

In vitro haemostatic effect of amphibian crude skin secretions in rabbit blood plasma

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Abstract

In this research we have studied whether the amphibian crude skin secretions can modify some parameters of haemostatic system. We prepared the samples of crude skin secretions of *Bombina bombina*, *Bombina variegata*, *Bufo bufo*, and *Bufo viridis* and investigated the plasma clotting function *in vitro* by performing activated partial thromboplastin time (aPTT), prothrombin time and thrombin time (TT) assays. The data indicate that the components of *B. bombina* and *B. variegata* skin secretions significantly prolonged aPTT clotting time, while the components of *B. viridis* skin secretions prolonged the time of plug formation in TT. The proteolytic activity and the ability of the skin secretions components to activate plasma proenzymes were

assayed *in vitro* using synthetic chromogenic substrates. The components of *B. variegata* skin secretions cleaved all studied chromogenic substrates, whereas the *B. viridis* secretions intensively cleaved thrombin specific substrate and protein C specific substrate. The components of *B. bombina* and *B. bufo* skin secretions activated prothrombin and protein C in plasma. The components of *B. variegata*, *B. bufo* and *B. viridis* skin secretions in a dose-dependent manner induced platelet aggregation.

Introduction

Over the past several decades, a great deal of emphasis has been placed on the search for the new natural sources of biologically active compounds for the development of novel pharmacologically effective agents.^{1,2} Considering the wide distribution of the amphibians throughout the mainland of Europe and the variety of the already recognized activities of the biologically active compounds derived from their dermal venoms, amphibian skin secretions seems to be a perfect raw material to search for the novel promising bioactive compounds and/or new effects of the already known constituents.^{3,4}

Since ancient times, amphibian venoms have always considered to be highly efficient in fighting different diseases.⁵ Extensive studies have been conducted to study the therapeutic potential of the components of skin secretions of various types of amphibians.⁶⁻⁸ The experimental data show that the amphibian glandular secretions are a rich source of various molecules with cardiotoxic,⁹ antidiabetic,¹⁰ immunomodulatory,¹¹ antimicrobial,^{12,13} and antiviral activities.^{14,15} A great number of researches also state that they have sedative and analgesic effects.^{16,17}

It is generally agreed that the haemostasis system disorders are a major cause of morbidity and mortality.¹⁸ Despite the wide arsenal of medicines, the prevalence rate is constantly increased. In recent years, the search for new pharmacological approaches to the prevention and treatment of these conditions is underway, although, the ideal drug has not yet been found. The bioactive components derived from the reptile venoms are actively used in the treatment of various haemostasis disorders.¹⁹ Furthermore, some similar studies regarding the effects of a poisonous species of amphibians were conducted in Asian countries.²⁰ However, little research has been undertaken to study the effects of the crude skin secretions constituents of the amphibian species that dwell Eastern Europe on some parameters of the haemostasis system.

In the present study, the amphibian crude skin secretions were

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Key words: Amphibian skin secretions; Plasma coagulation tests; Proteolytic activity; Plasma proenzymes; Platelet aggregation.

Contributions: the authors contributed equally.

Conflict of interest: the authors declare no potential conflict of interest.

Funding: this research was supported by the Ministry of Education and Science, Ukraine [n. 0116U002527, 2016-2018].

Received for publication: 7 August 2019.
Revision received: 24 September 2019.
Accepted for publication: 2 October 2019.

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Licensee PAGEPress, Italy
Journal of Biological Research 2019; 92:8475
doi:10.4081/jbr.2019.8475

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investigated for its haemostatic effect by determining the plasma clotting time and platelet aggregation. Also the proteolytic activity and the ability of the skin secretions components to activate plasma proenzymes were assayed. The components of all studied amphibian secretions had effects on different parameters. Currently the fractionation and the purification of an active component from the fractions and further molecular characterization of specific target proteins are in course.

