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Identification of biologically active fractions in the dermal secretions of the genus *Bombina* (Amphibia: Anura: Bombinatoridae)

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Abstract. Amphibian skin secretions have long been considered a convenient and useful natural source of bioactive compounds, but a comprehensive study of the effects of their dermal secretions on diverse parameters of the hemostasis system has not yet been carried out. This study aimed at identifying biologically active fractions in the skin secretions of *B. bombina*, *B. variegata*, and their hybrid – *B.bombina* × *B.variegata,* and to clarify whether their components can modify certain parameters of the hemostasis system. For the skin secretion analysis, we performed ion-exchange chromatography, electrophoresis, and zymography assays. Plasma coagulation tests, chromogenic assays, and platelet aggregation assays were also conducted *in vitro*. As a result of the fractionation, a number of fractions were identified, where the proteins with miscellaneous molecular weights were revealed. The data also suggested that some fractions have proteolytic enzymes with gelatinolytic, fibrinogenolytic and collagenolytic activities. The proteins present in the fraction #5 of *B. variegata* and #5 of the hybrid secretions are characterized by the ability to prolong the clotting plug formation in the aPTT. Proteins capable of inducing platelet aggregation in the rabbit PRP are present in the fraction #3 of *B. variegata* secretions. The ability of dermal components to activate plasma proenzymes is indicative of the fact that the non-protein components of fraction #9 of *B. variegata* and fraction #7 of hybrid secretions initiated the appearance of thrombin and activated protein C in plasma.

Keywords. Amphibian skin secretions, hemostasis system, fractionation.

INTRODUCTION

Recently, a great deal of emphasis has been placed on the study of the effects of biologically active compounds derived from the sources of natural origin (Dias et al., 2012; Veeresham, 2012). These agents have found their applications in the treatment of pathological conditions, diagnosis of diseases, and have served as valuable tools in laboratory studies. Moreover, the heightened need of society for new potent medicines with strong efficiency and high safety profile, along with the increased price for drugs based on synthetic compounds, places importance on the use of natural raw material to search for various biologically active substances.

Amphibian skin secretions are enriched with complex cocktails of bioactive molecules spanning a wide spectrum of biological actions (Erspamer and Melchiorri, 1980; Daly, 1995; Conlon and Leprince, 2010). Although recently there has been a spate of interest concerning the potential therapeutic effects of the biologically active compounds derived from amphibians' dermal secretions (Mor et al., 1994; Bevins and Zasloff, 1990), most of these substances are not yet widely implemented in medical and pharmaceutical industry, as the mechanism of their action and possible side effects remain insufficiently studied (König et al., 2015).

The results of our previous study showed that the crude skin secretions of ten species of amphibians: *B.*

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bombina, *B. variegata*, *B. bufo*, *B. viridis*, *R. temporaria*, *P. ridibundus*, *P. esculentus*, *P. fuscus*, *S. Salamandra*, and the hybrid of *B. bombina* and *B. variegata*, had a pronounced protease activity with wide substrate specificity (Nikolaieva et al., 2018). In fact, it was proved that these dermal venoms can be a potential source of proteolytic enzymes. In the other research we demonstrated that the whole skin secretions affect the parameters of clotting plug formation (Udovychenko et al., 2018). It was shown that the *B. bombina*, *B. variegata* and their hybrid, *R. temporaria*, and *P. ridibundus* crude skin secretions prolonged the time of fibrin clot formation in the APTT. In contrast, the components of *B. viridis*, *P. esculentus*, *P. fuscus*, and *S. salamandra* prolonged the TT. Another research of ours showed the presence of biologically active compounds in the *P. fuscus* crude skin secretions that affect some parameters of the hemostasis system (Udovychenko et al., 2019). Our findings confirm that amphibian skin secretions are a complex material which contains a variety of biologically active substances that differ in their biological effects. So, to identify the possible mechanism of action or/and to define the components present in the whole skin secretions, fractionation of the source material is required.

