded that the presence of this enzyme explains the ability of this fraction to slow down fibrin polymerization.

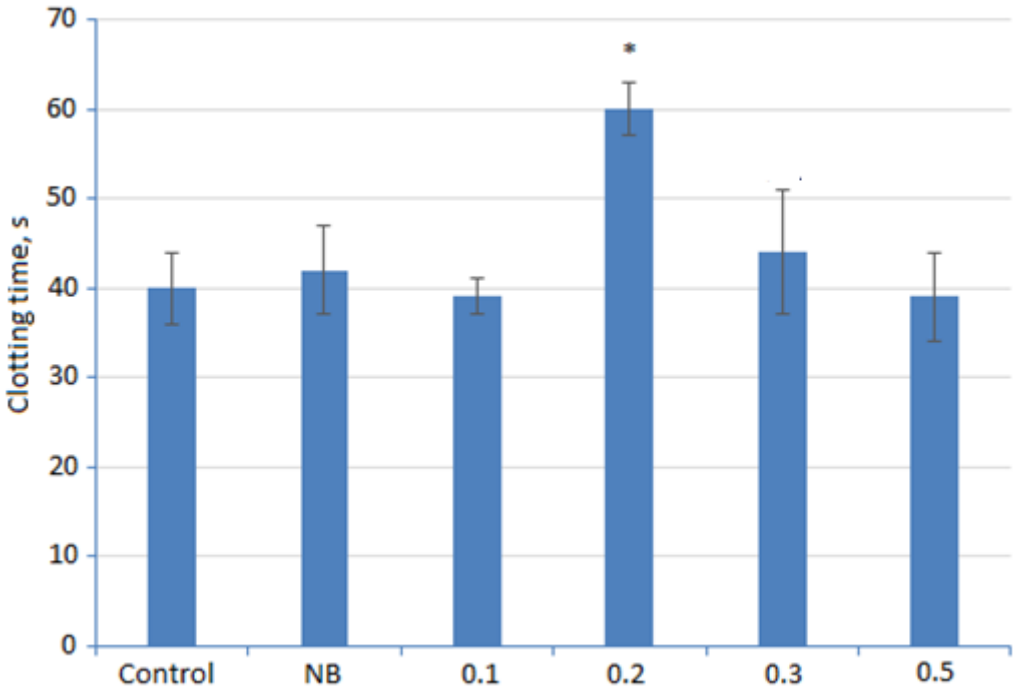

Figure 3. Time of thromboplastin-induced blood plasma coagulation after 5 minutes of incubation with Vipera berus berus venom fractions, obtained on Q Sepharose. NB – fraction which did not bind to the sorbent under present conditions. 0.1, 0.2, 0.3, 0.5 – fractions eluted by 0.1, 0.2, 0.3, 0.5 M NaCl, respectively. * – the result is significant in comparison to control at p < 0.05. The diagram is based on the results of three independent experiments.

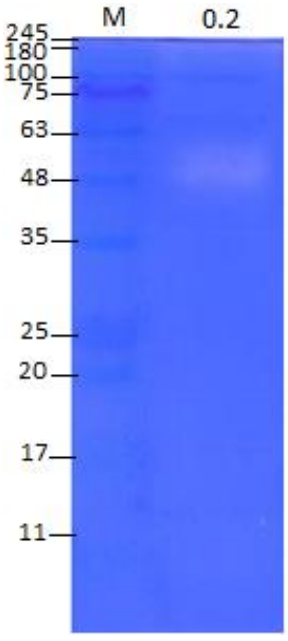

Figure 4. The enzymogram of Vipera berus berus venom fraction obtained on Q Sepharose and eluted with 0.2 M NaCl. M – molecular mass markers (245-11 kDa); 0.2 – fraction.