Materials and Methods

Collection of amphibian skin secretions

All animal procedures followed the European Directive 2010/63/EU (EC, 2010) on protecting animals used for experimental and other scientific purposes. All manipulations were approved by the Ethical Committee of Educational and Scientific Centre - Institute of Biology and Medicine, Taras Shevchenko National University of Kyiv, Ukraine.

Adult specimens (both sexes) of *Bombina bombina* ($n=20$), *Bombina variegata* ($n=20$), *Bufo bufo* ($n=15$), and *Bufo viridis* ($n=10$) were collected outdoors on the territory of Ukraine and were authenticated by the Department of Zoology and Ecology of Taras Shevchenko National University of Kyiv, Ukraine. Amphibians were kept in fauna boxes of different volumes and were fed 2-3 times a week with a frozen food on the basis of representatives of *Orthoptera* and *Blattoptera*, and larvae of *Diptera*. Water in the fauna boxes was changed once a week.

Skin secretions were collected as follows: frogs were put into a petri dish. Being mechanically stimulated with fingers for 1-2 min, frog skin surface was seen to exude copious secretions. Skin secretions were collected by washing the dorsal region of each frog with ultra-pure water. Water suspensions of skin secretions were centrifuged at 2,500 g for 15 min to remove debris. The supernatants were lyophilized (Telstar LyoQuest) and kept at 4 °C till use.

Samples preparation

The samples of lyophilized skin secretions (30 mg of dried material) were resuspended in 1 mL 0.05 M Tris-HCl buffer, pH 7.4, which contained 0.13 M NaCl and centrifuged at 7,000 g for 15 min. Protein concentration in the supernatant was assayed by Bradford (1976) method,²¹ using bovine serum albumin as a standard.

Preparation of platelet-rich plasma and platelet-pure plasma

Healthy adult rabbits were supplied by the vivarium of Taras Shevchenko National University of Kyiv, Ukraine. All animal experiments were approved by the Animal Care Committee of Taras Shevchenko National University of Kyiv, Ukraine. Platelet-rich plasma (PRP) and platelet-pure plasma were obtained following the standard protocol.²² All procedures were carried out at room temperature. Rabbit blood was collected from the ear artery into a polyethylene tube with 3.8% sodium citrate in the ratio 9:1. PRP was obtained by centrifugation of stabilized blood at 300 g for 10 min. The supernatant (PRP) was carefully separated and used further in the aggregation assay. Platelet-pure plasma was prepared by further centrifugation of the remained stabilized blood at 1,500 g for 30 min.

Plasma coagulation tests

Activated partial thromboplastin time (aPTT), thrombin time (TT) and prothrombin time (PT) were measured *in vitro* to assess the effects of the components of amphibian skin secretions on plasma clotting function. The tests were conducted using the coagulation analyser (Rayto RT-2201C, China) and the standard set of reagents (RENAM, Russian Federation). Briefly, to check the clot forming in aPTT test, 45 μ L of plasma was mixed with 5 μ L of the skin secretions sample and 50 μ L of aPTT reagent in the coagulometric cuvette and incubated for 3 min at 37°C. Clotting time in seconds (s) was recorded after the addition of 50 μ L of 0.025 M CaCl₂. For TT assay 45 μ L of plasma and 5 μ L of the skin secretions sample was mixed with 100 μ L of thrombin (final activity 3 U/mL) in the coagulometric cuvette and time necessary for clotting plug formation was recorded. The time in PT test was assessed by mixing 45 μ L of plasma and 5 μ L of skin secretions sample with 100 μ L of thromboplastin-Calcium mixture. All coagulation assays were performed in triplicate using plasma from three different rabbits. Plasma incubated with other components and equal amounts of ultra-pure water, instead of skin secretions samples, was used as controls.

