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PURIFICATION AND CHARACTERIZATION OF PLATELET AGGREGATION INHIBITOR FROM THE VENOM OF *BITIS ARIETANS*

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Disintegrins are the antagonists of integrin receptors that can be found mostly in snakes' venom. They can inhibit platelet aggregation, thus preventing the formation of blood clots. By blocking the integrin receptors of cancer cells, disintegrins can inhibit proliferation and metastasis. Thus, the search for new sources of disintegrins and development of methods of their purification is an important task of modern biotechnology. This work was dedicated to the purification and characterization of inhibiting polypeptides from Bitis arietans venom. Crude venom of B. arietans was fractionated using ion-exchange chromatography on Q Sepharose followed by size-exclusion chromatography on Superdex 75 using FPLC method. Analysis of molecular weight of protein components was performed using SDS-PAGE and MALDI-TOF analysis on Voyager-DE. Aggregation of platelet-rich plasma (PRP) in the presence of platelet aggregation inhibitor was investigated using aggregometry on the AR2110. MTT test was used for measuring HeLa cells proliferation and survival in vitro. Two-step chromatography allowed us to obtain fraction that contained polypeptides possessing the dose-dependent inhibitory action on adenosine diphosphate (ADP)-induced platelet aggregation in PRP. SDS-PAGE showed that obtained fraction contained two polypeptides with molecular weight 9.0 and 13.67 kDa according to MALDI-TOF analysis. Purified polypeptides inhibited ADP-induced platelet aggregation with IC_{50} 0.09 mg/ml. However, 0.005 mg/ml of fraction suppressed viability of HeLa cells according to MTT test on 20%. Discovered biological effects of fractions allowed us to conclude the possible use of these polypeptides as anti-aggregatory or anti-proliferative agents.

K e y w o r d s: snake venom, antithrombotic action, disintegrins, platelets, glycoprotein IIb/IIIa.

Snake venoms are a known source of biologically active proteins that can affect numerous physiological processes and can be a platform for the development of therapeutic agents [1]. As far as the main target of snake venom is the bloodstream of mammals, scientists are searching for snake venom compounds that can affect the hemostatic system [2]. Wide numbers of articles are focused on the purification of inhibitors of platelet aggregation from snake venom [3]. Such polypeptides were mostly found in the venom of *Viperidae* and *Crotalidae* families of snakes and later became a molecular platform for the development of anti-platelet drugs [4]. Mostly these polypeptides are disintegrins targeted to the glycoprotein IIb/IIIa (GPIIb/IIIa) receptor of platelets that are able to prevent interaction with fibrinogen and thus suppress platelet aggregation [5]. Also, the ability of snake venom disintegrins to block integrin functions allows the presumption of possible anti-cancer therapeutic potential, which

makes the exploration of such molecules even more interesting for biotechnology and clinical sciences [6, 7].

The venom of South African puff adder (*Bitis arietans* (*B. arietans*)) is less broadly investigated. Several studies reported the overall anticoagulant effect of B, arietans venom on human blood [8, 9]. Serine protease, able to cleave fibrinogen molecule, was also found in this venom [10]. In our study, we focused on the search of compounds of *B. arietans* venom with potential anti-aggregatory action. We had to find protein fractions possessing anti-aggregatory activity to identify their active compounds and test their ability to suppress the viability of cancer cells.

Materials and Methods

Chemicals. Q Sepharose, Superdex 75, crystal violet, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), RPMI medium, adenosine diphosphate (ADP), Amicon Ultra-0.5 ml centrifugal filters, SM1811 pre-stained protein markers (10-250 kDa) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Activated partial thromboplastin time (APTT)-reagent was purchased from Siemens (Munich, Germany). Gentamicin was from Arterium (Kyiv, Ukraine).

Platelet rich plasma and washed platelets. Platelet rich plasma (PRP) samples were obtained from the blood of healthy donors. This study was approved by the ethical committee of Palladin Institute of Biochemistry of NAS of Ukraine, 05.05.2021, N7. Volunteers signed informed consent prior to blood sampling according to the Declaration of Helsinki. PRP was prepared from human citrated blood by centrifugation at 1000 RPM for 20 min. Platelet poor plasma (PPP) was obtained from PRP by centrifugation for 15 minutes at 1900 RPM [11].

Chromatography. Q Sepharose column was pre-equilibrated with 0.05 M Tris-HCl buffer of pH 8.3. Crude venom of B. arietans was dissolved in the same buffer. The column volume was 3 ml, flow rate was 1 ml/min. Elution was performed by step gradient of NaCl. Fractions that contained anti-aggregatory activity were collected and prepared for size-exclusion chromatography by concentration and desalting using Amicon Ultra-0.5 ml centrifugal filters. The fraction that contained low-molecular weight and exhibited antiplatelet action was gel-filtered through a Superdex 75 column equilibrated with 0.05 M Tris-HCl buffer of pH 7.4 (TBS). The column volume was 30 ml, flow rate was 1 ml/min.

