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Purification and biochemical characterization of fibrino(geno)lytic enzymes from tissues of Antarctic hydrobionts

Abstract. Considering the continuing increase of morbidity and mortality rates associated with cardiovascular diseases, the search for novel compounds able to affect the hemostasis system is among the current trends of modern science and pharmacology. Fibrino(geno)lytic enzymes because of their role in dissolving blood clots as well as prevention of their formation attract special attention. The main goal of the current research was to develop the methodological approaches to obtain fibrino(geno) lytic enzymes from Antarctic hydrobionts and study their effects on the functioning of the hemostasis system. A complex approach which included affinity chromatography and size-exclusion chromatography was applied to isolate the fibrino(geno)lytic enzymes from the tissue of Antarctic nemertea (*Parborlasia corrugatus*), Antarctic sea urchin (*Sterechinus neumayeri*), and Antarctic sea star (*Odontaster validus*). The presence of proteolytic activity was monitored by zymographic technique. Fibrin(ogen)olytic activity was assessed by incubation of the samples with fibrinogen followed by 10% SDS-PAGE analysis. To test the substrate specificity of the enzymes, the chromogenic substrates such as H-D-Phe-Pip-Arg-*p*NA, pyroGlu-Pro-Arg-*p*NA, H-D-Val-Leu-Lys-*p*NA and Bz-IIe-Glu(γ-OR)-Gly-Arg-*p*NA were used. The influence of fibrino(geno)lytic enzymes on platelet aggregation was assessed in platelet-rich plasma. To analyze the effect of the fibrino(geno)lytic enzymes on coagulation the blood coagulation time was assessed. The obtained results clearly indicated the presence of enzymes with activity toward fibrinogen in the tissues of tested hydrobionts. Based on the results of SDS-PAGE and zymography the molecular weight of the fibrino(geno) lytic enzymes was in the range of 26—34 kDa. The fibrinogen cleavage pattern analyzed by SDS-PAGE revealed the susceptibility of fibrinogen chains to degradation by enzymes from tissues of Antarctic hydrobionts. The fibrino(geno)lytic enzymes from all tested hydrobionts cleaved preferentially the Aα-chain and more slowly the Bβ-chain of fibrinogen. The fibrino(geno)lytic enzymes mediated the significant prolongation of blood clotting time in chronometric tests and inhibition of ADP-induced platelet aggregation. The enzymes exhibit activity against chromogenic substrates, which was more expressed in case of pyroGlu-Pro-Arg-*p*NA — a specific synthetic substrate for activated protein C and factor XIa. The enzymes isolated from the tissues of Antarctic marine hydrobionts possess a fibrin(ogen)olytic activity and can be of medical interest as therapeutic agents in the treatment and prevention of thrombotic disorders.

Keywords: fibrino(geno)lytic enzymes, hemostasis, Antarctic hydrobionts

1 Introduction

The study of structural and functional characteristics of enzymes from various organisms adapted to function at low ambient temperatures is of considerable interest nowadays not only to the fundamental science but also to various branches of industry. At the current stage of economic development, the use of enzymes is extremely broad. The areas where the application of enzyme-based drugs is of great importance include among other things medicine. Considering that the thrombotic events caused by the impairment of blood coagulation play a major role in cardiovascular diseases, the search for novel com-

pounds which could affect hemostasis is among the important tasks of science. In this case, fibrino(geno)lytic enzymes because of their role in dissolving blood clots and prevention of their formation are potentially promising molecules for the treatment of diseases accompanied by excessive thrombus formation.

Marine organisms are known to be highly interesting objects to obtain substances with unique properties. A number of different compounds with antibacterial, antifungal, antiviral, anti-inflammatory, immunostimulatory, antitumor and anti-apoptotic effects have been found among their metabolites (Grienke et al., 2014; Lindequist, 2016; Carson, Clarke, 2018). However, in spite of the structural and functional diversity of metabolites, little research has been undertaken to study the effects of the compounds derived from the marine hydrobionts on hemostasis. For instance, only some anticoagulant proteins or peptides have been isolated from marine organisms (Jo et al., 2008; Jung, Kim, 2009; Kong et al., 2013).

In view of the foregoing, the aim of this research was development of methodological approaches to obtain fibrino(geno)lytic enzymes from the tissues of some representatives of Antarctic marine hydrobionts (sea urchin, nemertine, starfish), purification of these enzymes and study of their potential effects on the functioning of hemostasis system.

2 Materials and methods

Subjects and tissue extracts preparation. In this study, the frozen mass of hydrobionts collected in the Antarctic region, e.g. Antarctic nemertea (*Parborlasia corrugatus* (McIntosh, 1876)), Antarctic sea urchin (*Sterechinus neumayeri* (Meissner, 1900)), and Antarctic sea star (*Odontaster validus* Koehler, 1906), was used as the source material. The specimens (n = = 35 for each species) were collected near the Galindez Island (65°15′ S, 64°15′ W) of the Argentine Islands. The frozen mass of the hydrobionts' soft tissues was weighed and carefully ground to a powder state while adding liquid nitrogen. Then the substance was transferred to the homogenizer and the extracting solution was added, composed of 50 mL 0.1 M Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl, 1.0 mM EDTA, 0.25% sucrose, and 0.5% Triton X-100. The material was thoroughly homogenized for 10 min. The extraction solution was added at 5 mL of solution per 10 g of material. Then the samples were incubated for 15 min at 4 °C and centrifuged at 10000 g for 40 min. The supernatant was re-centrifuged and the resultant supernatant was purified by size exclusion chromatography on a Sephadex G25 column (2.5 × 80 cm) (GE Healthcare). The fractions were lyophilized and used for further studies.

Affinity Chromatography. Blue Sepharose (Bio Rad, USA) was used for affinity chromatography. The lyophilized samples (50 mg/mL) were dissolved in 10 mM Tris-HCl, pH 7.4. After centrifugation at 10000 g for 5 min, the supernatant was loaded to the Blue Sepharose column (flow rate of 30 mL per hour), which was preequilibrated with 10 mM Tris-HCl, pH 7.4. Elution of the adsorbed proteins was carried out in a stepwise manner (flow rate of 1 mL/min) using elution buffer composed of 10 mM Tris-HCl (pH 7.4) containing 1 M NaCl.

Size Exclusion Chromatography (SEC). The flow-through fractions after affinity chromatography were applied to size exclusion chromatography on a Superdex 200 PG column (GE Healthcare), which was preequilibrated with distilled water buffered by NaOH to pH 8.0. The peaks were collected at a flow rate 45 mL per hour.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymography assay (substrate SDS-PAGE). SDS-polyacrylamide gel electrophoresis was performed as described in (Laemmli, 1970), using 4% (w/v) stacking gel and 10% (w/v) separating gel. Samples were prepared by mixing the fractions at 1 : 1 ratio (v/v) with sample buffer (62.5 mM Tris-HCl, pH 8.3, 2% SDS, 5% sucrose, and 0.02% bromophenol blue). Samples were heated at 95 °C for 2 min before the experiment. Gels were stained with 2.5% Coomassie Brilliant Blue R-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. The molecular weight of proteins was estimated using low-molecular weight calibration kit (Bio Rad, USA). The total amount of proteins applied per well was 20 μg.