Hemolytic activity

To reveal phospholipases in the obtained fractions, their ability to provoke red blood cell hemolysis was studied. For this purpose, a homogeneous suspension of human red blood cells was prepared. As the positive control, the hemolytic agent Triton X-100 was used. The samples were incubated at 37 ˚C for 60 min, then the red blood cells were precipitated by centrifugation, and relative hemolytic activity was estimated by absorbance under 543 nm.

Red blood cell hemolysis was observed in the presence of the Vipera berus berus venom fraction eluted by 0.5 M NaCl. It is curious that the gel-electrophoretic data showed the presence of a single protein component of 70 kDa mass in this fraction.

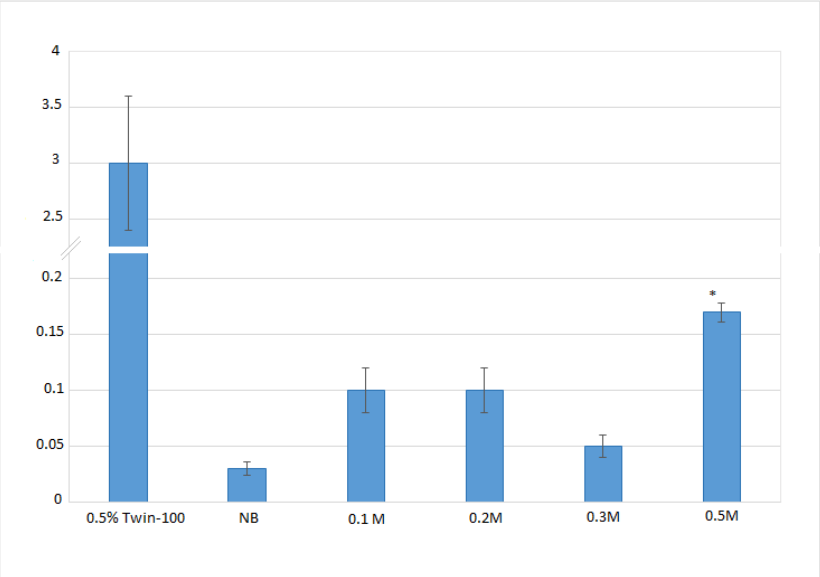

Figure 5. Time of erythrocyte hemolysis under previous incubation with Vipera berus berus venom fractions. Extinction of supernatant after hemolysis was estimated under the 543 nm light wave. As the control, extinction of supernatant of erythrocyte solution with PBS buffer was taken. 0.5% Twin-100 – erythrocyte solution with 0.5% Twin-100 detergent (positive control). NB – fraction which did not bind to the sorbent under present conditions. 0.1, 0.2, 0.3, 0.5 – fractions eluted by 0.1, 0.2, 0.3, 0.5 M NaCl, respectively.

*** – the result is significant at $p < 0.05$. The diagram is based on the results of three independent experiments.**

Impact on platelets

To estimate the impact of obtained fractions on hemostasis, we used the original approach with modified aggregometry, which allowed us to consider the ability of fractions to activate the platelets, initiate blood coagulation, or inhibit platelet aggregation.

For this purpose, 50 mL of the fraction (in the final quantity of 0.05 o.u./mL) was added to 200 µL of platelet-rich blood plasma and incubated in the aggregometer cuvette for 3 min, recording the platelet aggregation process. After that, 25 µL of 0.025 M CaCl2 was added, and recording was continued for another 3 min. Then, 25 µL of 25 µM ADP was added, and platelet aggregation was recorded. Decrease of the platelet aggregation level at this stage of measurement may indicate inhibition of aggregation or the damaging of platelets after contact with the studied fractions.

The analysis of aggregation curves presented in Figure 6 allowed us to conclude that the fraction, which did not bond to the sorbent, and the fraction eluted by 0.1 M NaCl were able to activate the platelets moderately.

At the same time, the platelet aggregation inhibitor was found in the fraction eluted with 0.2 M NaCl. The ADP addition did not cause platelet aggregation. It is important to underline that the same fraction had the fibrinogen-specific protease, according to our data. Fibrinogen hydrolysis may be one of the causes of inhibition of platelet aggregation, since this process is mediated by fibrinogen.