SDS-PAGE. The molecular weights and purity of obtained fractions were determined by SDS-PAGE in 10% gel according to Laemmli [12].

APTT. Activated partial thromboplastin time (APTT) was performed according to the following procedure. First, 0.09 ml of blood plasma was mixed with equal volume of APTT-reagent and 0.02 ml of studied solution or TBS and incubated for 3 min at 37°C. Then, the coagulation was initiated by adding of 0.1 ml of 0.025 M solution of $CaCl_2$. Clotting time was monitored by the Coagulometer CGL-2410 (Solar, Minsk, Belarus). We assumed that the fraction possessed fibrinogenolytic activity if it prolonged the time of clotting of blood plasma on 15% or more.

MALDI-TOF. MALDI-TOF analysis of purified platelet aggregation inhibitor was performed using a Voyager-DE (Applied Biosystems, USA). H^+-matrix ionization of polypeptides with sinapinic acid (Sigma-Aldrich, St. Louis) was used. Results were analyzed by Data Explorer 4.0.0.0 (Applied Biosystems) [13].

Aggregometry. Platelet aggregation measuring was based on the changes in the turbidity of platelet rich human plasma [14]. In typical experiment, 0.2 ml of platelet rich plasma was incubated with 0.05 ml of studied fraction or TBS and then activated by 0.025 M $CaCl_2$ and ADP in final concentration 12.5 μM at 37°C. Aggregation was detected for 10 minutes using Aggregometer AP2110 (Solar, Minsk, Belarus). In preliminary studies, we assumed that the fraction possessed anti-aggregatory action if it decreased the rate of platelet aggregation on 30% or more.

Cell culturing. The HeLa cells (cervical cancer cells) were grown in RPMI medium supplemented with 10% FBS and gentamicin (0.05 mg/ml). Cells were incubated and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced next day, cells were seeded in same concentration – 20000 cells/cm^2. Control group of cells were incubated 48 hours in RPMI medium containing 10% FBS and gentamicin (0.05 mg/ml). After reaching a confluence of 70% to 80%, HeLa cells were trypsinized (0.25% trypsin, Sigma-Aldrich, St. Louis, USA). Trypsin was stopped by FBS adding to dissociated cells. For the experimental group in complete medium platelet aggregation inhibitor from *B. arietans*, venom was added in concentrations of 0.0005, 0.0001 and 0.005 mg/ml. Cells were cultivated in the presence of polypeptides for 72 h.

MTT test. To perform MTT [3-(4,5-diethylthiazoly-2-yl)-2,5-diphenyltetrazolium bromide] cell viability assay, cells were plated at a density of 6000 HeLa cells per well in 96-well plates. On the next day, the cells were incubated with 100 µl complete medium containing 1 mg/ml MTT (thiazolyl blue tetrazolium bromide) at 37°C for 4 hours followed by solubilisation with DMSO. For this purpose, cell medium was aspirated and 100 µl DMSO was added for 15 min until complete dissolution. Eight replicates were conducted for control at each disintegrin concentration (0.0005, 0.0001, 0.005 mg/ml). The absorbance of the dissolved formazan crystals was measured at 540 nm with a microplate reader Multiscan EX (Thermo Fisher, Waltham, MA, USA). Cell proliferation was compared between control (non-treated cells) and treated cells [15].

Crystal violet staining. Crystal violet staining was carried out for attached cells visualization in the presence of disintegrin taken in high concentration (0.02 mg/ml). For this experiment, HeLa cells were cultivated in 24-well plates for 72 hours. After this, cells were washed from culture medium and incubated for 20 min with 0.5% crystal violet staining solution, which was replaced by 0.9% NaCl solution.

Protein concentration determination. Protein concentration in the sample of enzyme was assayed by Bradford method using bovine serum albumin as a standard protein and measuring the absorbance of the samples at 595 nm [16].

Statistics. Statistical data analysis was performed using Microsoft Excel. All assays were performed in series of three replicates and the data were fitted with standard errors using STATISTICA 7.0. Student *t*-test was used. Results are presented as means ± standard deviation. Data were considered significant when $P < 0.05$.

Results

Purification and characterization of platelet aggregation inhibitor from the venom of B. arietans. Crude venom of *B. arietans* was fractionated by ion-exchange chromatography on Q Sepharose. Fractions were eluted using step gradient of NaCl, collected separately, and analysed by SDS-PAGE and functional tests.

We obtained fraction that did not bind to the Q Sepharose at current conditions and main fractions eluted at 0.1, 0.2, 0.5 M NaCl (Fig. 1). SDS-PAGE analysis demonstrated the presence of low-molecular weight proteins (below 17 kDa) that could correspond to disintegrins [17] in all obtained fractions (Fig. 2).