Zymography (substrate SDS-PAGE) was performed according to the method suggested (Ostapchenko et al., 2011). Separating gel (12% w/v) was polymerized in the presence of fibrinogen (1 mg/mL). Samples were applied to the wells without heating. After SDS-PAGE was done, gels were incubated for 30 min at room temperature on a rotary shaker in 2.5% Triton X-100 for SDS removal and re-naturation of the enzyme. The gels were then washed with distilled water for 10 min to remove Triton X-100 and incubated in 50 mM Tris-HCl (pH 7.5) at room temperature for 12 hours. Gels were stained with 2.5% Coomassie Brilliant Blue R-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, 15% (v/v) isopropanol for 30 min. Areas of substrate digestion were visualized as clear patches on dark background.

Blood coagulation time assay. Activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were measured *in vitro* using a coagulation analyzer (Rayto, Shenzhen, China) and the standard set of reagents (RENAM, Russian Federation) according to manufacturer's instructions.

Fibrino(geno)lytic activity determination. Fibrino-(geno)lytic activity was assayed according to (Raksha et al., 2016). Fibrinogen was obtained from plasma by precipitation with 16% ammonium sulfate followed with cryofibrinogen separation according to the study (Varetskaya, 1960). Fibrinogen (1 mg/mL) and enzyme were mixed 1 : 10 (w/w) and the mixture was incubated at 37 °C for different intervals of time (0—48 h). Aliquots were taken from the reaction mixture at various time points (0, 1, 2, 3, 5, 24, and 48 h) and boiled with β-mercaptoethanol for 1 min. Then an equal volume of a sample buffer was added. The samples were analyzed by 10% SDS-PAGE to examine the cleavage pattern of the fibrinogen.

Platelet aggregation assay. The effect of fibrino-(geno)lytic enzymes on platelet aggregation was assessed using a photo-optical aggregometer AT-02 (Medtech, Russia). The blood was collected into a polyethylene tube with 3.8% sodium citrate (9 : 1). Platelet-rich plasma (PRP) for platelet analysis was obtained by blood centrifugation at 150 g for 10 min at room temperature. Platelet-poor plasma (PPP) was prepared by further centrifugation of the remaining blood at 2500 g for 20 min at room temperature. PPP was stored at −20 °C until further analysis. To prevent spontaneous platelet activation, PRP was placed in a water bath at 37 °C for 30 min before the assay that was performed within the first 3 h after blood sampling. Before the assessment, the platelet count in PRP was adjusted with PPP to 230×10^3–250×10^3 cells $\cdot\ \mu L^{-1}$. PRP was pre-incubated with samples of fibrinogenolytic enzyme at final concentration 200 μg per 1 mL of PRP at 37 °C with continuous stirring at 600 rpm. To induce platelet aggregation, ADP (Sigma, USA) in the final concentration of 5×10^{-6} M was added to the samples and the aggregation process was monitored for 10 min. The aggregation curves were analyzed using AT-02 software (Medtech, Russia).

Hydrolysis of chromogenic substrates. The substrate specificity of fibrino(geno)lytic enzymes was measured using chromogenic peptide substrates such as H-D-Phe-Pip-Arg-*p*NA (S2238), pyroGlu-Pro-Arg-*p*NA (S2366), H-D-Val-Leu-Lys-*p*NA (S2251) and Bz-IIe-Glu(γ-OR)-Gly-Arg-*p*NA (S2222) (Sigma, USA). The substrates were dissolved in 50.0 mM Tris–HCl buffer (pH 7.4) containing 0.13 M NaCl. The enzyme activity was studied by mixing the 50 μL of the enzyme (20 μg in 50.0 mM Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl) with 175 μL of 50 mM Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl. The reaction was initiated by addition of 25 μL of 3 mM appropriative chromogenic substrate. The production of *p*-nitroaniline (*p*NA) from the substrates was measured by monitoring the increment at 405 nm every 60 s for 60 min. The amount of released *p*NA was calculated using molar extinction coefficients for free *p*NA. The enzyme activity was calculated using molar extinction coefficient for free *p*-nitroaniline (8800 $M^{-1} \cdot cm^{-1}$). In each experiment ($n = 5$) all samples were tested in triplicate.

Statistical analysis. Data were analyzed for variance by ANOVA followed by Tukey's test at $p < 0.05$ and expressed as means values ± SD.

Protein Concentration. Protein concentration was assayed by Bradford method (Bradford, 1976), using bovine serum albumin as a standard protein and measuring the absorbance of the samples at 595 nm.

3 Results and discussions

To obtain the fibrino(geno)lytic enzymes from the tissues of hydrobionts a complex approach which combined several chromatographic steps was used, including affinity chromatography and the subsequent fractionation of the flow-through fractions by size-exclusion chromatography.

For the first step of purification of fibrino(geno)-lytic enzymes the affinity sorbent Blue Sepharose 6 FF was used. This sorbent was selected based on the results of previous studies (Fořtová et al., 1990) which suggested its high efficiency in protein isolation from snake venoms. The fractions were then applied to zymography assay with fibrinogen polymerized into the gel to study the presence of the enzymes able to cleave fibrinogen. In general, the analysis confirmed the presence of active enzymes in the bound fractions that was evidenced by the appearance of the light areas in the gel due to fibrino(geno)-lytic activity. Based on the results, the most active enzymes were found in the tissues of *S. neumayeri*, while the enzymes from *P. corrugatus* were the least active against fibrinogen (Fig. 1).

Unbound fractions were also analyzed, as this may be the indicator of the effectiveness of the enzyme purification procedure. Electrophoresis profiles shown in Fig. 1 demonstrate that all unbound fractions contained only trace amounts of enzymes with fibrino-(geno)lytic activity, since no pronounced hydrolysis zones were revealed in the tracks. The absence of expressed activity in the unbound fractions demonstrates the effectiveness of using Blue Sepharose as a sorbent in the purification of fibrino(geno)lytic enzymes. To obtain a pure fibrino(geno)lytic enzyme, the fractions after affinity chromatography were subjected to the subsequent purification step with size exclusion chromatography application.

After the separation conditions were optimized, the Superdex 200 PG carrier was selected for further experiments. This type of carrier enables efficient separation of proteins in a wide range of molecular weights. All flow-through fractions at this stage of chromatographic separation were also analyzed for fibrino(geno)lytic activity.