Several research projects have been conducted concerning the study of the effects of skin secretion components of the toads that belong to the genus *Bombina*. A multitude of antimicrobial peptides from their dermal secretions have been discovered (Simmaco et al., 1998). For example, peptides called bombenins were isolated and found to possess antimicrobial activities, which have not been detected in other amphibian genera (Simmaco et al., 1991). In addition, bombenins are among the most studied amphibian constituents, and numerous studies have been published describing the various pharmacological activities of bombesin and its homologues (Gonzalez et al., 2008). The in vitro antitumor assay conducted by Zhou et al. (2018) showed that the bombinin-like peptide and bombinin H type peptide possessed obvious antiproliferative activity on three human hepatoma cells (Hep G2/SK-HEP-1/Huh7) at the nontoxic doses. This indicates that the peptide family of bombinins could be a potential source of drug candidates for anti-infection and anticancer therapy. A few studies also report on the antidiabetic activity of the compounds derived from the *Bombina* skin secretions: several insulin-releasing peptides have been isolated (Marenah et. al., 2004). The mechanism underlying their insulinotropic actions suggests possible involvement of a cAMP dependent G-protein insensitive pathway. Secretary glands of some representatives of genus *Bombina* also produce antimicrobial peptides called maximins. According to Lai et al. (2002), maximin 3 possesses a significant anti-HIV activity. Furthermore, maximins tend to have a potent antimicrobial activity, cytotoxicity against tumor cells and spermicidal action.

Although considerable amount of research has been conducted to study the composition of the skin secretions of the *Bombina* toads, and many biological effects of the venom constituents have been revealed, it still remains unclear whether the components of their dermal secretions affect the functioning of the hemostasis system. Moreover, considering the huge amount of research that has been conducted based on the study of the similar effects of the reptile venoms (Meier and Stocker, 1991; Markland, 1998; Manjunatha, 2006), such studies in the context of the toad skin secretions are a highly promising area for investigations. In view of these facts, the aim of the present study was to identify the biologically active fractions in the dermal secretions of the genus *Bombina* and to study their effects on some parameters of the hemostasis system. This work will provide a better understanding of the role of the proteins and enzymes present in the skin secretion of these species and might give a background for further potential medical and pharmaceutical applications of the components of amphibian skin secretions.

MATERIALS AND METHODS

Collection of amphibian skin secretions

Adult specimens of *Bombina bombina* (n = 20, females = 2), *Bombina variegata* (n = 15, females = 1), and their hybrid – *B.bombina* × *B.variegata* (n = 5, females = 0) were collected in Kyiv, Transcarpathian and Khmelnitsky regions of Ukraine, respectively. Amphibians were authenticated by the Department of Zoology and Ecology of Taras Shevchenko National University of Kyiv, Ukraine. Skin secretions were collected as follows: frogs were put into a petri dish. After mechanically stimulated with fingers for 1-2 min, the 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 2500 g for 15 min to remove debris. The supernatants were lyophilized (Telstar LyoQuest) and kept at 4 °C till use.

Fractionation

Lyophilized skin secretions samples of *B. bombina*, *B. variegata*, and their hybrid (0.2 g) were dissolved in 1 mL 0.05 M Tris-HCl buffer (pH 7.4), containing 0.2 M NaCl and centrifuged at 10,000 g for 5 min. Protein concentration in the supernatant was assayed by Bradford (1976) method, using bovine serum albumin as a standard. Chromatographic assays were performed using a system for liquid chromatography (BioRad,

USA). The samples were applied to a Superdex 200 pg (GE Healthcare, HiLOad™ 16/60) gel filtration column (flow rate 1 ml / min) equilibrated with 0.05 M Tris-HCl buffer (pH 7.4), containing 0.2 M NaCl. The elution was performed with the same buffer. The appearance of peaks was monitored, and the corresponding fractions were collected in the plastic tubes. The absorbance of the eluate was monitored at 280 nm.

SDS-Polyacrylamide gel electrophoresis

Flow-through fractions were concentrated as follows: the aliquots of the fractions were mixed with 25% trichloroacetic acid (1:1) and centrifuged for 5 min at 10,000 g. The precipitate was diluted with 1 ml of acetone and centrifuged. The procedure was repeated twice. The precipitate was then incubated at 37 °C for 15 minutes. After that, the precipitate was mixed (1:1) with the standard SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% sucrose, and 0.002% bromophenol blue) without heating.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed as reported (Laemmli, 1970), using 4% (w/v) stacking gel and 15% (w/v) separating gel. SDS-PAGE was conducted using Mini-Protean Tetra System (Bio Rad, USA) at 19 mA for stacking and 36 mA for separating gels. Fraction samples were applied in 15 μL volume per well. Gels were stained with 2.5% Coomassie brilliant blue G-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, 15 % (v/v) isopropanol and the background of the gel was destained with 7% (v/v) acetic acid for 30 min. Apparent molecular weights of proteins were estimated using protein calibration mixture (Bio Rad, USA) containing myosin, β-galactosidase, phosphorylase b, serum albumin, ovalbumin (Mr 97; 66; 45; 31; 21; 14 kDa).