Then, obtained fractions were desalted using Amicon Ultra-0.5 ml and their ability to inhibit

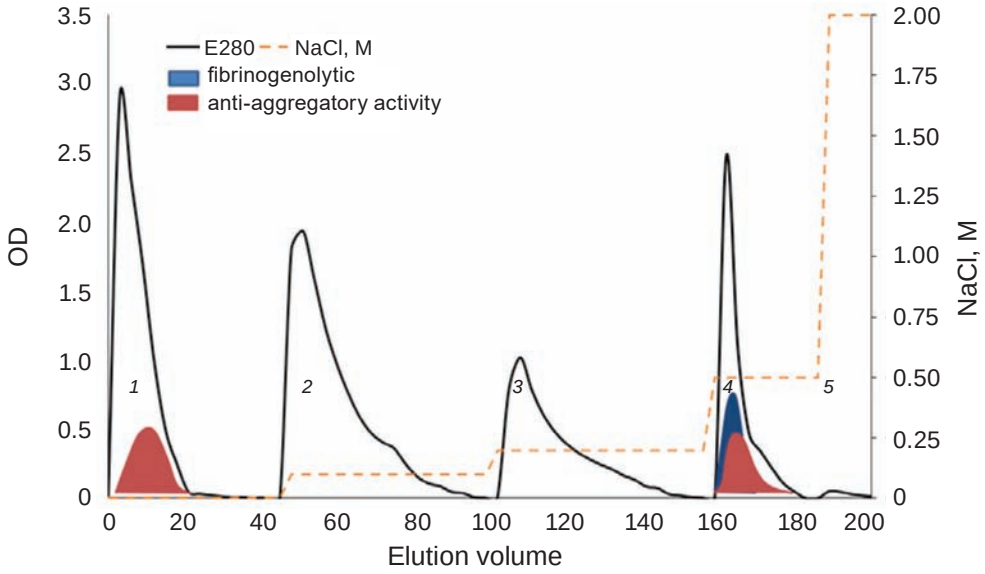

Fig. 1. Ion-exchange chromatography of crude venom of Bitis arietans on Q Sepharose using Akta Prime. 1 – non-binded fraction; 2 – fraction eluted at 0.1 M NaCl; 3 – fraction eluted at 0.2 M NaCl; 4 – fraction eluted at 0.5 M NaCl; 5 – fraction eluted at 2 M NaCl. Red band – fractions with anti-aggregatory activity; blue band – fibrinogenolytic activity. OD – optical density at 280 nm

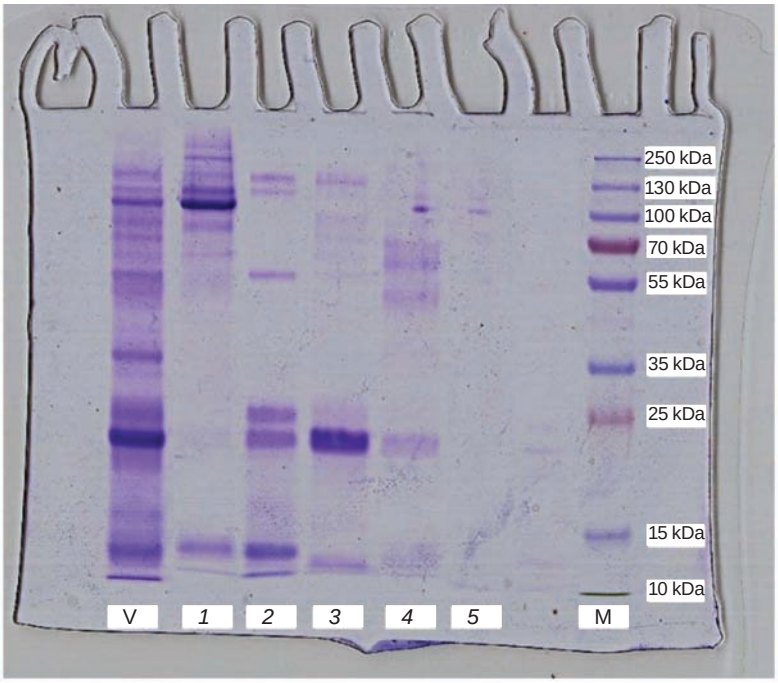

Fig. 2. SDS-PAGE of fractions obtained using ion-exchange chromatography of crude venom of Bitis arietans on Q Sepharose. V – crude venom; 1 – non-binded fraction; 2 – fraction eluted at 0.1 M NaCl; 3 – fraction eluted at 0.2 M NaCl; 4 – fraction eluted at 0.5 M NaCl; 5 – fraction eluted at 2 M NaCl; M – markers of molecular weight

platelet aggregation or blood plasma clotting were estimated as it was described above. Fraction 4 eluted by 0.5 M NaCl possessed anti-aggregatory and anti-clotting activity that can be a consequence of action of protease specific to fibrinogen that was found in studied venom previously [5]. Such proteases cleave fibrinogen, which also inhibits the platelet aggregation that is strongly dependent on fibrinogen [18]. However, non-binded fraction possessed anti-aggregatory action exclusively, which is why it was selected for further purification on Superdex 75.