Fig. 2 illustrates the separation of the *S. neumayeri* fraction into 7 peaks. Since the applied conditions failed to separate the source material into individual peaks, only the apexes of the peaks were selected for further analysis. Fractions № 6 and № 7 were not analyzed due to the high conductivity level, which indicates the exit of non-protein material from the column. The obtained fractions were applied to zymography assay, which revealed the expressed hydrolysis zone in the third fraction (Fig. 2b). The molecular weight of the enzyme present in this fraction was approximately 34 kDa.

The application of this methodological approach for the separation of the fraction from the *O. validus* proved to be more effective and made it possible to get 5 clearly defined peaks (Fig. 3a). The first peak was yielded at the 20th min of the chromatographic process compared to the 60th min while analyzing *S. neumayeri* fraction, which indicates the presence of proteins with higher molecular weight in the tissues of *O. validus*. Zymography profile illustrated in Fig. 3b demonstrates a pronounced hydrolysis zone in the 5th track which corresponds to the fraction № 5. The molecular weight of the active enzyme was about 28 kDa.

Chromatographic separation of the fraction from the *P. corrugatus* also yielded 5 fractions. The most pronounced fibrino(geno)lytic activity was shown in fraction № 4, which manifested in the emergence of the hydrolysis zone localized in the region of 26 kDa (Fig. 4b).

To examine the purity of the obtained enzymes and to estimate their precise molecular weight, fractions containing the active enzymes were applied to SDS-PAGE. As shown in Fig. 5a, the fraction from tissues of *S. neumayeri* contained only one clearly defined protein band with molecular weight about 34–35 kDa. This result coincides with the data we obtained in the result of zymography assay of this fraction.

The analysis of fraction № 5 of *O. validus* revealed two protein bands with molecular weights 28 and 44 kDa (Fig. 5b). Since zymography of this fraction revealed only one active enzyme with a molecular weight 28 kDa we can assume that a protein with molecular weight 44 kDa is either an enzyme with a non-fibrino(geno)lytic activity or a non-enzyme protein.

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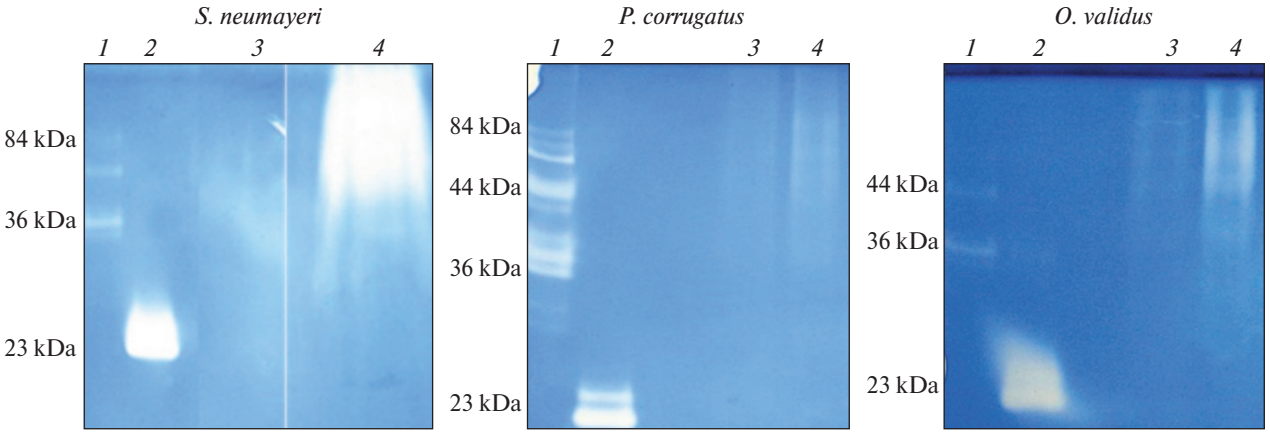

Figure 1. Substrate SDS-PAGE of the fractions obtained by affinity chromatography on Blue-sepharose: lane *1–2* — molecular weight markers; lane *3* — the unbound fraction; *4* — the bound fraction. Zymography assay was performed using fibrinogen (1 mg/ml) polymerized in 12% separating gel. 20 μg of total protein was loaded into each well and electrophoresis was run at 39 mA per gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. The gels were washed with 2.5% Triton-X 100 for 60 min and then incubated in 50 mM Tris-HCl (pH 7.5) for 12 hours. Gels were stained with Coomassie Blue G-250

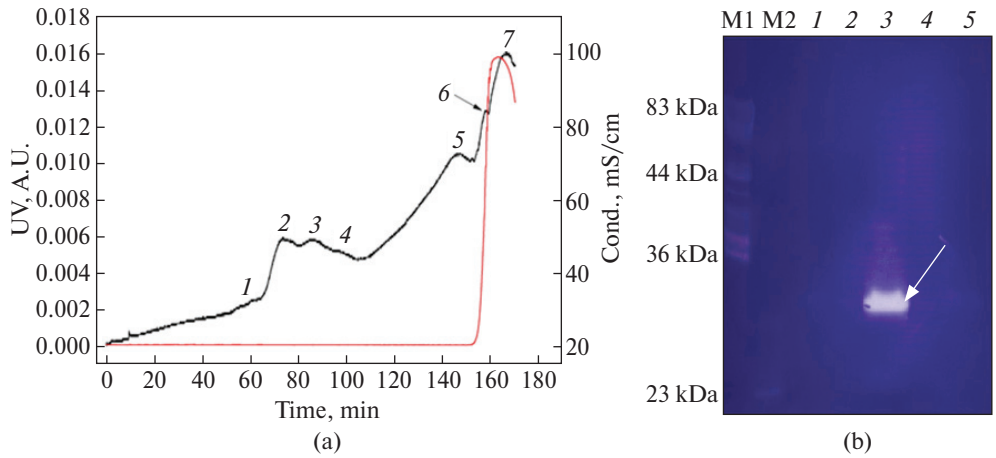

Figure 2. Purification procedure of the *Sterechinus neumayeri* fibrino(geno)lytic enzyme: (a) size exclusion chromatography on a Superdex 200 PG column: *1–7* — fraction numbers; (b) substrate SDS-PAGE of the obtained fractions: lanes M1–M2 — molecular weight markers; lane *1–5* — the fractions obtained by size exclusion chromatography. The Superdex 200 PG column was equilibrated with distilled water buffered by NaOH to pH 8.0, and the fractions were eluted with the same buffer at a flow rate of 45 mL per hour. The elution profiles were monitored by spectrophotometry at 280 nm. Zymography assay was performed using fibrinogen (1 mg/mL) polymerized in 12% separating gel. 20 μg of total protein was loaded into each well and electrophoresis was run at 39 mA per gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. The gels were washed with 2.5% Triton-X 100 for 60 min and then incubated in 50 mM Tris-HCl (pH 7.5) for 12 hours. Gels were stained with Coomassie Blue G-250

As illustrated in Fig. 5c, fraction № 4 of *P. corrugatus* contained a number of protein bands with molecular weights ranging from 14 to 40 kDa. The most remarkable band was observed in the region of 26 kDa. According to the zymography results, this molecular weight corresponds to the molecular weight of an active enzyme, so it might be the same protein. The presence of protein bands in the fraction after size-exclusion chromatography suggests the need to modify our methodological approach to analyze this hydrobiont.