The fraction eluted with 0.5 M NaCl did not have an intense impact on the platelets. Apparently, 6 minutes of incubation of fractions with platelets was not enough for a manifestation of phospholipase activity, which was predicted by the results of red blood cell hemolysis (60 min incubation).

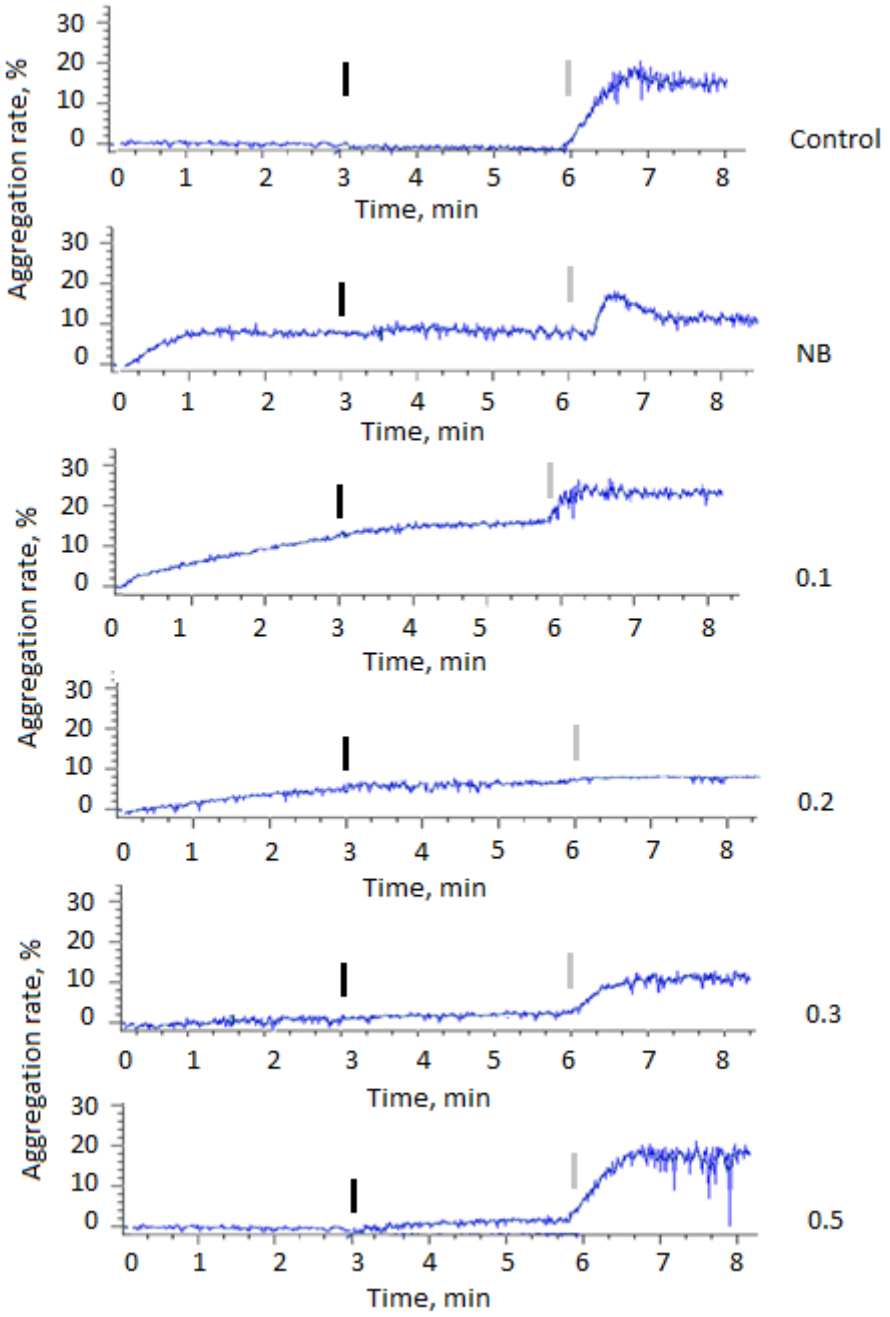

Figure 6. The plot of platelet aggregation in platelet-rich blood plasma after incubation with Vipera berus berus venom fractions. Control – under the equal buffer volume. NB – fraction which did not bind to the sorbent under present conditions. 0.1, 0.2, 0.3, 0.5 – fractions eluted by 0.1, 0.2, 0.3, 0.5 M NaCl, respectively. Typical curves for three independent experiments are shown.

Black lines – points of addition of 25 mkl of 0.025 M CaCl2; Grey lines – points of addition of 25 µl of 25 µM ADP.

Discussion

The fractioning of Vipera berus berus venom allowed us to obtain separate fractions, which differ in their protein compositions and in their action on the hemostasis system. In particular, we obtained the fractions containing phospholipase. We also found a protease capable of hydrolyzing fibrinogen, leading to the

loss of its ability to maintain platelet aggregation. The presence of components capable of moderately activating the platelets is expected for some venom fractions. Further purification and study of these components may represent a promising research direction.

Phospholipases, the enzymes that selectively destroy phospholipids, have been obtained from a few snake venoms. In particular, the phospholipases of 12.5, 13.9 and 14.2 kDa were obtained from the Crotalus molossus nigrescens venom. Authors suggest that they should be used in oncological medicine due to their potential as anticancer agents and their lower neurotoxic effects compared to conventional drugs [13]. Phospholipases were also found in the Naja sumatrana and Bothrops alternatus venoms, and methods for their purification were suggested [14,15]. We expect that the application of newly purified phospholipase may be useful for researching the structural-functional features of lipid membranes.