Size-exclusion chromatography allowed obtaining fraction that inhibited platelet aggregation and purifying it from high-molecular weight and low-molecular weight admixtures (Fig. 3). SDS-PAGE demonstrated the presence of two main protein bands in the obtained fraction (Fig. 4). For accurate determination of molecular weight of these proteins, we used MALDI-TOF-MS analysis. Mass-spectrometry showed accurate molecular weights of 13.7 and 9 kDa (Fig. 5). 6.8 and 4.5 kDa peaks seen on the spectra corresponded to the half-charged molecules.

Thus, as a result of two-step chromatography, we obtained fraction that possessed strong anti-aggregatory activity and contained two proteins and was used for further studies.

Anti-aggregatory action of polypeptides from the venom of B. arietans. To study the peculiarities of anti-aggregatory action of platelet aggregation inhibitor from the venom of *B. arietans,* we used ADP-induced platelet aggregation in PRP. Studied compound was analyzed in the range of concentrations from 0.025 to 0.4 mg/ml. The change of the rate and speed of platelet aggregation was estimated (Fig. 6, Fig. 7). We demonstrated that anti-aggregatory action of platelet aggregation inhibitor from the venom of *B. arietans* was strongly dose-dependent (Fig. 6, *A*). Calculations of dependence of the rate of platelet aggregation from the concentration of inhibitor allowed estimating IC_{50} of its inhibitory action (Fig. 6, *B*). We showed that IC_{50} = 0.09 mg/ml.

Preincubation of PRP with inhibitor from the venom of *B. arietans* caused the propagation of its inhibitory action (Fig. 7). The stabilization of the rate of inhibition after 10 minutes of preincubation allowed us to conclude that platelet aggregation inhibitor from the venom of *B. arietans* is the reverse inhibitor [12] (Fig. 7).

Anti-proliferative action of polypeptides from the venom of B. arietans. For the determination of

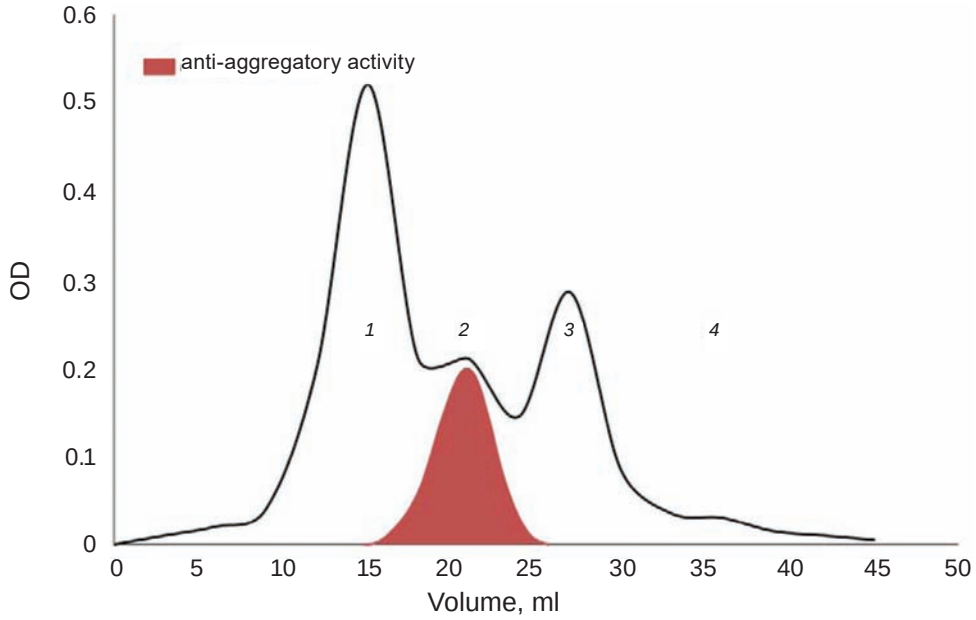

Fig. 3. Size-exclusion chromatography using Superdex 75 of non-binded fraction obtained from crude venom of Bitis arietans on Q Sepharose. 1 – high-molecular weight compounds; 2 – fraction of anti-aggregatory agent; 3 – low-molecular weight compounds and admixtures; 4 – admixtures. OD – optical density at 280 nm