Thus, a combination of several steps of chromatographic separation (affinity chromatography

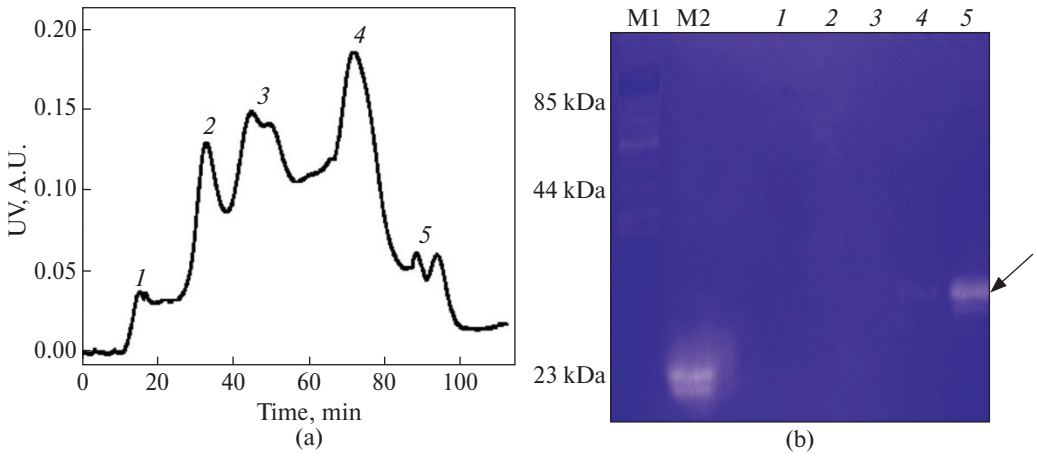

Figure 3. Purification procedure of the *Odontaster validus* fibrino(geno)lytic enzyme: (a) size exclusion chromatography on a Superdex 200 PG: *1–5* — fraction numbers; (b) substrate SDS-PAGE of the obtained fractions: lanes M1–M2 — molecular weight markers; lane *1–5* — the fractions obtained by size exclusion chromatography. The Superdex 200 PG column was equilibrated with distilled water buffered by NaOH to pH 8.0, and the fractions were eluted with the same buffer at a flow rate of 45 mL per hour. The elution profiles were monitored by spectrophotometry at 280 nm. Zymography assay was performed using fibrinogen (1 mg/mL) polymerized in 12% separating gel. 20 μg of total protein was loaded into each well and electrophoresis was run at 39 mA per gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. The gels were washed with 2.5% Triton-X 100 for 60 min and then incubated in 50 mM Tris-HCl (pH 7.5) for 12 hours. Gels were stained with Coomassie Blue G-250

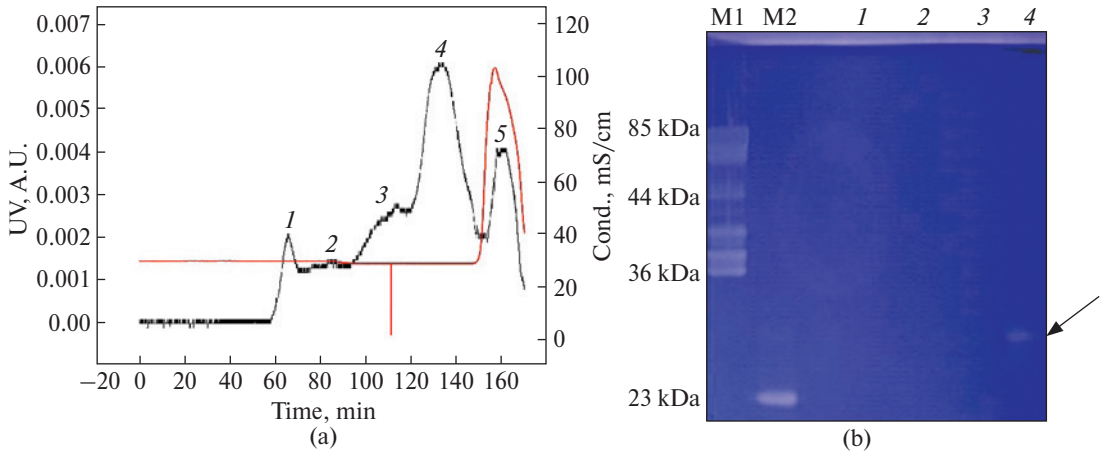

Figure 4. Purification procedure of the *Parborlasia corrugatus* fibrino(geno)lytic enzyme: (a) size exclusion chromatography on a Superdex 200 PG: *1–5* — fraction numbers; (b) substrate SDS-PAGE of the obtained fractions: lanes M1–M2 — molecular weight markers; lane *1–4* — the fractions obtained by size exclusion chromatography. The Superdex 200 PG column was equilibrated with distilled water buffered by NaOH to pH 8.0, and the fractions were eluted with the same buffer at a flow rate of 45 mL per hour. The elution profiles were monitored by spectrophotometry at 280 nm. Zymography assay was performed using fibrinogen (1 mg/mL) polymerized in 12% separating gel. 20 μg of total protein was loaded into each well and electrophoresis was run at 39 mA per gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. The gels were washed with 2.5% Triton-X 100 for 60 min and then incubated in 50 mM Tris-HCl (pH 7.5) for 12 hours. Gels were stained with Coomassie Blue G-250

on a Blue Sepharose column and size exclusion chromatography on a Superdex 200 column) yielded fractions of fibrino(geno)lytic enzymes with defined molecular weights: 34 kDa for *S. neumayeri*, 28 kDa for *O. validus*, and 26 kDa for *P. corrugatus*.