Chromogenic assays

The effects of crude skin secretions of studied amphibian species on key haemostasis enzymes were assayed using chromogenic substrates with detectable leaving groups: thrombin specific substrate Phe-Pip-Arg-pNA (S 2238), plasmin specific substrate Val-Leu-Lys-pNA (S 2251), factor Xa specific substrate Ile-Glu-Gly-Arg-pNA (S 2222) and activated protein C specific substrate pyroGlu-Pro-Arg-pNA (S 2366) (RENAM, Russian Federation).

To determine the proteolytic activity of the components of crude skin secretions of *B. bombina*, *B. variegata*, *B. bufo* and *B. viridis*, *in vitro* experiments were performed. The incubation medium with a final volume of 250 μ L contains: 0.05 M Tris-HCl buffer, pH 7.4 and a sample of skin secretions containing 20 μ g of the total protein. After 5 min of incubation at 37°C in a 96-well plate, the reaction was initiated by adding corresponding specific chromogenic substrate (in a final concentration of 0.3 mM). The control sample contained the same components but with an equal volume of 0.05 M Tris-HCl, pH 7.4 instead of the sample of amphibian skin secretions. Incubation was performed at 37°C for 2 hours. The intensity of the proteolytic activity was proportional to the colour that revealed in the result of the gradual release of *p*-nitroaniline from the chromogenic substrate. The amount of *p*-nitroaniline hydrolyzed from the substrate was recorded at equal intervals of time (every 15 min for 120 min) at 405 nm using the kinetic microplate spectrophotometer (μ Quant, BioTek).

To study the ability of the components of crude skin secretions of *B. bombina*, *B. variegata*, *B. bufo* and *B. viridis* to activate plasma proenzymes *in vitro* experiments were carried out. Incubation medium (250 μ L) of the similar composition was used: 0.05 M Tris-HCl buffer, pH 7.4 and the samples of skin secretions (20 μ g of total protein) were mixed with 20 μ L of plasma for 5 min at 37°C and then the corresponding specific chromogenic substrate (in a final concentration of 0.3 mM) was added. Incubation was performed at 37°C for 2 hours. The intensity of *p*-nitroaniline release was measured at the equal intervals of time (every 15 min). The control sample contained the same components but with an equal volume of 0.05 M Tris-HCl, pH 7.4 instead of amphibian skin secretions. During the computation of the results, the optical density of plasma was subtracted from the optical density of plas-

ma that was incubated with the samples of skin secretion. Then the values of the optical density of the previous experiment were also subtracted to show the effect of crude skin secretions on plasma proenzymes.

Platelet aggregation assays

Platelet aggregation assay was undertaken within the first 3 h after blood sampling using photo-optical aggregometer AT-02 (Medtech, Russia). Before the assessment, the number of platelets in PRP was determined ($230\text{--}250 \times 10^3$ cells/ μL). The effects of the components

of the skin secretions on platelet functions were performed *in vitro*. Briefly, 380 μL PRP and 20 μL of the samples with 50 μg of total protein were incubated at 37°C in the aggregometer cuvette and the aggregation process was monitored for 5 min. The maximum degree of aggregation was recorded and compared with the degree of aggregation in response to one of the platelet physiological inducers – ADP (Sigma, USA) in the final concentration 5×10^{-6} M.

When the direct effect of the skin secretions samples on platelet aggregation was observed the concentration dependence study was carried out. Thus in the same experiment we have studied the effects

Table 1. The clotting time (sec) of the rabbit blood plasma in the coagulation tests after incubation with the amphibian skin secretions.

	aPTT	PT	TT
Control	18.6±2.6	7±0.1	28.2±4.3
<i>B. bombina</i>	62.7±14.3*	7.1±0.2	29.1±1.3
<i>B. variegata</i>	41.9±15.2*	6.9±0.1	29.1±0.7
<i>B. bufo</i>	20.8±3.1	6.8±0.5	30.9±1.6
<i>B. viridis</i>	21.0±0.8	7.2±0.2	39.1±1.6*

aPTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time. * $P \leq 0.05$, the difference is comparable to the control.

