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Original Article

Isolation of collagenolytic enzymes from general skin secretions of *Bombina variegata*: Methodological approaches for protocol optimization

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ABSTRACT

Amphibian skin secretions are rich sources of biomolecules with diverse biotechnological applications. In this study, the general skin secretions of *Bombina variegata* is suggested as an alternative source of collagenolytic enzymes applied in different areas of industry such as the pharmaceutical, textile and leather sectors. Collagenolytic proteases were partially purified from skin secretions by ion-exchange chromatography on a DEAE-Sepharose column and several methodological approaches were tested to optimize the purification protocol. The salt concentration, and pH of mobile phase were varied to determine their effects on the resolution of the collagenolytic enzyme separation. Thus, time and resolution of purification were optimized yielding the required purity and amounts of active enzymes that can be useful for several research areas and biotechnology.

KEYWORDS: amphibian skin secretions, collagenolytic enzymes, purification, ion-exchange chromatography.

INTRODUCTION

Recently, at a time of scientific and technological progress, there has been a spate of interest concerning the use of biologically active compounds of natural origin in the various sectors of medicine and industrial activities. A great number of studies conducted in the past several decades have illustrated the considerable importance of enzymes isolated from natural raw materials in pharmaceutical and biotechnological scenarios [1, 2]. The use of such enzymes has been extended to the cosmetics industry and many other fields, such as meat production, dairy industry, brewing, along with leather and fur manufacturing [3-5].

Collagenolytic enzymes are generally recognized to be promising target molecules of both practical and scientific interest. These proteases are used as non-toxic and eco-friendly biocatalysts facilitating the collagen degradation, and have a wide application spectrum with high biotechnological potential [6].

Microorganisms represent a large group of living beings which for a long time have occupied a central place in the technology of collagenolytic enzymes' production and isolation. Historically, the first collagenases were isolated from *Clostridium histolyticum* [7]. Later, several studies illustrated the isolation of collagenases from various representatives of the genus Bacillus (e.g., *Bacillus pumilus* [8], *Bacillus cereus* [9], etc.), Streptomyces (e.g., *Streptomyces exfoliate* [10]), and Vibrio (e.g., *Vibrio alginolyticus*,

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Vibrio vulnificus [11]). Fungi appear to be another important source of collagenolytic enzymes. Thus, a few studies reported on the collagenase isolation from some representatives of the genus Penicillium (*Penicillium sp*. [12]), a mosquito's parasitic fungus *Lagenidium giganteum* [13], and *Aspergillus tamarii* [14]. Recently, due to the popularization of the concept of waste-free living, another source of collagenolytic enzymes has become in demand, namely the fish processing waste. Thus, several studies have proved that the organic solid residues produced by the fish processing industry are a promising source of biomolecules with diverse biotechnological applications, including collagenolytic enzymes [15]. Thus, a considerable number of raw materials for collagenolytic enzyme isolation is commercially available, but due to the high cost often involved in their production and purification, the search for new promising natural sources is underway. Furthermore, collagenases derived from representatives of different taxonomic categories are recognized to be interesting objects in terms of finding specific enzymes with unique properties, such as ability to work in extreme pH, thermal stability, inhibitor resistance, etc.

The results of our recent studies have proven the presence of active collagenolytic enzymes in the skin secretions of some representatives of the European batrachofauna [16]. Our findings showed the pronounced collagenolytic potential of the components of *Bombina variegata* general skin secretions. Although there have been a few studies that have reported on the isolation of collagenolytic enzymes from the amphibian tadpoles' tails [17, 18], to date, the skin secretions of amphibians remain insufficiently studied as a source for collagenases isolation.

In this context, the present study aimed to optimize the chromatographic approach for purification of proteases, capable to cleave the collagen, from the *B. variegata* general skin secretions as well as to investigate these enzymes' properties for future research and biomedical applications.

MATERIALS AND METHODS

Materials

Protein calibration mixture that contained phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), and lysozyme (14 kDa) were purchased from Bio Rad, USA. DEAE-Sepharose was purchased from GE Healthcare, USA. Commercial kit for Bradford microplate assay was purchased from BioRad, USA. Phenylmethylsulphonyl fluoride (PMSF), a serine-protease inhibitor; benzamidine, a trypsin inhibitor; soybean trypsin inhibitor (STI); iodoacetamide, a cysteine peptidase inhibitor; and thylenediamine tetra-acetic acid (EDTA) were purchased from Sigma-Aldrich Inc., USA. Chromogenic peptide substrates; Phe-Pip-Arg-*p*NA (S_{2238}), pyroGlu-Pro-Arg-*p* NA (S_{2366}), Val-Leu-Lys-*p*NA (S_{2251}) and Bz-IIe-Glu-Gly-Arg-*p*NA (S_{2222}) were purchased from RENAM, Russian Federation.