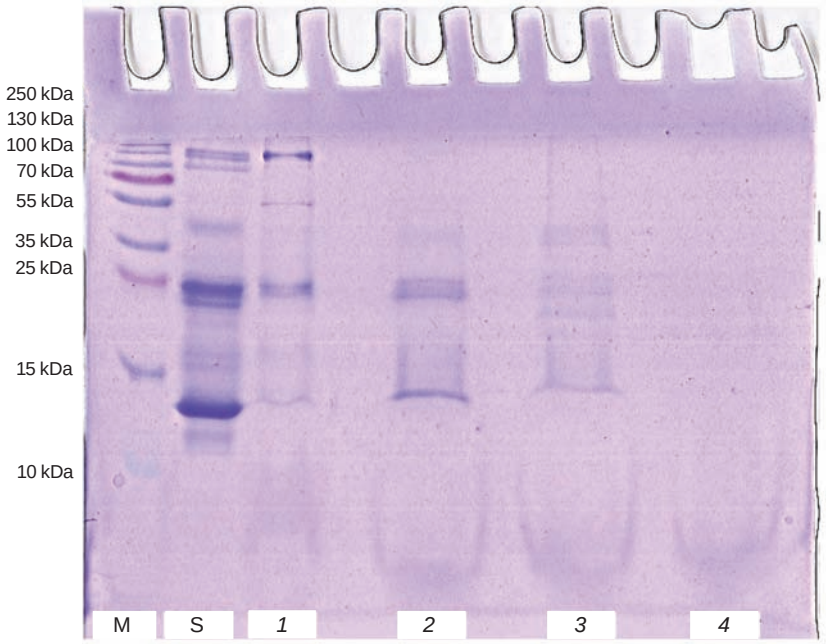

Fig. 4. SDS-PAGE of fractions obtained using Superdex 75. S – non-binded fraction obtained from crude venom of Bitis arietans on Q Sepharose; M – markers of molecular weight; S – sample that was fractionated; 1 – high-molecular weight compounds; 2 – fraction of anti-aggregatory agent; 3 – low-molecular weight compounds and admixtures; 4 – admixtures

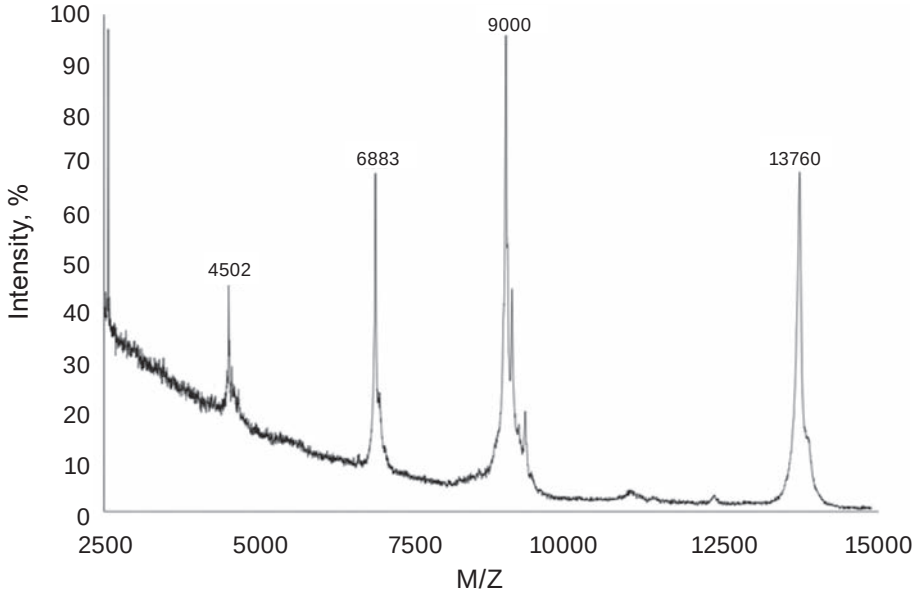

Fig. 5. MALDI-TOF mass-spectrometry analysis of platelet aggregation inhibitor from the venom of Bitis arietans. M/Z – ratio of mass and charge

the effects of platelet aggregation inhibitor from the venom of *B. arietans* on proliferation of cell line HeLa, we performed an MTT test that is commonly used for testing the effect of snake venom disintegrins on cells viability [19, 20]. Polypeptides were added to wells simultaneously with cells. Cells were grown for 72 hours, which is a standard time for analysis of disintegrins effects on cells viability [21, 22]. It was shown that all experimental concentrations of protein inhibited proliferation activity of HeLa cells and the effect was dose-depended. The highest inhibition was approximately 20% and observed at protein concentration 0.005 mg/ml (Fig. 8). Inhibitor from the venom of *B. arietans* taken in concentrations 0.005 and 0.001 mg/ml significantly inhibited viability of HeLa cells.

The crystal violet staining was applied for visualization of the effect of platelet aggregation inhibitor from the venom of *B. arietans* on HeLa cells. We compared the morphology of cell culture in the presence or absence of studied solution. In the presence of 0.02 mg/ml of platelet aggregation inhibitor from the venom of *B. arietans*, we observed the decreased number of cells that did not form the monolayer. The difference with untreated cells was obvious (Fig. 9).