Zymography

The aliquots of the flow-through fractions were mixed with **β**-mercaptoethanol solution (9:1) and sample buffer (1:1) (0.01 M tris-HCl buffer, pH 6.8, 2% sodium dodecyl sulfate, 10% sucrose and 0.01% bromophenol blue) without heating.

Zymography was performed according to the method suggested by Ostapchenko et al. (2011). Separating gel (12% w/v) was polymerized in the presence of gelatin (1 mg/mL), fibrinogen (1 mg/mL) or collagen (1 mg/ml). Fraction samples were applied in 15 μL volume per well. After SDS-PAGE, gels were incubated for 30 min at room temperature on a rotary shaker in 2.5% Triton X-100. The gels were then washed with distilled water to remove Triton X-100 and incubated in 50 mM Tris-HCl (pH 7.4) at room temperature for 12 hours. Gels were stained with 2.5% coomassie brilliant blue G-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, 15 % (v/v) isopropanol for 30 min.

Preparation of platelet-rich plasma and platelet-poor plasma

Platelet-rich plasma (PRP) and platelet-poor plasma were obtained following the standard protocol. All procedures were carried out at room temperature. Healthy adult rabbits were supplied by the vivarium of Taras Shevchenko National University of Kyiv, Ukraine. The blood was collected from the ear artery into a polyethylene tube with 3.8% sodium citrate (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-poor plasma was prepared by further centrifugation of the remaining stabilized blood at 1,500 g for 30 min.

Plasma coagulation tests

Activated partial thromboplastin time, thrombin time and prothrombin time were measured *in vitro* to assess the effects of the components of flow-through fractions of skin secretions on plasma clotting function. The tests were conducted using the coagulation analyzer (Rayto RT-2201C, China) and the standard set of reagents (RENAM, Russian Federation). To measure the activated partial thromboplastin time (aPTT), 45 μL of rabbit plasma was mixed with 5 μL of fraction sample and 50 μL aPTT reagent in the coagulometric cuvette. After 3 min of incubation at 37°C the 50 μL of 0.025 M $CaCl_2$ was added and the time necessary for the clotting plug formation was recorded. For the *in vitro* TT assay, the 45 μL of plasma and 5 μL of fraction sample were incubated in the coagulometric cuvette at 37°C for 2 min. Clotting time was immediately recorded after the addition of 100 μL thrombin (final activity 3 U/mL). The prothrombin time (PT) was assessed by mixing 45 μL of plasma and 5 μL of fraction sample in the coagulometric cuvette at 37°C for 2 min. Clotting time was immediately recorded after the addition of 100 μL 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 0.05 M Tris-HCl buffer (pH 7.4), containing 0.2 M NaCl instead of fractions samples, was used as controls.

Chromogenic assays

The effects of flow-through fractions of the studied skin secretions on key hemostasis enzymes were assayed using *p*-Nitroaniline chromogenic substrates: thrombin specific substrate Phe-Pip-Arg-*p*NA (S-2238), plasmin specific substrate Val-Leu-Lys-*p*NA (S-2251), factor Xa specific substrate Ile-Glu-Gly-Arg-*p*NA (S-2222) and activated protein C specific substrate pyroGlu-Pro-Arg-*p*NA (S-2366) (RENAM, Russian Federation). The direct proteolytic activity of the components of the fractions and their ability to activate plasma proenzymes was measured *in vitro*. Assays were performed in 0.05 M Tris-HCl buffer, pH 7.4 in a total volume 250 μL. To analyze the direct activity, the fractions samples with 20 μg of total protein in 0.05 M Tris-HCl buffer were mixed with the corresponding chromogenic substrates. To study the ability to activate plasma, proenzymes 20 μL of plasma was additionally added to the incubation medium. The formation of *p*-nitroaniline was monitored at equal intervals of time at 405 nm. The amount of *p*-nitroaniline, which was hydrolyzed from the substrate, was calculated by

using molar extinction coefficient of 10,000 M^{-1} x cm^{-1} for free *p*-nitroaniline.

Platelet aggregation assay

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 (230-250 × 10^3 cells/μL). The effects of the flow-through fractions of the studied skin secretions on platelet functions were performed *in vitro*. Briefly, 380 μL PRP and 20 μL of the samples 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 of 5 × 10^{-6} M.