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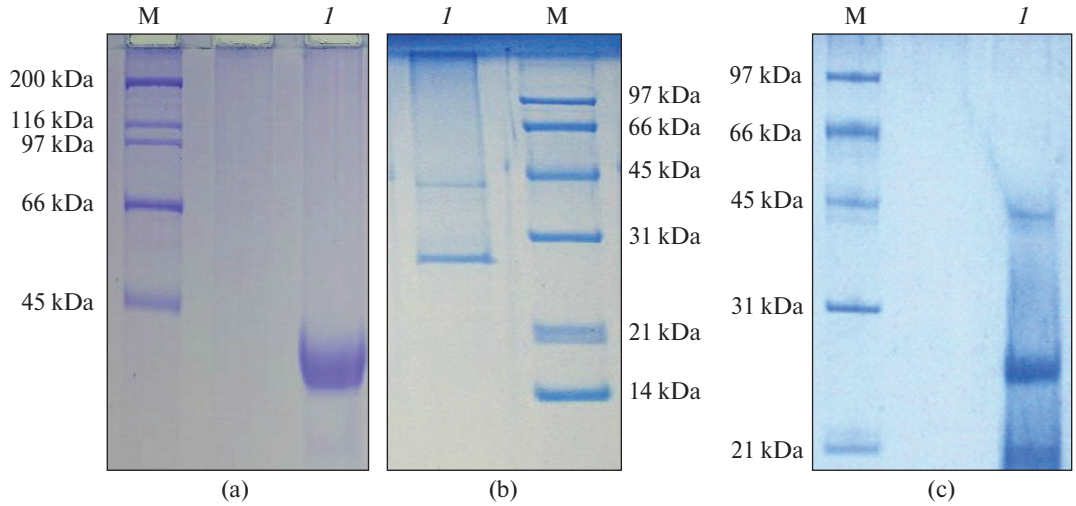

Figure 5. SDS-PAGE of the fraction № 3 *Sterechinus neumayeri* (a), fraction №5 *Odontaster validus* (b), and fraction № 4 *Parborlasia corrugatus* (c): line M — molecular weight markers; lane *1* — the fraction. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed in 10% (w/v) separating gel. The samples were mixed at 1 : 1 ratio (v/v) with sample buffer (62.5 mM Tris-HCl, pH 8.3, 2% SDS, 5% sucrose, and 0.02% bromophenol blue) and heated at 95 °C for 2 min. 20 μg of total protein was loaded into each well. Electrophoresis was carried out at 39 mA per gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. Gels were stained with Coomassie Blue G-250

An important characteristic of fibrino(geno)lytic enzymes is their specificity to fibrinogen chains. The enzymes that first cleave the Aα-chain are known as α-fibrinogenases, and those that act on the Bβ-chain as β-fibrinogenases, respectively (Cortelazzo et al., 2010). The specificity of the action of fibrinogenolytic enzymes derived from hydrobionts tissues on fibrinogen was studied by SDS-PAGE.

The results presented in Fig. 6–8 illustrate the splitting of both the Aα- and Bβ-chains of fibrinogen by fibrino(geno)lytic enzymes that depended on the duration of incubation and the object. The enzymes from the tissues of *S. neumayeri* turned to be the most active, as one hour of incubation of fibrinogen with the fibrino(geno)lytic enzyme led to complete cleavage of both Aα- and Bβ-chains of fibrinogen. Since we did not take samples during the first hour of incubation, we cannot determine which of the fibrinogen chains was cleaved first. The fibrino(geno)lytic enzyme from the tissues of the studied hydrobionts also cleaved the γ-chain of fibrinogen — the intensity of the band that corresponds to this chain decreased with the increasing of incubation time, and it completely disappeared after 6 hours of incubation.

As demonstrated by the electrophoresis profile shown in Fig. 7, the fibrino(geno)lytic enzyme from tissues of *O. validus* also effectively cleaved fibrinogen. In this case, the Aα-chain was cleaved first. The analysis of fibrinogen samples after one hour of incubation failed to reveal the presence of this chain.

The rate of cleavage of the fibrinogen Bβ-chain by the *O. validus* fibrino(geno)lytic enzyme was lower than the same while studying *S. neumayeri* tissue enzyme. The intensity of the band that corresponds to the Bβ-chain was slightly reduced only after 3 hours of incubation with the enzyme, and the complete cleavage of this band was observed after 6 hours of incubation. Such results do not contradict other studies, since even despite the pronounced specificity to one of the fibrinogen chains the prolonged incubation of the fibrino(geno)lytic enzymes results in the hydrolisation of the other chain also. The ability of the *O. validus* enzyme to affect the fibrinogen γ-chain failed to be detected — the intensity of the band that corresponds to the γ-chain remained unchanged throughout the 48-hour study period.

The analysis of fibrinogen samples after incubation with the fibrino(geno)lytic enzyme from *P. cor-*

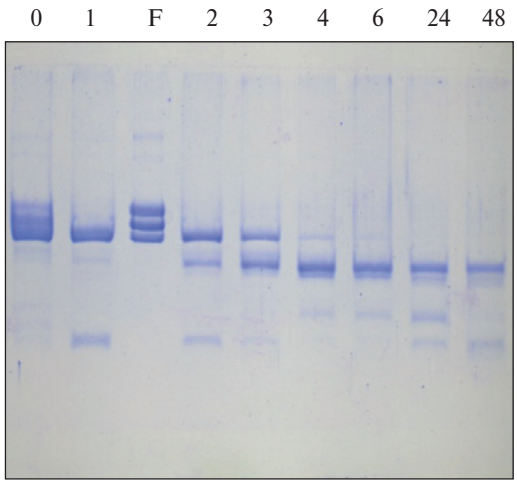

Figure 6. SDS-PAGE analysis of the fibrinogen after incubation with *Sterechinus neumayeri* fibrino(geno)lytic enzyme: F — intact fibrinogen; 0–48 — hrs of incubation. Fibrinogen (1 mg/mL) and fibrino(geno)lytic enzyme were mixed at 1 : 10 (w/w) ratio. The mixture was incubated at 37 °C. Aliquots were taken at 0, 1, 2, 3, 5, 24 and 48 hrs and boiled with β-mercaptoethanol for 1 min. Then the samples were mixed with equal volumes of buffer (62.5 mM Tris-HCl, pH 8.3, 2% SDS, 5% sucrose, and 0.02% bromophenol blue). The reaction products were analyzed by 10% SDS-PAGE. Electrophoresis was carried out at 39 mA per gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. Gels were stained with Coomassie Blue G-250

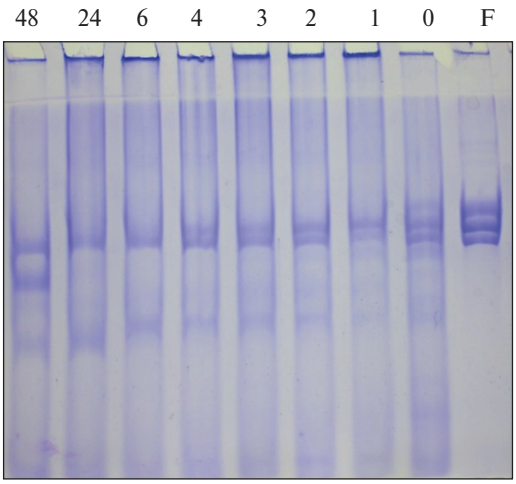

Figure 7. SDS-PAGE analysis of the fibrinogen after incubation with *Odontaster validus* fibrino(geno)lytic enzyme: F — the intact fibrinogen; 0–48 — hrs of incubation. Fibrinogen (1 mg/mL) and fibrino(geno)lytic enzyme were mixed at 1 : 10 (w/w) ratio. The mixture was incubated at 37 °C. Aliquots were taken at 0, 1, 2, 3, 5, 24, 48 hrs and boiled with β-mercaptoethanol for 1 min. Then the samples were mixed with equal volume of a sample buffer (62.5 mM Tris-HCl, pH 8.3, 2% SDS, 5% sucrose, and 0.02% bromophenol blue). The reaction products were analyzed by 10% SDS-PAGE. Electrophoresis was carried out at 39 mA per gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. Gels were stained with Coomassie Blue G-250