Recent studies have discussed the practical application of phospholipases. In particular, by inducing Treg cells, these enzymes can be effective as potential therapeutic agents in atherosclerosis [16]. Antibacterial activity is another promising feature of phospholipases [17,18]. Targeting the cell membrane, phospholipases can disrupt the cellular integrity of bacteria. In addition to their potential biomedical use, phospholipases can be applied in studying liposomes and lipid rafts [19].

Further study of the fibrinogen-specific protease from the Vipera berus berus venom will let us establish its specificity concerning certain chains of the fibrinogen molecule. Enzymes capable of selectively hydrolyzing fibrinogen are used in studying its structure and function [20] and obtaining partly hydrolyzed fibrinogen forms [21]. The possibility of fibrinogenase use for thrombolysis is also being discussed [22]. For this purpose, several recombinant analogs for certain fibrinogenases were obtained [23,24].

The mechanism of anticoagulant action of fibrinogen-specific proteases is obvious – cleaving of fibrinogen chains by these enzymes decreases its ability to polymerize [25]. In contrast to other anticoagulants that inhibit coagulation factors (mainly thrombin or factor Xa), fibrinogen-specific proteases act in the final step of the clotting process, preventing the formation of a clot [26]. Likewise, these enzymes can often effectively cleave polymerized fibrin, thus promoting the dissolution of intravascular thrombus [27]. cardiovascular and renal disease in non-diabetic individuals with arterial hypertension remains a question to be answered.

Conclusion

Proteins from vipers' venoms do not only cause death; they can also be used for treatment of thrombosis, arthritis, cancer and many other diseases [ ]. As snake venom contains a wide spectrum of biological compounds, research conducted for the purpose of identifying their structure, biological activity, and pharmaco-clinical application is now of great significance [28].

We have developed a novel method of fractionation of Vipera berus berus venom, which enabled us to obtain fractions with different action on the human blood coagulation system. Our data corresponded to the recently summarized results presented in [2]. Further purification of bioactive proteins that we found in Vipera berus berus venom will allow the performance of their full characterization. These molecular effectors can be used for studying regulatory mechanisms of blood coagulation system regulation.