Calculation of the results

TotalLab 2.04 program was used to analyze the resultant electrophorograms and zymograms. 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 StatSoft Statistica version 7.0 for Windows. The data were analyzed for statistical significance of difference by Student's t-test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Fractionation of the whole skin secretions

The supernatants of skin secretions of *B. bombina*, *B. variegata* and their hybrid were fractionated into several fractions by Superdex 200 pg column as illustrated in Fig. 1. The purification was followed by determination of various activities for each fraction.

SDS-Polyacrylamide gel electrophoresis and zymography

SDS-PAGE analysis was applied to get information about the protein composition of the flow-through fractions of the studied skin secretions (Table 1 and Figs. S1, S2, S3, S4). The results of protein separation revealed the presence of proteins with molecular weights ranging from 3 to 128 kDa. Zymography with gelatin, fibrinogen and collagen as substrates was conducted to evaluate the proteolytic potential of the flow-through fractions of the studied skin secretions. Key results are presented in Table 1. It was revealed that some fractions have proteolytic enzymes with gelatinolytic, fibrinogenolytic, and collagenolytic activities.

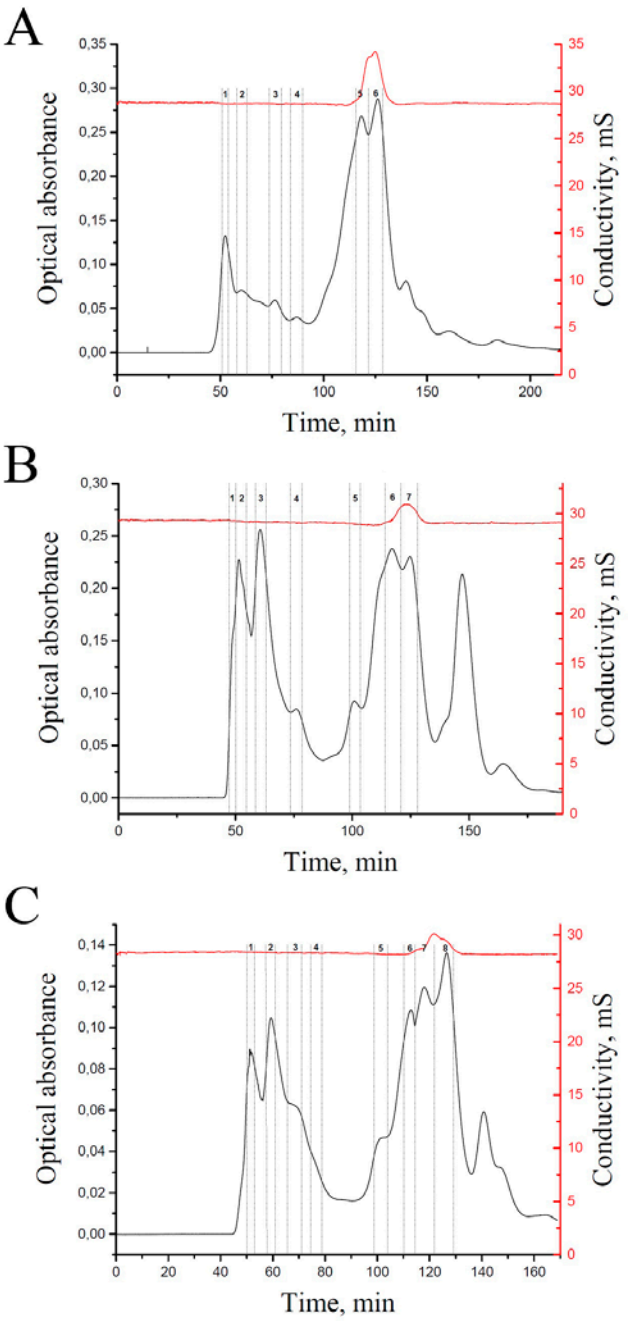

Fig. 1. Fractionation of *B. bombina* (A), *B. variegata* (B) and their hybrid (C) whole skin secretions.

aPTT, PT, and TT in vitro assays

Table 2 summarizes the effects of the flow-through fractions of the studied skin secretions on the time of clot-

Table 1. Molecular weights (MW) of proteins and the presence of proteolytic enzymes with certain substrate specificity in the flow-through fractions of skin secretions of studied amphibian species. The sign "+" - the manifestation of proteolytic activity, and the sign "-" - the absence.