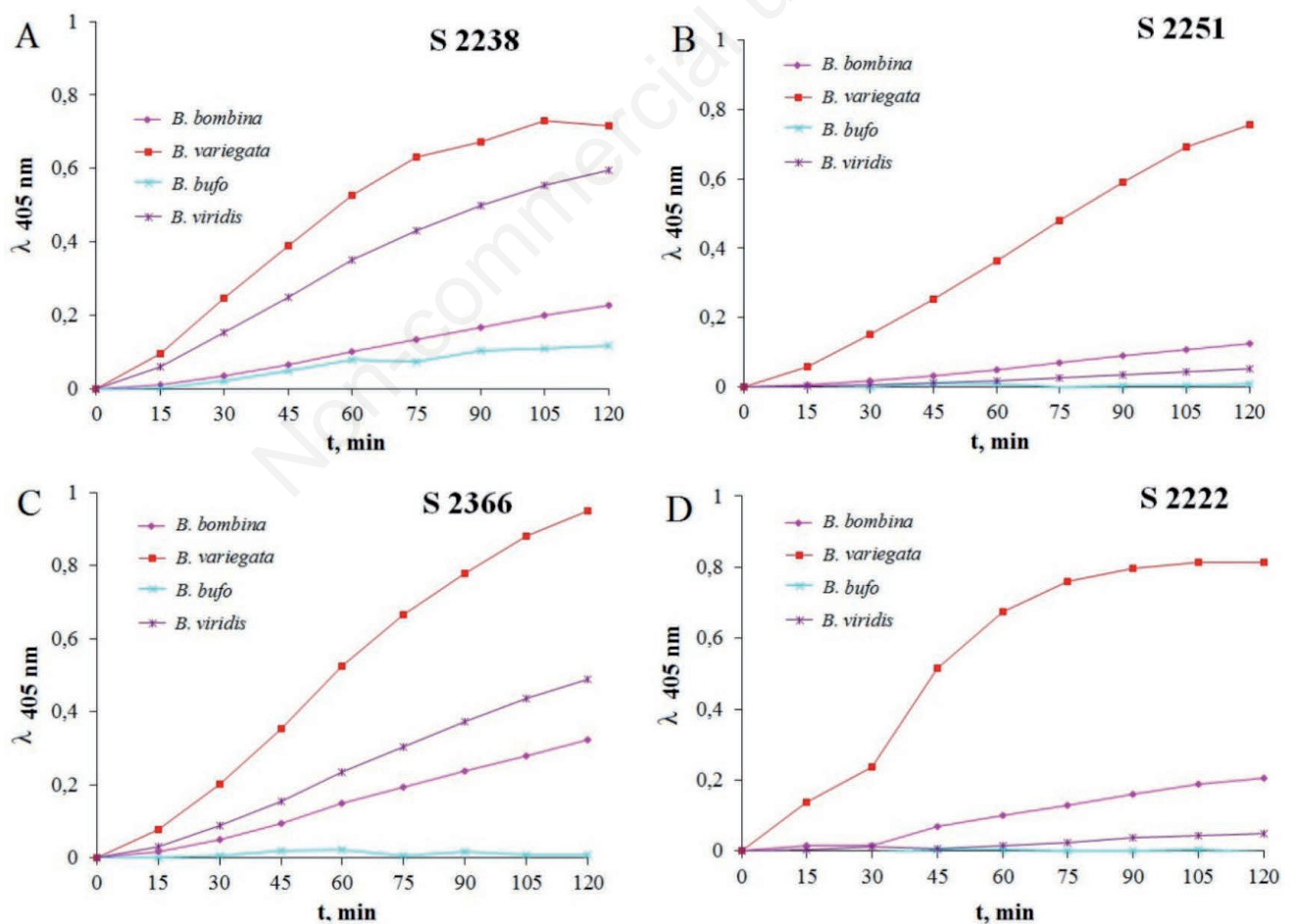


Figure 1. Proteolytic activity of the components of amphibian crude skin secretions to the thrombin specific substrate (A), plasmin specific substrate (B), protein C specific substrate (C) and factor Xa specific substrate (D).

of amphibian skin secretions in the final concentrations of 250 μg , 125 μg , 50 μg and 25 μg of total protein in 1 mL of PRP.