Discussion

A combination of ion-exchange and size-exclusion chromatography allowed us to obtain the fraction able to inhibit platelet aggregation. MALDI-TOF mass-spectrometry detected the distinct molecular weights of two protein compounds of fraction: 13.7 and 9 kDa. Taking into account the similarity of charge (according to the behavior during ion-exchange chromatography) and molecular weights, we suggest that these two proteins are likely isoforms of one protein.

Molecular weight and the action on ADP-induced platelet aggregation of studied compounds allowed us to conclude that studied polypeptides were typical disintegrins that were found in different snake venoms. In particular, different snake venoms were a source of such disintegrins as: halysin from *Agkistrodon halys* venom (7.5 kDa) [23], multisquamatin from *Echis multisquamatis* venom (5.7 kDa) [24], PAIEM from the same venom (14.7 kDa) [25] or contortrostatin from the venom of *Agkistrodon contortrix* (13.5 kDa) [26].

Action of studied compounds on ADP-induced platelet aggregation was rather moderate. Taking into account the average molecular weight of studied compounds, we can conclude that platelet aggregation inhibitor from the venom of *B. arietans* was much less effective in comparison to saxatilin from the venom of *Gloydius saxatilis* (IC_{50} = 0.13 μM) [27] or leucurogin from the venom of *Bothrops leucurus* (IC_{50} = 0.65 μM) [28]. Specificity of inhibitory action of the studied inhibitor from the venom of *B. arietans* was close to TFV-3 from *Trimeresu-*