	Fraction #	SDS-PAGE MW, kDa	Zymography		
			gelatin	collagen	fibrinogen
B. bombina	1	103; 78; 46; 31	+	+	+
	2	41	+	-	-
	3	74; 56; 44; 37; 33; 29; 22; 16; 13; 12; 11	+	+	-
	4	55; 48; 39; 36; 33; 27; 24; 21; 18; 12	+	+	-
	5	54	+	-	-
	6	55	+	-	-
B. variegata	1	76; 43; 39; 27; 3	+	-	-
	2	141; 129; 118; 101; 79; 57; 51; 49; 43; 39; 31; 27; 22; 17; 16; 3	+	-	+
	3	121; 109; 79; 42; 39; 27; 23; 22; 3	+	-	+
	4	124; 103; 80; 61; 48; 42; 36; 34; 29; 26; 22; 19; 17; 13; 9; 3	+	-	+
	5	16; 3	+	-	-
	6	16; 3	-	-	-
	7	3	-	-	-
Hybrid	1	100; 53; 43; 29; 22	-	-	-
	2	128; 108; 81; 53; 43; 39; 30; 27; 25; 23	+	+	+
	3	93; 65; 47; 44; 36; 32; 30; 28; 24; 23	-	+	+
	4	120; 88; 51; 42; 36; 31; 27; 24; 1	+	+	+
	5	21; 9	+	+	+
	6	8	+	-	-
	7	-	-	-	-
	8	-	-	-	-

ting plug formation, which corresponds to the plasma coagulation tests results (aPTT, TT and PT). As shown by the data, the whole skin secretions of all the studied amphibians prolonged aPTT clotting time: *B. bombina* – to 62.7 ± 0.5 s, *B. variegata* – to 73.6 ± 0.5 s, and their hybrid – to more than 90 s, compared to the control values – 21.35 ± 1.2 s. Fraction #6 of *B. variegata* skin secretions prolonged aPTT to 56.9 ± 1.1 s, and fraction #5 of hybrid skin secretions prolonged aPTT to 74.1 ± 0.8 s vs 21.35 ± 1.2 s in control. PT and TT assays showed no changes in the time of clotting plug formation while incubating with all studied whole skin secretions and flow-through fractions.

Chromogenic assays

The specific proteolytic activity of the components of flow-through fractions of the studied skin secretions was determined by the ability to hydrolyze the amide bond in the synthetic chromogenic substrates specific to thrombin (S-2238), plasmin (S-2251), factor Xa (S-2222) and protein C (S-2366). Table 3 demonstrates the results of this assay.

The ability of the studied fractions to activate plasma proenzymes was determined *in vitro*. While performing this experiment, rabbit blood plasma was additionally introduced into the incubation medium with the appropriate substrate and the skin secretion fraction sample. Table 3 indicates the emergence of activated thrombin, plasmin, and factor X in plasma while incubating with *B. bombina* whole skin secretions. Only one fraction of this amphibia – #1 – initiated the appearance of factor Xa in plasma. Fraction #9 of *B. variegata* skin secretions activated prothrombin, factor X, and protein C in plasma, whereas its whole secretions had no effects on plasma proenzymes. The hybrid *B. bombina* × *variegata* whole skin secretions and fraction 8 initiated the emergence of active prothrombin and protein C in plasma.

Platelet aggregation assay

This part of the research was aimed at investigating the potential effects of the flow-through fractions of the studied skin secretions on the process of platelet aggregation. Fraction #3 of *B. variegata* skin secretions induced platelet aggregation. As illustrated by Fig. 2, the degree of aggregation in the final concentration of 250 μg of

Table 2. The clotting time of rabbit blood plasma (sec) in the coagulation tests after incubation with the flow-through fractions of studied skin secretions.