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Disclosure

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Competing interests. None to declare.

References

1. Nițescu GV, Ulmeanu CE, Crăciun MD, Ciucă AM, Ulici A, Ghira I, Lonati D. Neurotoxicity and Other Clinical Manifestations of a Common European Adder (Vipera berus) Bite in Romania. Toxins (Basel) 2022; 18;14(7):500.
2. Siigur J, Siigur E. Biochemistry and toxicology of proteins and peptides purified from the venom of Vipera berus berus. Toxicon X 2022 Jun 12;15:100131. doi: 10.1016/j.toxcx.2022.100131
3. Op den Brouw B, Ghezellou P, Casewell NR, Ali SA, Fathinia B, Fry BG, Bos MHA, Ikonomopoulou MP. Pharmacological Characterisation of Pseudocerastes and Eristicophis Viper Venoms Reveal Anticancer (Melanoma) Properties and a Potentially Novel Mode of Fibrinogenolysis. Int J Mol Sci. 2021; 22(13):6896. doi: 10.3390/ijms22136896.
4. Cortelazzo A, Guerranti R, Bini L, Hope-Onyekwere N, Muzzi C, Leoncini R, Pagani R. Effects of snake venom proteases on human fibrinogen chains. Blood Transfus 2010 Jun;8 Suppl 3(Suppl 3):s120-5. doi: 10.2450/2010.019S.
5. Chernyshenko V, Shteinberg K, Lugovska N, Ryzhykova M, Platonova T, Korolova D, Lugovskoy E. Preparation of highly-concentrated autologous platelet-rich plasma for biomedical use. Ukr. Biochem. J. 2019; 91:2.
6. Varetska T, Microheterogeneity of fibrinogen. Cryofibrinogen Ukr. Biochem. J. 1960; 32:13-24.
7. Cummins PM, Rochfort KD, O'Connor BF. Ion-Exchange Chromatography: Basic Principles and Application. Methods Mol Biol. 2017; 1485:209-223.
8. Bradford MM. A rapid and sensitive method for quantities of utilizing the principle of protein binding. Analytical Biochemistry 1976; 86:193-200.
9. Ostapchenko L, Savchuk O, Burlova-Vasilieva N. Enzyme electrophoresis method in analysis of active components of haemostasis system. ABB 2011 ;2:20-26.
10. Koroleva DS, Vynogradova RP, Chernyshenko TM, Platonova TM, Volkov GL. The use of ekamulin - a prothrombin activator from the poison of the Echis multisquamatus in clinical laboratory diagnostics. Laboratory diagnostics 2006; 3:18-21.
11. Kozynets GI, Makarov VA. Blood system research in clinical practice. Triada-X 1997: 480.
12. Born GV. Changes in the distribution of phosphorus in platelet-rich plasma during clotting. Biochem J. 1958; 68(4):695-704.
13. Lazcano-Pérez F, Rangel-López E, Robles-Bañuelos B, Franco-Vásquez AM, García-Arredondo A, Navarro-García JC, Zavala-Moreno A, Gómez-Manzo S, Santamaría A, Arreguín-Espinosa R. Chemical structure of three basic Asp-49 phospholipases A2 isolated from Crotalus molossus nigrescens venom with cytotoxic activity against cancer cells. Toxicon 2022; 210:25-31.
14. Abdullah NAH, Rusmili MRA, Zainal Abidin SA, Shaikh MF, Hodgson WC, Othman I. Isolation and Characterization of A2-EPTX-Nsm1a, a Secretory Phospholipase A2 from Malaysian Spitting Cobra (Naja sumatrana) Venom. Toxins (Basel) 2021; 13(12):859.
15. Dias EHV, Dos Santos Paschoal T, da Silva AP, da Cunha Pereira DF, de Sousa Simamoto BB, Matias MS, Santiago FM, Rosa JC, Soares A, Santos-Filho NA, de Oliveira F, Mamede CCN. BaltPLA2: A New Phospholipase A2 from Bothrops Alternatus Snake Venom with Antiplatelet Aggregation Activity. Protein Pept Lett. 2018; 25(10):943-952.
16. Kang GH, Lee S, Choi DB, Shin D, Kim J, Yang H, Bae H. Bee Venom Phospholipase A2 Ameliorates Atherosclerosis by Modulating Regulatory T Cells. Toxins (Basel). 2020; 12(10):609. doi: 10.3390/toxins12100609.
17. Nunes E, Frihling B, Barros E, de Oliveira C, Verbisck N, Flores T, de Freitas Júnior A, Franco O, de Macedo M, Migliolo L, Luna K. Antibiofilm Activity of Acidic