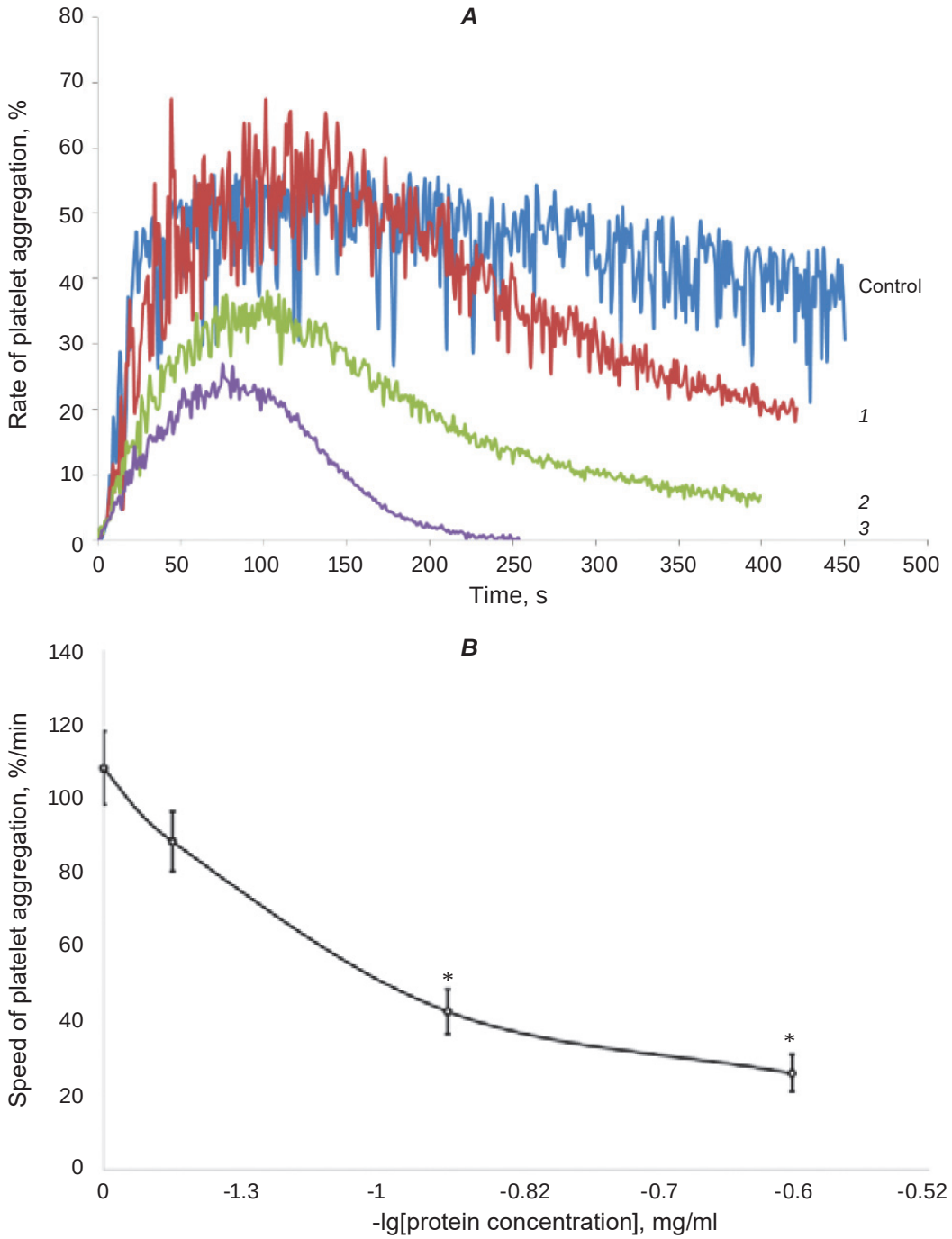

Fig. 6. Platelet aggregation induced in PRP by 12 μM ADP in the presence of inhibitor from the venom of Bitis arietans. ***A****. Platelet aggregation curves obtained in the presence of different amounts of inhibitor from the venom of Bitis arietans. C – control; 1 – 0.025 mg/ml; 2 – 0.125 mg/ml; 3 – 0.250 mg/ml. Data are typical for three independent experiments.* ***B****. Dependence of the initial speed of platelet aggregation from the concentration of inhibitor from the venom of Bitis arietans. *Data are statistically significant for P ≤ 0.05 in comparison to point zero, n = 3*

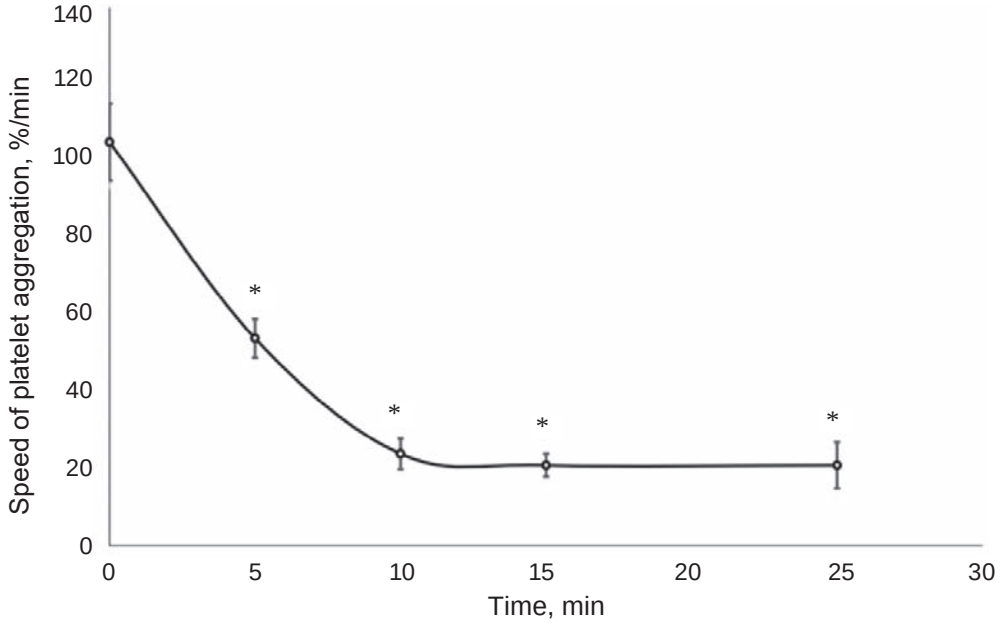

*Fig. 7. Dependence of the initial speed of platelet aggregation induced in PRP by 12 μM ADP from the time of preincubation (5, 10, 15, 25 min) with 0.025 mg/ml of inhibitor from the venom of Bitis arietans. *Data are statistically significant for P ≤ 0.05 in comparison to point zero, n = 3*

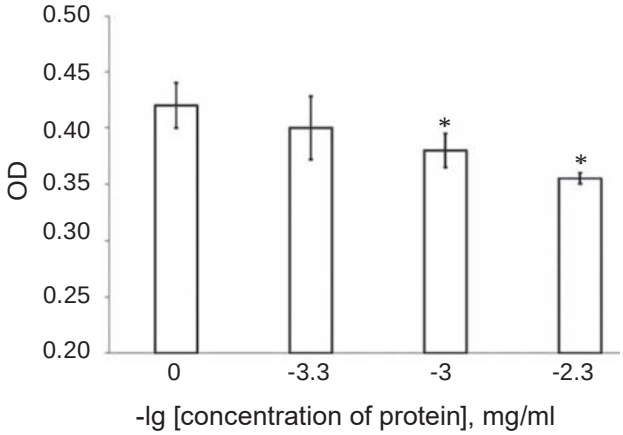

*Fig. 8. Proliferation activity of HeLa cancer cells in the presence of platelet aggregation inhibitor from the venom of Bitis arietans. *Data are statistically significant for P ≤ 0.05 in comparison to point zero, n = 8. OD – optical density at 540 nm*