If the direct activation effect of the test sample on the process of platelet aggregation was not observed within the 3-min period, the ADP (5×10^{-6} M) was added and the process of aggregation was monitored. The degree of ADP-dependent aggregation, after 3 minutes of incubation of PRP with Tris-buffered saline, pH=7.4 (instead of studied samples), was used as a control for this experiment.

Calculation of the results

All experiments were performed in parallels and repeated at least three times each using the blood of different rabbits. The statistical analysis was performed using Stat Soft Statistica version 7.0 for Windows. The data were analysed for statistical significance of difference by Student's t-test. Differences were statistically significant when $P < 0.05$.

Results

Activated partial thromboplastin time, prothrombin time and thrombin time *in vitro* assays

The effects of amphibian crude skin secretions on the time of clotting plug formation, which corresponds the results of plasma

coagulation tests (aPTT, TT and PT), are demonstrated in Table 1. The prolongation of aPTT by 3.4 times and 2.25 times was observed during the incubation of plasma with *B. bombina* and *B. variegata* crude skin secretions, respectively. The skin secretions of *B. viridis* prolonged TT clotting time to 39.1 ± 1.6 seconds vs 28.2 ± 4.3 in control. The PT assay showed none changes in the time of clotting plug formation during incubation of plasma with crude skin secretions of all studied species.

Chromogenic assays

As shown in Figure 1A, the components of *B. variegata* and *B. viridis* skin secretions intensively cleaved thrombin specific substrate – S 2238. The *B. variegata* skin secretions also actively cleaved plasmin specific substrate – S 2251 (Figure 1B). Skin secretions of *B. bombina* and *B. viridis* showed mild proteolytic activity to activated protein C specific substrate – S 2366, whereas the *B. variegata* skin secretions significantly cleaved this chromogenic substrate (Figure 1C). The incubation of factor Xa specific substrate – S 2222 with skin secretions of *B. variegata* also resulted in the progressive increase of the optical density of the samples (Figure 1D).

Figure 2 demonstrates the results of the ability of the components of *B. bombina*, *B. variegata*, *B. bufo* and *B. viridis* skin secretions to activate plasma proenzymes. To conduct this experiment rabbits' blood plasma was additionally added to the incubation