rugatus indicates low activity of fibrinogenolytic enzyme from this hydrobiont. Fig. 8 demonstrates the absence of pronounced specificity of this fibrino(geno)-lytic enzyme to any of the fibrinogen chains. Thus, the presence of fibrinogen Aα-chain was detected after one hour of incubation as well as at a distant time (4, 6 hours) of incubation. Despite the rather low enzyme activity, some Aα-chain cleavage still occurred, since the intensity of the band that corresponds to this chain slightly decreased during the incubation. Only the trace amounts of fibrinogen Aα-chain were revealed after 24 hours of incubation with fibrino-(geno)lytic enzyme from *P. corrugatus*.

It is important to mention that the incubation of fibrinogen with fibrino(geno)lytic enzymes from tissues of the studied hydrobionts was not accompanied by formation of a fibrin clot either at the initial stages of the study or after the 24 hours of incubation. So, we can state that the action of enzymes is not associated with the release of fibrinopeptides A and/or B from fibrinogen molecule required to initiate the process of fibrin polymerization and clot formation.

Thus, summarizing the results, we can conclude that the fibrino(geno)lytic enzymes from the tissues of the three Antarctic hydrobionts significantly differ in their ability to cleave fibrinogen chains.

It is generally known that fibrino(geno)lytic enzymes isolated from the snake venoms are able to cleave proteins involved in the coagulation step of the hemostasis system, thus causing the bleeding (Gardiner, Andrews, 2008). To study whether the fibrino(geno)-lytic enzymes from the tissues of the Antarctic hydrobionts can affect the blood coagulation system, the chronometric tests which are widely used in clinic were performed. The most common tests are activated partial thromboplastin time (APTT), prothrombin time

(PT), and thrombin time (TT). Table 1 summarizes the effects of the fibrino(geno)lytic enzymes from the tissues of the Antarctic hydrobionts on the time of clotting plug formation. As shown by the data, the incubation of plasma with fibrino(geno)lytic enzymes from *S. neumayeri* and *O. validus* tissues caused a significant prolongation of plasma clotting times.

Coagulation tests were performed using a coagulation analyzer RT-2201C and the corresponding commercial kits. The final concentration of the fibrino(geno)lytic enzyme was 1 mg per 1 mL of plasma. Plasma incubated for 5 min with TBS, pH 7.4 (instead of the fibrino(geno)lytic enzyme) was used as the control. All coagulation tests were performed in triplicate using plasma from three different rabbits.

APTT was used to evaluate the coagulation factors such as XII, IX, XI, VIII, X, V, prothrombin, and fibrinogen in the intrinsic coagulation pathway. The prolongation of APTT clotting time from 18.6 ± 2.6 s in the control to 55.8 ± 8.3 s and to 31.9 ± 11.2 s was observed during the incubation of plasma with the fibrino(geno)lytic enzyme from *S. neumayeri* and *O. validus* tissues, respectively. We failed to record any statistically significant changes of APTT while incubation of plasma with the fibrino(geno)lytic enzyme from *P. corrugatus*. It is commonly recognized that the prolongation of APTT clotting time indicates the development of a hypocoagulation state, which may be related to deficiencies or abnormalities of the factors of the intrinsic coagulation pathway, as well as the factors of the common pathway particularly those involved in the prothrombinase complex activation. On the next step of our research PT was measured, which 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. According to the results, only the fibrino(geno)lytic enzyme from *S. neumayeri* prolonged the time of clotting plug formation in this test to 9.2 ± 0.3 *vs* 7.2 ± 0.3 in control. TT measures the time required to fibrinogen to form fibrin strands in the presence of thrombin. This test only reveals disturbances in the final stages of coagulation. The fibrino(geno)lytic enzymes from *S. neumayeri*

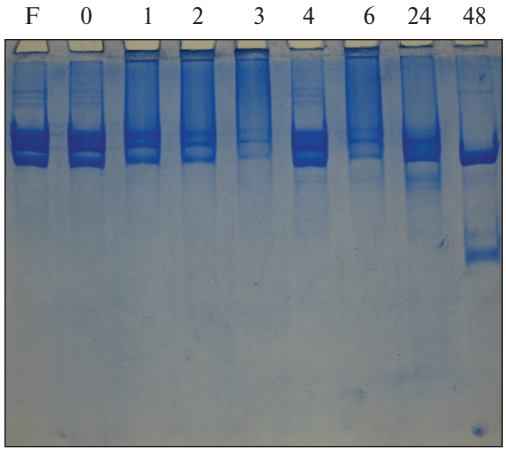

Figure 8. SDS-PAGE analysis of the fibrinogen after incubation with *Parborlasia corrugatus* fibrino(geno)lytic enzyme: F — the intact fibrinogen; 0–48 — hrs of incubation. Fibrinogen (1 mg/mL) and fibrino(geno)lytic enzyme were mixed at 1 : 10 (w/w) ratio. The mixture was incubated at 37 °C. Aliquots were taken at 0, 1, 2, 3, 5, 24, 48 hrs and boiled with β-mercaptoethanol for 1 min. Then the samples were mixed with equal volumes of buffer (62.5 mM Tris-HCl, pH 8.3, 2% SDS, 5% sucrose and 0.02% bromophenol blue). The reaction products were analyzed by 10% SDS-PAGE. Electrophoresis was carried out at 39 mA per gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. Gels were stained with Coomassie Blue G-250

Table 1. The time of clotting plug formation of blood plasma in the coagulation tests after incubation with the fibrino(geno)lytic enzymes from Antarctic hydrobionts (M ± m, n = 6)

Test name	Control	*S. neumayeri*	*O. validus*	*P. corrugatus*
APTT, s	18.6 ± 2.6	55.8 ± 8.3*	31.9 ± 11.2*	20.8 ± 3.1
PT, s	7.2 ± 0.3	9.2 ± 0.3*	7.1 ± 0.2	6.8 ± 0.5
TT, s	28.7 ± 0.7	49.1 ± 1.6*	34.3 ± 0.3*	30.9 ± 1.6

* – $p \leq 0.05$ the difference is comparable to the control.