		aPTT	PT	TT
	Control	**21.35 ± 1.2**	**6.75 ± 0.1**	**30.3 ± 2.2**
B. bombina	***Whole secretions***	***62.7 ± 0.5****	***7.1 ± 0.2***	***31.9 ± 0.4***
	1	20.3 ± 0.6	7.5 ± 0.3	32.6 ± 0.2
	2	21.3 ± 1.2	7.2 ± 0.2	32.8 ± 0.5
	3	20.7 ± 0.5	7.4 ± 0.5	32.2 ± 0.2
	4	22.5 ± 0.6	7.5 ± 0.3	32.4 ± 0.5
	5	20.8 ± 0.8	5.7 ± 1.2	33.2 ± 0.9
	6	21.7 ± 1.0	7.2 ± 0.4	32.7 ± 0.7
B. variegata	***Whole secretions***	***73.6 ± 0.5****	***6.8 ± 0.2***	***32.1 ± 0.2***
	1	23.6 ± 0.6	6.9 ± 0.4	29.7 ± 0.5
	2	19.3 ± 1.5	7.4 ± 0.3	29.0 ± 0.7
	3	25.6 ± 0.3	7.4 ± 0.2	29.8 ± 0.1
	4	28.1 ± 0.8	8.4 ± 1.2	32.2 ± 0.9
	5	56.9 ± 1.1*	6.9 ± 0.1	32.2 ± 0.7
	6	29.8 ± 0.6	7.5 ± 0.4	32.4 ± 0.7
	7	33.4 ± 0.3	6.9 ± 0.2	31.5 ± 0.2
Hybrid	***Whole secretions***	***>90****	***6.7 ± 0.3***	***31.7 ± 0.5***
	1	18.3 ± 0.6	7.5 ± 0.4	31.3 ± 0.5
	2	18.6 ± 1.2	7.4 ± 0.5	31.3 ± 0.6
	3	19.4 ± 0.3	6.8 ± 0.4	30.1 ± 0.4
	4	19.2 ± 0.4	6.8 ± 0.2	32.2 ± 0.5
	5	74.1 ± 0.8*	7.2 ± 1.1	32.2 ± 0.2
	6	17.1 ± 1.1	6.6 ± 0.2	29.7 ± 0.5
	7	18.7 ± 0.4	7.0 ± 0.6	31.0 ± 0.6
	8	18.4 ± 0.8	5.8 ± 0.5	31.6 ± 0.5

* – p ≤ 005 the difference is comparable to the control.

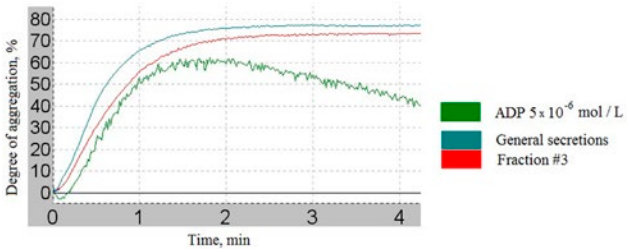

Fig. 2. Whole skin secretions and fraction #3 of *B. variegata* induce platelet aggregation in rabbit PRP.

total protein in 1 ml of PRP were 73% and 65%, respectively, which corresponds to the degree of aggregation in response to the action of $5x10^{-6}$ M ADP. Even though the components of whole skin secretions of hybrid *B. bombina* × *variegata* induced platelet aggregation in the rabbit PRP with the degree of aggregation 52%, the fraction that might have this effect was unidentified. This might be the result of the dilution of the fractions while performing the chromatography assay. Both the whole skin secretions and flow-through fractions of *B. bombina* had no effect on the process of platelet aggregation.

DISCUSSION

To adequately undertake the fractionation, a chromatographic carrier Superdex 200 pg (GE Healthcare, HiLOad™ 16/60) gel filtration column was used in our research. The prerequisite for the use of this carrier was its wide zone of separation (from 3 to 70 kDa), high stability, and inactivity to form nonspecific interactions with the fraction samples. As a result of fractionation, six protein fractions were identified in the *B. bombina* dermal secretions, seven protein fractions were determined in the *B. variegata* skin secretions, and eight protein fractions were defined in the hybrid *(B. bombina* × *B. variegata*) whole secretions. The data on SDS-PAGE assay suggests the presence of the mixture of the proteins with similar molecular weights in each identified fraction. A review of the biological effects of the fractions was conducted and compared to those of the whole skin secretions. Results of our research into the proteolytic potential indicate the presence of enzymes with gelatinase activity in all *B. bombina* (Bb) fractions, whereas the proteolytic enzymes with collagenolytic activity were determined in fractions #1, #3, and #4 of these amphibians. The fibrinogenolytic enzymes were detected only in the Bb#1 fraction. The data indicate the presence of enzymes with gelatinase activity in the fractions #1, #2, #3, #4, and #5 of *B. variegata* (Bv) skin secretions. Fractions Bv#2, Bv#3, and Bv#4 are characterized by the presence of fibrinogenolytic enzymes. No proteolytic potential was indicated in the *B. variegata* skin secretion fractions while conducting zymography assay with collagen as a substrate. The results of the study on the proteolytic potential of the hybrid fractions (Bh) indicate the presence of gelatinolytic enzymes in the fractions #2, #4, #5, #6, and #7, collagenolytic enzymes in the fractions #2, #3, #4, #5, and #7, and the presence of fibrinogenolytic enzymes in fractions #2, #3, #4, and #5. The data clarify the relationship between the emergence of specific type of activity and the release of high molecular weight proteins while performing the SDS-PAGE assay.