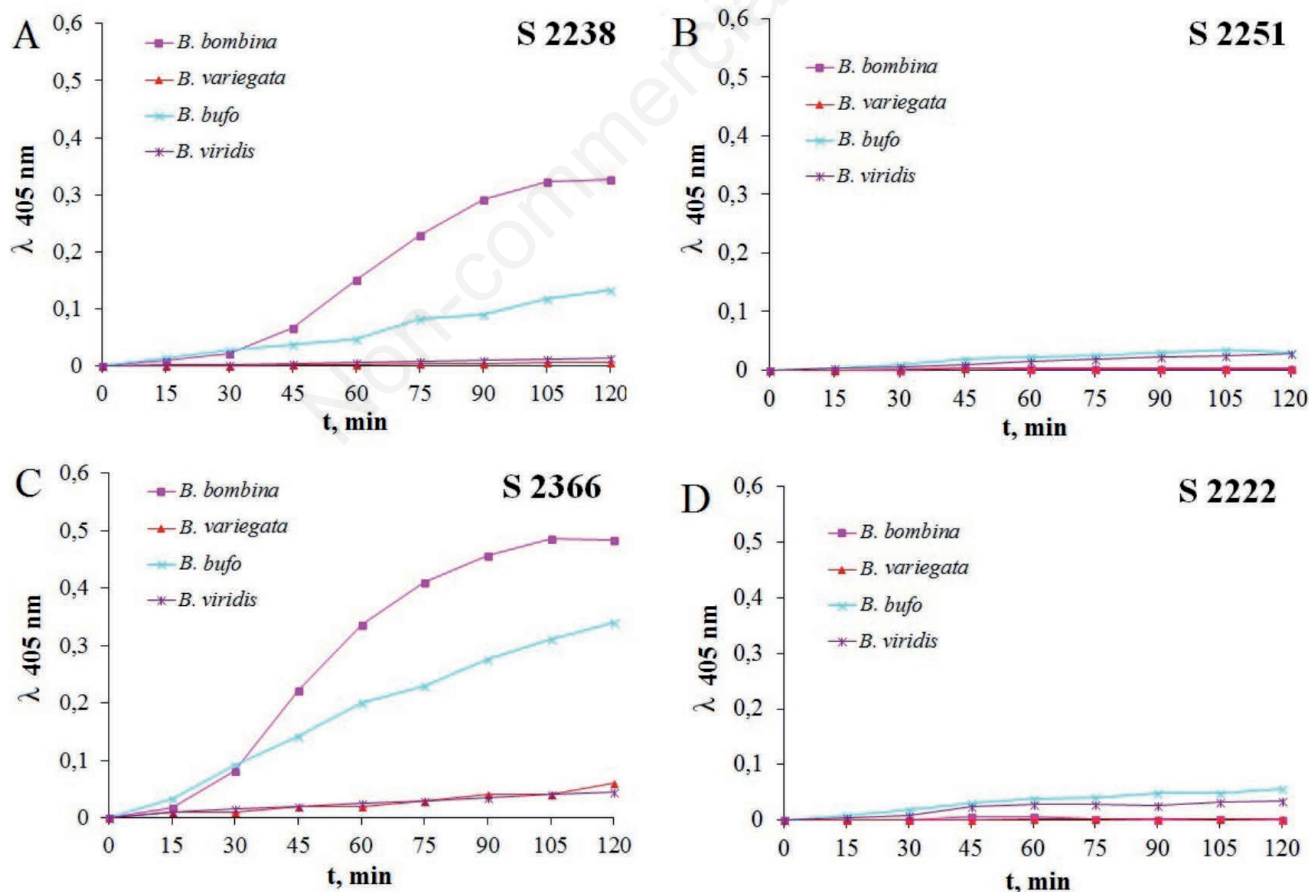


Figure 2. The ability of the components of the crude skin secretions of *B. bombina*, *B. variegata*, *B. bufo* and *B. viridis* to activate prothrombin, plasminogen, protein C and factor X.

medium with the appropriate substrate and samples of the amphibians' skin secretions. The optical density of plasma was subtracted from the values of the optical density of plasma, which was incubated with the samples of skin secretion. Then the results of the previous experiment were also subtracted. The intensive cleavage of the S 2238 and S 2366 substrates evidenced that the components of *B. bombina* and *B. bufo* crude skin secretions activate prothrombin and protein C in plasma (Figure 2A and B).

Platelet aggregation *in vitro* assay

The results of the potential effects of the crude skin secretions on the process of platelet aggregation are shown in Figure 3. The components of the *B. bombina* skin secretions had no effect on platelets and did not influence the ADP-dependent aggregation after 3 min of incubation with PRP (Figure 3A). The crude skin secretions of *B. variegata*, *B. bufo*, and *B. viridis* in a dose-depen-

dent manner induced platelet aggregation in the rabbit PRP (Figure 3B-D). The degree of platelet aggregation under the influence of the three studied amphibian skin secretions, in the final concentration of 250 µg of total protein in 1 mL of PRP, was identical to the values of the degree of aggregation in response to the action of 5×10^{-6} M ADP and was $60 \pm 3\%$.