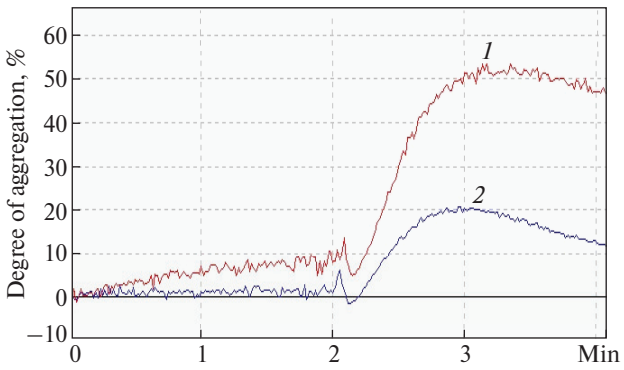

Figure 9. Effect of *Sterechinus neumayeri* fibrino(geno)lytic enzyme on platelet aggregation in platelet-rich plasma: *1* — the control sample; *2* — the fibrino(geno)lytic enzyme. Platelet-rich plasma was pre-incubated at 37 °C with sample of the fibrinogenolytic enzyme at final concentration 200 μg per 1 mL of plasma. After two minutes, the aggregation was initiated by the addition of ADP ($5 \cdot 10^{-6}$ M) and the aggregation process was monitored for 10 min

and *O. validus* significantly prolonged the clotting time in TT test to 49.1 ± 1.6 and 34.3 ± 0.3, respectively, *vs* 7.2 ± 0.3 in control.

The comparison of the coagulation tests results showed that the fibrino(geno)lytic enzymes might act on the common pathway, in particular, the conversion of fibrinogen into fibrin. Moreover, the prolongation of the clotting time formation in all tests may be due to the fibrinogen deficiency. Taking into account the ability of fibrino(geno)lytic enzymes from tissues of *S. neumayeri* and *O. validus* to cleave fibrinogen chains that we showed in our study, the results we obtained are quite regular and can be explained by the reducing of functionally active fibrinogen concentration.

Table 2. The effect of the fibrino(geno)lytic enzymes from Antarctic hydrobionts on the ADP-induced platelet aggregation (M ± m, n = 6)

Hydrobiont	Aggregation degree (%)	Inhibition degree (%)
Control	51.00 ± 5.00	—
S. neumayeri	20.00 ± 3.00*	61.00
O. validus	31.00 ± 3.00*	40.00
P. corrugatus	38.00 ± 4.00*	26.00

* — $p \le 0.05$ the difference compared to the control.

The next part of the research was aimed at investigating the potential effects of the fibrino(geno)lytic enzymes of the studied hydrobionts on ADP-induced platelet aggregation. The results illustrated in Fig. 9–11 indicate the ability of fibrino(geno)lytic enzymes to inhibit the process of platelet aggregation. As demonstrated by the data in Table 2, the *S. neumayeri* fibrino-(geno)lytic enzyme turned to be the most effective. Thus, the degree of aggregation after the incubation of PRP with the enzyme was 20%, compared with the same in control that was 51%.

The inhibition of ADP-induced platelet aggregation was also observed while incubation with *O. validus* fibrino(geno)lytic enzyme. The level of aggregation in this experiment was 1.64 times lower compared with the control values. Such results correlate with the ability of enzymes from the tissues of Antarctic hydrobionts to cleave Aα-chains of fibrinogen that we proved in the previous stage of work. Furthermore, the prior studies suggest that the ability to suppress platelet aggregation is typical mainly of α-fibrinogenases, which are specific to the Aα-chain of fibrinogen.

P. corrugatus fibrino(geno)lytic enzyme appeared to be the least effective. The degree of ADP-induced platelet aggregation during the incubation of this enzyme with PRP was 38%, which indicates the inhibition of this process by 26%.

The inhibition of the ADP-induced platelet aggregation during the incubation of PRP with fibrino-(geno)lytic enzymes on the one hand may be due to the cleavage of fibrinogen, which is an important cofactor of this process, and on the other, due to the blocking of the platelet receptor in the result of the accumulation of fibrinogen degradation products or as a result of the direct interaction of the enzyme with platelet receptors (Laraba-Djebari, Chérifi, 2014; Sanchez et al., 2017).

To further characterize the obtained fibrino(geno)-lytic enzymes the substrate analysis was performed using synthetic substrates, which are widely used in the study of the effects of the hemostasis system enzymes. The results demonstrated in Table 3 indicate

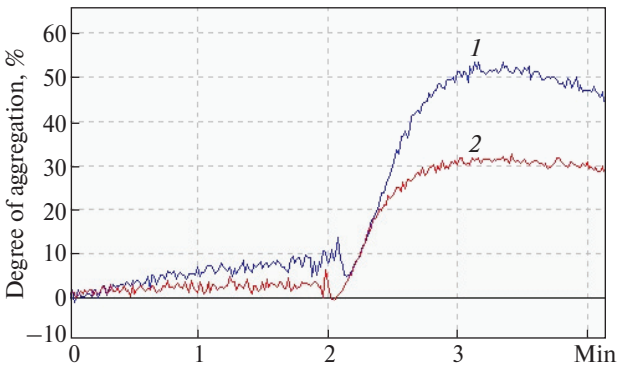

Figure 10. Effect of *Odontaster validus* fibrino(geno)lytic enzyme on platelet aggregation in platelet-rich plasma: *1* — the control sample; *2* — the fibrino(geno)lytic enzyme. Platelet-rich plasma was pre-incubated at 37 °C with sample of the fibrinogenolytic enzyme at final concentration 200 μg per 1 mL of plasma. After two minutes, the aggregation was initiated by the addition of ADP ($5 \cdot 10^{-6}$ M) and the aggregation process was monitored for 10 min

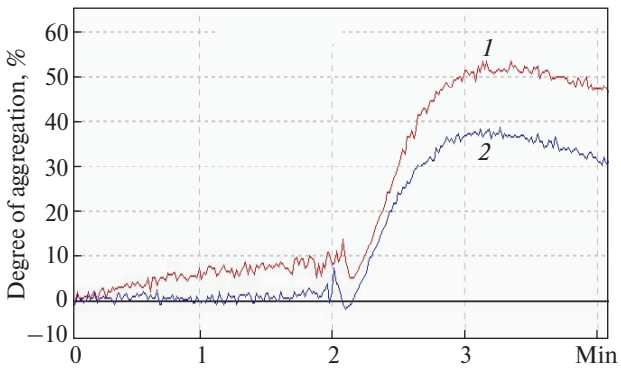

Figure 11. Effect of *Parborlasia corrugatus* fibrino(geno)lytic enzyme on platelet aggregation in platelet-rich plasma: *1* — the control sample; *2* — the fibrino(geno)lytic enzyme. Platelet-rich plasma was pre-incubated at 37 °C with sample of the fibrinogenolytic enzyme at final concentration 200 μg per 1 mL of plasma. After two minutes, the aggregation was initiated by the addition of ADP (5×10^{-6} M) and the aggregation process was monitored for 10 min

that the studied fibrino(geno)lytic enzymes showed different activity against all the applied substrates.