To assess the effects of the components of the fractions on plasma clotting function, aPTT, PT, and TT were measured. The data indicate that the *B. variegata* and hybrid whole skin secretions prolonged the clotting plug formation in the aPTT. The proteins that are responsible for the manifestation of this effect were identified in the fraction Bv#5 and Bh#5. Thus, according to the results,

Table 3. The amount of hydrolyzed *p*-NA (nmol) from the chromogenic substrates under the action of whole skin secretions and the flow-through fractions samples of studied amphibian species. S-2238 – thrombin specific substrate; S-2251 – plasmin specific substrate; S-2222 – factor Xa specific substrate; S-2366 – activated protein C specific substrate. The sign "-" - the absence of the effect.

		Proteolytic activity				Plasma enzymes activity			
		S-2238	S-2251	S-2222	S-2366	trombin	plasmin	factor Xa	protein Ca
B. bombina	***Whole secretions***	***0***	***0***	***3.73*** ± **0.07**	***23.76*** ± **0.71**	***14.48*** ± **0.72**	***3.81*** ± **0.32**	***8.61*** ± **0.45**	-
	1	9.32 ± 0.18	2.21 ± 0.04	3.62 ± 0.07	9.11 ± 0.07	-	-	1.09 ± 0.12	-
	2	13.79 ± 0.27	2.06 ± 0.04	5.98 ± 0.17	24.63 ± 0.62	-	-	-	-
	3	13.52 ± 0.07	6.53 ± 0.19	16.97 ± 0.34	25.32 ± 0.23	-	-	-	-
	4	1.03 ± 0.39	11.62 ± 0.31	17.26 ± 0.51	22.63 ± 0.43	-	-	-	-
	5	4.61 ± 0.09	-	-	4.73 ± 0.05	-	-	-	-
	6	5.85 ± 0.18	-	-	6.04 ± 0.17	-	-	-	-
B. variegata	***Whole secretions***	***3.41*** ± **0.13**	***0.73*** ± **0.02**	***1.95*** ± **0.08**	***1.47*** ± **0.07**	-	***0.35*** ± **0.02**	-	-
	1	-	-	-	-	1.52 ± 0.08	-	-	-
	2	-	-	-	-	0.78 ± 0.03	-	-	-
	3	6.01 ± 0.17	-	1.51 ± 0.07	3.57 ± 0.12	-	-	-	-
	4	9.49 ± 0.09	3.76 ± 0.12	4.42 ± 0.15	6.67 ± 0.16	-	-	-	
	5	-	-	-	-	-	-	-	1.97 ± 0.08
	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	7.62 ± 0.23	-	2.91 ± 0.13	11.21 ± 0.31
Hybrid	***Whole secretions***	***3.75*** ± **0.18**	***0.75*** ± **0.05**	***1.76*** ± **0.12**	***1.60*** ± **0.09**	***6.11*** ± **0.12**	-	-	***9.71*** ± **0.35**
	1	2.37 ± 0.12	-	-	2.31 ± 0.04	-	-	-	-
	2	2.31 ± 0.09	-	-	-	-	-	-	-
	3	2.37 ± 0.11	-	-	2.27 ± 0.07	-	-	-	-
	4	6.82 ± 0.34	-	1.78 ± 0.15	3.78 ± 0.25	-	-	-	-
	5	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
	8	-	-	-	-	6.34 ± 0,15	-	-	10.89 ± 0,43

the Bv#5 fraction were characterized by the presence of two proteins with the molecular weight 16 kDa and 3 kDa and had a gelatinolytic activity. Fraction Bh#5 was characterized by the presence of proteins with molecular weight 21 kDa and 9 kDa and had a pronounced protease activity with wide substrate specificity. These results may be attributed to the presence of the inhibitors of certain factors of coagulation hemostasis that are present in the fractions of skin secretions, or, to the degradation of the factors of coagulation hemostasis by the active components of crude skin secretions. Another test – PT – is used to evaluate the functioning of the coagulation factors V, VII, and X and the time necessary to generate fibrin after activation of factor VII in the extrinsic coagulation pathway (Azevedo et al., 2007). TT measures the time required for fibrinogen to form fibrin strands in the presence of thrombin. This test only reveals disturbances in the final stages of coagulation (Koch and Biber, 2007). Although the data shows no potential effect of the components of the flow through fractions on these coagulation tests, mention should be made of the specificity of action.