Discussion

The results of our study showed that the crude skin secretions of some amphibian species significantly prolonged the time of clotting plug formation in the aPTT and TT. The prolongation of these tests may be due to the presence of inhibitors of certain factors of coagulation haemostasis. On the other hand, this may be

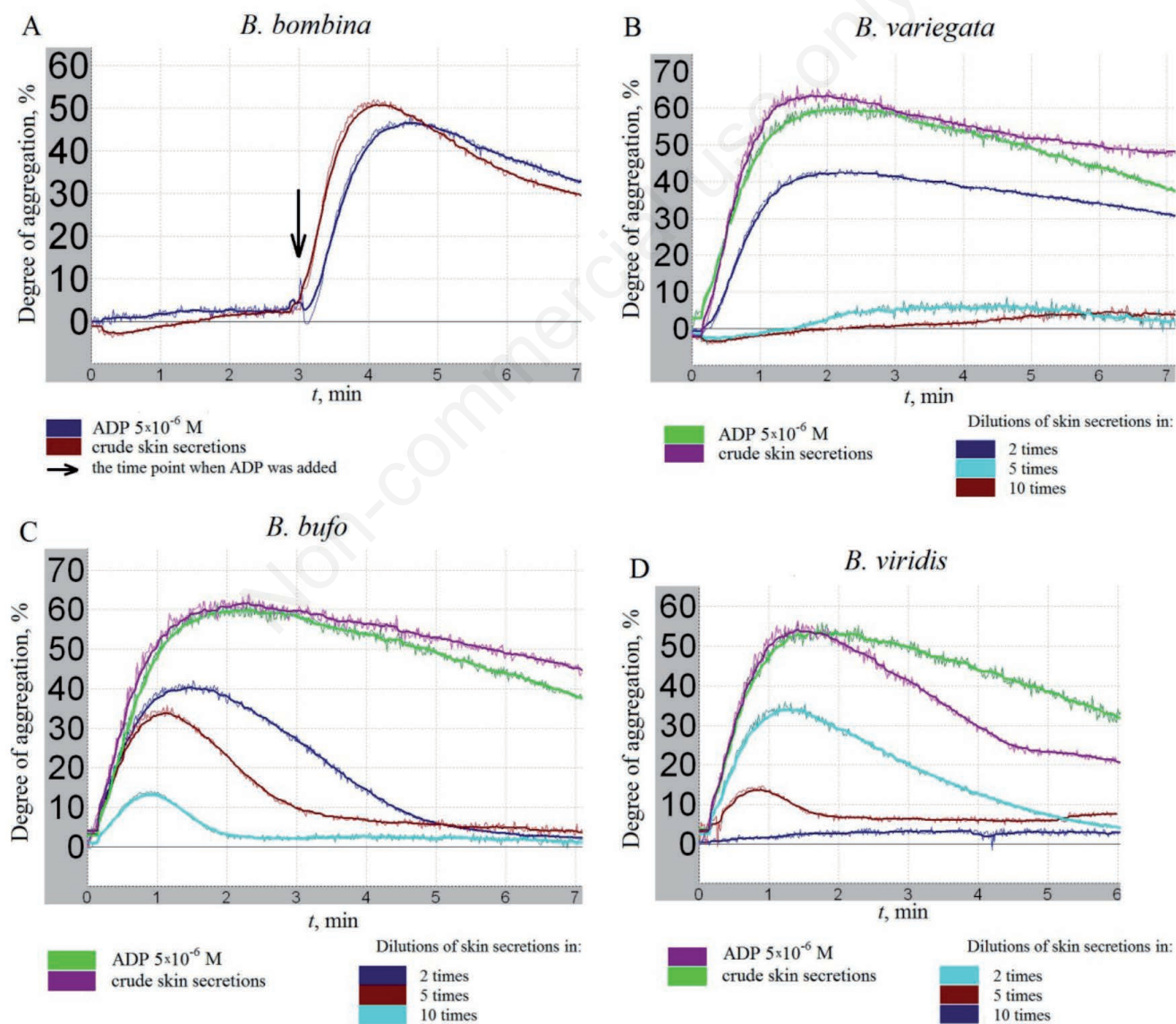


Figure 3. The degree of platelet aggregation in rabbit platelet-rich plasma under the influence of skin secretions of studied amphibian species: *B. bombina* (A), *B. variegata* (B), *B. bufo* (C), and *B. viridis* (D).

due to the degradation of the factors of coagulation haemostasis by active components of crude skin secretions. The last hypothesis corresponds to our previous results where we have stated that some crude skin secretions have a pronounced protease activity with specificity to different substrates.²³

To further investigate the proteolytic activity of the studied amphibian crude skin secretions chromogenic assays were conducted. We used four synthetic chromogenic substrates – thrombin specific substrate, plasmin specific substrate, factor Xa specific substrate and activated protein C specific substrate. Unfortunately, currently we cannot make a precise conclusion on the fact that the components of *B. variegata* skin secretions cleaved all studied chromogenic substrates as it might be an action of a single enzyme, which has a wide substrate specificity, or the presence of several enzymes, each acting with very narrow specificity on one of the substrates. The corresponding researches should be made. The results of the effects of the *B. viridis* skin secretions during the incubation with studied chromogenic substrates were different: only two substrates were cleaved, that could be the evidence of the presence of the thrombin-like or protein C-like enzymes. *B. bombina* and *B. bufo* skin secretions had no significant changes in the optical density during the incubation with all studied chromogenic substrates. This represents the absence of active enzymes in their skin secretions that could affect the studied chromogenic substrates.