The fibrino(geno)lytic enzyme (20 μg per 250 μL of incubation medium) was incubated in TBS, pH 7.4 for 5 min at 37 °C. The reaction was initiated by addition of chromogenic substrate (with the final concentration of 0.3 mM). The change in absorbance was recorded for 60 min at a 405 nm. The activity of enzyme was calculated using a molar extinction coefficient of *p*-nitroaniline — 8800 $M^{-1} \cdot cm^{-1}$.

As shown by the data, the *S. neumayeri* fibrino(geno)lytic enzyme appeared to be the most active. Its highest activity was recorded while using the substrates containing the Arg residue in P1-position compared with the activity against the substrate which contained the Lys residue. Thus, the activity of the enzyme from *S. neumayeri* while using the pyroGlu-Pro-Arg-*p*NA substrate was 1.85 times higher than when using the H-D-Val-Leu-Lys-*p*NA substrate.

The efficiency of the chromogenic substrates hydrolysis by the *O. validus* fibrino(geno)lytic enzyme was reduced in a number of substrates, such as pyroGlu-Pro-Arg-*p*NA, H-D-Phe-Pip-Arg-*p*NA, H-D-Val-Leu-Lys-*p*NA, Bz-Ile-Glu(γ-OR)-Gly-Arg-*p*NA.

It should be noted that the fibrino(geno)lytic enzyme obtained from the *P. corrugatus* tissues turned to be the least effective. This result correlates with our previous findings which showed the lower activity

Table 3. The activity of fibrino(geno)lytic enzymes obtained from the tissues of Antarctic hydrobionts against synthetic chromogenic substrates (M ± m, n = 6)

Hydrobiont	Enzyme activity, μmol *p*NA · min^{-1} · mg $protein^{-1}$			
	pyroGlu-Pro-Arg-*p*NA	H-D-Phe-Pip-Arg-*p*NA	H-D-Val-Leu-Lys-*p*NA	Bz-Ile-Glu(γ-OR)-Gly-Arg-*p*NA
S. neumayeri	3.31 ± 0.064	2.94 ± 0.028	1.78 ± 0.035	1.96 ± 0.057
O. validus	1.93 ± 0.024	1.78 ± 0.019	1.53 ± 0.035	1.42 ± 0.017
P. corrugatus	1.78 ± 0.025	0.87 ± 0.015	1.05 ± 0.017	1.24 ± 0.027

of this enzyme to fibrinogen, its ability to affect the plasma clotting time in chronometric tests and its effect on platelet aggregation.

4 Conclusion

In the current study the purification procedure, which included affinity chromatography on Blue Sepharose column followed by size-exclusion chromatography on Superdex 200 PG column was described to isolate the fibrino(geno)lytic enzymes from the tissues of Antarctic hydrobionts. Based on the results of SDS-PAGE and zymography assay the molecular weight of the fibrino(geno)lytic enzymes was in the range of 26–34 kDa. The enzymes showed a strong fibrino-(geno)lytic activity, cleaving preferentially the Aα-chain and more slowly the Bβ-chain of fibrinogen. The fibrino(geno)lytic enzymes caused the significant prolongation of blood clotting time in APTT, PT, and TT tests and mediated the inhibition of ADP-induced platelet aggregation. They also actively cleaved several synthetic colored peptide substrates. The activity was more expressed in case of using pyroGlu-Pro-Arg-*p*NA — a specific substrate for activated protein C and factor XIa. Considering the obtained results, further studies are required to provide a more detailed characteristic of the fibrino-(geno)lytic enzymes.

Author contributions. NR carried out the experiments; wrote the article; IU, TV carried out the experiments; TH drew up the manuscript; OS designed the experiment and interpreted the data; LO supervised the research. All authors have read and agreed with the published version of the manuscript.

Competing interests. The authors declare that they have no conflict of interest.

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Очищення та біохімічна характеристика фібрино(гено)літичних ферментів з тканин антарктичних гідробіонтів

Реферат. Беручи до уваги невпинне зростання поширеності серцево-судинних захворювань, пошук сполук та розробка нових ефективних лікарських засобів, здатних впливати на систему гемостазу, належать до актуальних напрямків сучасної науки та фармакології. Фібрино(гено)літичні ферменти можуть бути потенційно перспективними як профілактичні засоби розвитку ускладнень, обумовлених активацією прокоагулянтної ланки, а також при терапії захворювань, що супроводжуються надмірним тромбоутворенням. Метою дослідження було розробити методологічні підходи щодо одержання фібрино(гено)літичних ферментів з тканин гідробіонтів Антарктичного регіону та дослідити їх вплив на систему гемостазу. Схема одержання ферментів включала декілька хроматографічних етапів — афінну хроматографію та хроматографію, що поділяє за розмірами. Наявність ферментів, здатних розщеплювати фібриноген, оцінювали методом ензим-електрофорезу. Специфічність фібрино(гено)літичних ферментів щодо ланцюгів фібриногену визначили методом електрофорезу у поліакриламідному гелі після інкубації одержаних ферментів з фібриногеном. Для оцінки субстратної специфічності ферментів було використано хромогенні субстрати H-D-Phe-Pip-Arg-*p*NA, pyroGlu-Pro-Arg-*p*NA, H-D-Val-Leu-Lys-*p*NA та Bz-IIe-Glu(γ-OR)-Gly-Arg-*p*NA. Також було досліджено вплив фібрино(гено)літичних ферментів з тканин гідробіонтів Антарктичного регіону на АДФ-індуковану агрегацію тромбоцитів та на час зсідання плазми крові у хронометричних тестах. Отримані результати доводять присутність у тканинах досліджуваних гідробіонтів фібрино(гено)літичних ферментів. З огляду на результати електрофоретичного аналізу молекулярна маса одержаних ферментів знаходиться у межах 26–34 кДа. Здатність фібрино(гено)літичних ферментів розщеплювати у першу чергу Aα-ланцюг молекули фібриногену опосередковано свідчить про їх належність до α-фібриногеназ. При тривалій інкубації ферментів з фібриногеном спостерігалося розщеплення також і Bβ-ланцюга. Одержані нами фібрино(гено)літичні ферменти обумовлювали подовження часу зсідання плазми крові у базових хронометричних тестах та пригнічували агрегацію тромбоцитів. Відповідно до результатів субстратного аналізу, фібрино(гено)літичні ферменти виявляли найвищу активність щодо pyroGlu-Pro-Arg-*p*NA, який є специфічним субстратом для протеїну С та активованого фактору XI. Проведені дослідження свідчать про перспективність використання гідробіонтів Антарктичного регіону як потенційного джерела для одержання фібрино(гено)літичних ферментів.

Ключові слова: фібрино(гено)літичні ферменти, система гемостазу, гідробіонти