The data on the dermal components ability to activate plasma proenzymes indicate that the whole secretions of the hybrid initiated the appearance of thrombin and activated protein C in plasma. This effect may be attributed to the action of the components of fraction Bh#7. Although the fraction Bv#9 initiated the appearance of thrombin and activated protein C in plasma, no effect was observed under the action of the whole secretions of this amphibian. According to the SDS-PAGE assay, the components responsible for the manifestation of these effects have a low molecular weight, e.g., fraction Bv#9 – 3 kDa, or, are non-protein nature and have little interest in the context of studying of the effects of the amphibian skin secretions. These results have failed to support our hypothesis that the appearance of thrombin and activated protein C may be due to the presence of active forms of proteolytic enzymes in the crude skin

secretions that can directly activate the cleavage of the corresponding proenzymes of thrombin and protein C or affect the cofactors which can promote a cascade of reactions that result in the formation of zymogens, as the data of SDS-PAGE shows no proteolytic activity in the active fractions Bv#7 and Bv#9. The data indicate that the whole skin secretions of *B. bombina* initiated the appearance of thrombin, plasmin, and factor Xa in plasma, whereas the results have failed to define the fractions that were responsible for these effects. We can assume that such effects of the whole *B. bombina* skin secretions might be the result of the sequential activity of the proteins, when two proteins from different fractions initiated the appearance of thrombin. Or this may also be due to the action of non-protein components or might be related to the high dilution of the fractions as a result of gel filtration chromatography.

The results of the study on the platelet aggregation capability suggest that the *B. variegata* whole skin secretions induced platelet aggregation in the rabbit PRP. The proteins that were responsible for this effect are concentrated in the fraction Bv#3. Mention should be made of the fact that the effect of the whole secretion and the protein fraction corresponds to the effect of ADP, which is known as one of the physiological inducers of platelet aggregation. Previous results of SDS-PAGE assay indicate the presence of several proteins in the fraction Bv#3 with various molecular weights: 121 kDa, 109 kDa, 79 kDa, 42 kDa, 39 kDa, 27 kDa, 23 kDa, 22 kDa, and 3 kDa. Moreover, presence was also indicated of proteolytic enzymes in this fraction with specificity to gelatin and fibrinogen. The proteins present in fraction Bv#3 could be a prominent source of inducers of platelet aggregation. Although inducers that can modulate platelets function are not relevant in the treatment of hemostasis system disorders, they could be a useful tool for studying platelets functions and their signaling pathways. Despite the activity of the components of the hybrid *B. bombina* × *variegata* whole skin secretions to induce platelet aggregation (data is not shown), the results have failed to identify the fractions that were responsible for this effect. This may be due to the significant dilution of the fractions resulting from gel filtration chromatography.

In conclusion, the results of our study demonstrate that the studied amphibian skin secretions of the genus *Bombina* are a potential source of bioactive constituents that can affect different stages of the hemostasis system. It was defined that certain flow-through fractions have active molecules that demonstrate some specific effects. It was established that these molecules are proteins by nature and some of them have pronounced proteolytic activities. Furthermore, the data showed that the proteins with the ability to prolong the clotting plug formation in the aPTT *in vitro* assay are present in the fraction #5 of *B. variegata* and #5 of the hybrid skin secretions. The ability of dermal components to activate plasma proenzymes indicates that the non-protein components of fraction Bv#9 and fraction Bh#7 skin secretions initiated the appearance of thrombin and activated protein C in plasma. The proteins capable of inducing platelet aggregation in the rabbit PRP are present in the fraction #3 of *B. variegata* skin secretions.

Our research has made a contribution into the study of the genus *Bombina* dermal secretions, in particular, the effect of the venom constituents on the hemostasis system and the nature of the active components; however, the mechanism of their action still requires additional research. Such findings may be used by biomedical researchers as a source of potential novel drug leads or pharmacological agents with a direct therapeutic effect and can rapidly provide a basis for related scientific studies such as those involved in systematic or the evolution of species.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www.unipv.it/webshi/appendix> manuscript number 7858

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