The next our experiment was to study the ability of the components of crude skin secretions to activate plasma proenzymes. It was revealed that the *B. variegata* and *B. viridis* skin secretions did not affect the activation of prothrombin, plasminogen, protein C and factor X in plasma. Such a conclusion was made as the substrates remained uncleaved after the incubation with the skin secretions. The intensive cleavage of thrombin specific substrate and protein C specific substrate indicated that the components of *B. bombina* and *B. bufo* skin secretions activate prothrombin and protein C in plasma. There is some evidence to support our hypothesis stating that the appearance of thrombin and activated protein C may be due to the presence of an active forms of proteolytic enzymes in the skin secretions that directly activate the cleavage of the corresponding proenzymes or affect the cofactors that launch the cascade of the reactions and thus result in the formation of zymogens. We can assume two different ways of how the components of crude skin secretions can affect the thrombin molecules. The first can be carried out when the components of skin secretions directly affect the prothrombin molecules: initiate the cleavage of prothrombin and, therefore, stimulate the thrombin formation. The second is realized when the components can activate the factor X and affect the prothrombin activation.

In our *in vitro* study of platelet aggregation activity we demonstrated that the crude skin secretions of *B. variegata*, *B. bufo* and *B. viridis* in a dose-dependent manner induced platelet aggregation in the rabbit PRP. The components of these skin secretions could be the prominent source of inducers of platelet aggregation. Agents that can modulate platelets functioning are extremely relevant these days, as the available medications are characterized by a large number of side effects and unpredictable actions. Moreover, the search for inducers that can modulate platelets functions and their signalling pathways.

Conclusions

The results of our study demonstrate that the components of the studied amphibian crude skin secretions of *B. bombina*, *B. var-*

iegata, *B. bufo* and *B. viridis* are a potential source of various active molecules that affect different stages of haemostasis system. However, the mechanism of their action and the nature of these compounds require additional researches. The continuation of the study of the amphibian skin secretions composition in this context may lay the foundations of the creation of novel biologically active substances with a direct therapeutic effect.

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