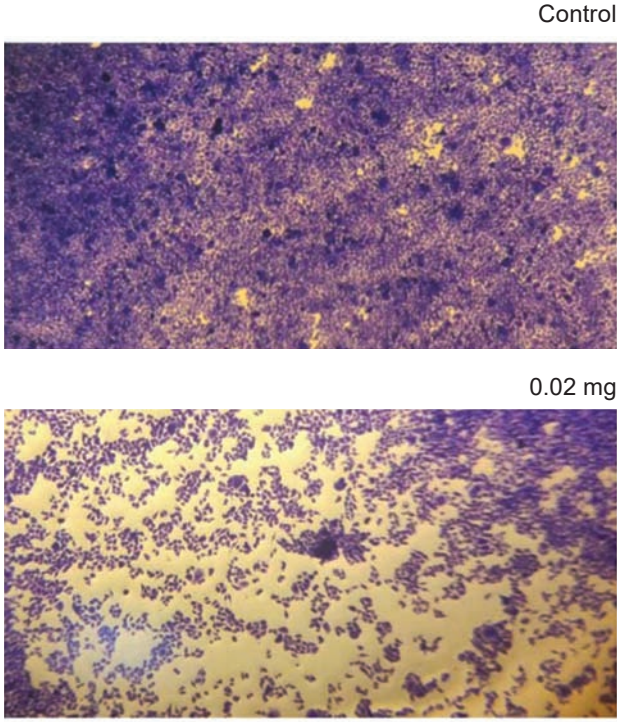

Fig. 9. Crystal violet staining of HeLa cells after 72 h of cultivation with platelet aggregation inhibitor from the venom of Bitis arietans. Control – in the presence of equivalent volume of sterile filtered buffer; 0.02 – in the presence of sterile filtered solution of inhibitor from the venom of Bitis arietans (0.02 mg/ml)

rus flavoviridis venom (IC_{50} = 0.133 mg/ml) [29]. However, direct action of the obtained polypeptide on the viability of HeLa cells makes it a promising object for further studies.

In conclusion, polypeptides possessing anti-aggregatory and anti-proliferative effects were purified from the venom of *B. arietans* by two-step chromatographic protocol. Inhibition of platelet aggregation as well as the suppression of the viability of cancer

cells by newly discovered polypeptides allowed us to conclude that they belong to the disintegrin family and are able to block integrin GPIIb/IIIa-receptor of platelets or other integrin receptors of cancer cells.

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Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ОЧИСТКА ТА ХАРАКТЕРИСТИКА ІНГІБІТОРА АГРЕГАЦІЇ ТРОМБОЦИТІВ З ОТРУТИ *BITIS ARIETANS*

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Дезінтегрини – антагоністи інтегринових рецепторів, які отримують з отрути змій. Вони інгібують агрегацію тромбоцитів, тим самим перешкоджаючи формуванню кров'яного згустку. Відомо, що дезінтегрини, блокуючи інтегрин-опосередковані взаємодії пухлинних клітин, можуть пригнічувати їхню проліферацію та метастазування. Отже, пошук нових джерел дезінтегринів та розробка методів їх очистки є важливим завданням сучасної біотехнології. Цю роботу присвячено очистці та характеристиці дезінтегринів отрути *Bitis arietans.* Цільну отруту *B. arietans* фракціонували методом іонообмінної хроматографії на Q Sepharose з наступною очисткою гель-фільтрацією на Superdex 75, використовуючи FPLC. Аналіз молекулярної маси протеїнових компонентів виконаний методами гель-електрофорезу та MALDI-TOF аналізу на Voyager-DE (Applied Biosystems, США). Агрегацію збагаченої тромбоцитами плазми крові (ЗТПК) за присутності інгібітора агрегації тромбоцитів проводили методом агрегатометрії на AR2110. МТТ-тест було виконано для оцінки проліферативної активності та життєздатності клітин лінії HeLa *in vitro.* Двоетапна хроматографія дозволила нам отримати фракції, які містять поліпептид із дозозалежною інгібіторною дією на ADP-індуковану агрегацію тромбоцитів у ЗТПК. Гель-електрофорез показав, що отримана фракція містить 2 поліпептиди з молекулярною масою 9,0 та 13,67 кДа відповідно до результатів MALDI-TOF аналізу. Очищені поліпептиди інгібували ADP-індуковану агрегацію тромбоцитів (IC_{50} = 0,09 мг/мл). Відповідно до результатів МТТ-тесту, фракція дезінтегринів (0,005 мг/мл) пригнічувала життєздатність пухлинних клітин лінії HeLa на 20%. Зроблено висновок про можливість використання отриманих поліпептидів як антитромботичних та антипроліферативних агентів.

Ключові слова: отрута змій, антитромботичний ефект, дезінтегрини, тромбоцити, глікопротеїн IIb/IIIa.

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