Phospholipase Isoform Isolated from Bothrops erythromelas Snake Venom. Toxins (Basel). 2020; 12(9):606. doi: 10.3390/toxins12090606.
18. Corrêa EA, Kayano AM, Diniz-Sousa R, Setúbal SS, Zanchi FB, Zuliani JP, Matos NB, Almeida JR, Resende LM, Marangoni S, da Silva SL, Soares AM, Calderon LA. Isolation, structural and functional characterization of a new Lys49 phospholipase A2 homologue from Bothrops neuwiedi urutu with bactericidal potential. Toxicon. 2016; 115:13-21. doi: 10.1016/j.toxicon.2016.02.021.
19. Staneva G, Angelova MI, Koumanov K. Phospholipase A2 promotes raft budding and fission from giant liposomes. Chem Phys Lipids. 2004; 129(1):53-62. doi: 10.1016/j.chemphyslip.2003.11.005.
20. Weisel JW, Litvinov RI. Fibrin Formation, Structure and Properties. Subcell Biochem 2017; 82:405-456.
21. Mihalyi E. Kinetics and molecular mechanism of the proteolytic fragmentation of fibrinogen. Ann N Y Acad Sci. 1983; 408:60-70.
22. Kumar SS, Sabu A. Fibrinolytic Enzymes for Thrombolytic Therapy. Adv Exp Med Biol. 2019; 1148:345-381.
23. He J, Chen S, Gu J. Identification and characterization of Harobin, a novel fibrino(geno)lytic serine protease from a sea snake (Lapemis hardwickii). FEBS Lett. 2007; 581(16):2965-73.
24. Wagstaff AJ, Gillis JC, Goa KL. Alteplase. A reappraisal of its pharmacology and therapeutic use in vascular disorders other than acute myocardial infarction. Drugs 1995; 50(2):289-316.
25. Gardiner EE, Andrews RK. The cut of the clot(h): snake venom fibrinogenases as therapeutic agents. J Thromb Haemost. 2008; 6(8):1360-2. doi: 10.1111/j.1538-7836.2008.03057.x.
26. Chernyshenko V. O, Lugovska N. E. Molecular mechanisms of intravascular inhibition and stimulation of extravascular thrombosis. Biotechnologia Acta. 2021, 14(6), pp. 5–22. https://doi.org/10.15407/biotech14.06.005
27. Altaf F, Wu S, Kasim V. Role of Fibrinolytic Enzymes in Anti-Thrombosis Therapy. Front Mol Biosci. 2021; 8:680397. doi: 10.3389/fmolb.2021.680397.
28. Pal SK, Gomes A, Dasgupta SC, Gomes A. Snake venom as therapeutic agents: from toxin to drug development. Indian J Exp Biol. 2002; 40(12):1353-8.
29. Cristina RT, Kocsis R, Tulcan C, Alexa E, Boldura OM, Hulea CI, Dumitrescu E, Radulov I, Muselin F. Protein structure of the venom in nine species of snake: from bio-compounds to possible healing agents. Braz J Med Biol Res. 2020; 53(1):e9001.

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