Methods

Collection of amphibian skin secretions: Adult specimens of yellow-bellied toad *B. variegata* of both sexes (n = 30; weight range 30-50 g) were collected from the Transcarpathian region of Ukraine. Skin secretions were collected as follows: toads were put into a Petri dish. The dorsal surface of the toads was mechanically stimulated with fingers. After being massaged for 1-2 min, toad skin surface was seen to exude copious secretions. Skin secretions were collected by washing the dorsal region of each toad with ultra-pure water (5 mL of total volume). The water suspensions of skin secretions were quickly centrifuged at 2500 g for 15 min to remove debris. The supernatants were lyophilized (Telstar LyoQuest) and kept at 4 °C till use. All the experimental protocols were approved by the local institutional committee for animal ethics and were performed in accordance with the international rules on animal rights (Directive 2010/63/EU).

Chromatographic separation of general skin secretions: Lyophilized skin secretions of *B. variegata* (25 mg of dried material) were dissolved in 1 mL of 20 mM Tris-HCl buffer, containing 0.36 mM $CaCl_2$ and centrifuged at 3000 g for 10 min. Then 1 mL sample of the supernatant with total protein concentration of 3.8 mg/ml was applied to a DEAE-Sepharose column (1 × 1 cm) attached to a Bio-Rad BioLogic LP chromatography system. A stepwise

NaCl gradient elution was carried out between buffer A (20 mM Tris-HCl, 0.36 mM $CaCl_2$) and buffer B (20 mM Tris HCl, 0.36 mM $CaCl_2$, with 1 M NaCl), at a flow rate of 2.0 mL min. The column eluate was monitored with a UV detector set at 280 nm. The stationary phase, column temperature, injection volume and flow rate were kept unchanged during analysis. Mobile phase pH and NaCl gradient profile were varied to observe the impact on peak shape, resolution and retention time, in order to optimize the chromatographic conditions.

Protein determination: Protein concentration in the samples was determined using commercial kit for Bradford microplate assay and bovine serum albumin as a standard.

SDS-Polyacrylamide gel electrophoresis: Protein samples for SDS-PAGE were concentrated as follows: the aliquots of the flow-through fractions were mixed with equal volume of 25% trichloroacetic acid and centrifuged for 5 min at 10,000 g. The precipitated protein pellets were washed twice with acetone, and dried. After that, the protein precipitates were mixed with the SDS-PAGE sample buffer that contained 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% sucrose, and 0.002% bromophenol blue, without heating.

SDS-PAGE was performed according to Laemmli (1970), using Mini-Protean Tetra vertical electrophoresis system (Bio Rad, USA). Fraction samples with equal amount of total protein (20 µg) were applied in 15 µL volume per line. Gels were stained with 2.5% coomassie brilliant blue G-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, and 15 % (v/v) isopropanol. Gels destaining was carried out using 7% (v/v) acetic acid for 30 min. The apparent molecular weights of the separated proteins were estimated using the protein calibration mixture that contained phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), and lysozyme (14 kDa).

Zymography: Zymography (substrate SDS-PAGE) was performed according to the method suggested by Ostapchenko *et al*. (2011). Separating gel (12% w/v) was polymerized in the presence of collagen (1 mg/ml), gelatin (1 mg/mL) or fibrinogen (1 mg/mL). Fraction 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. 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, and 15% (v/v) isopropanol for 30 min. Areas of substrate digestion were visualized as clear patches on dark background.

Characterization of some properties of the purified collagenolytic enzyme fraction

Hydrolysis of chromogenic substrates: Substrate specificity of collagenolytic enzymes was determined using chromogenic peptide substrates such as Phe-Pip-Arg-*p*NA (S_{2238}), pyroGlu-Pro-Arg-*p* NA (S_{2366}), Val-Leu-Lys-*p*NA (S_{2251}) and Bz-IIe-Glu-Gly-Arg-*p*NA (S_{2222}).

The enzyme activity was studied by mixing 50 μL of the purified enzymes or *B. variegata* general skin secretion sample (20 μg of protein in 20 mM Tris-HCl buffer, pH 10, containing 0.36 мМ $CaCl_2$) with 175 μL of 20 mM Tris-HCl buffer, pH 10, containing 0.36 mM $CaCl_2$. The reaction was initiated by adding 25 μL of the corresponding specific chromogenic substrate in a final concentration of 3 mM. The control sample contained the same components but with an equal volume of buffer instead of the corresponding samples. Incubation was performed at 37 °C for 1 hour. The optical density of the samples at the wavelength 405 nm was determined at equal intervals of time using the microplate reader µQuant (BioTek). The amount of released *p*-nitroaniline (*p*NA) was calculated using molar extinction coefficient for free *p*NA (8,800 $M^{-1} \times cm^{-1}$). In each experiment ($n = 5$) all samples were tested in triplicate.

Effect of inhibitors: The sensitivity of the collagenolytic enzymes to inhibitors was evaluated by using phenylmethylsulphonyl fluoride (PMSF), a serine-protease inhibitor; benzamidine, a trypsin inhibitor; soybean trypsin inhibitor (STI); iodoacetamide, a cysteine peptidase inhibitor; and ethylenediamine tetra-acetic acid (EDTA),

a chelating compound at final concentrations of 2 мМ, 20 мМ, 1 mg/mL, 1 мМ, and 5 мМ, respectively. Each inhibitor was incubated with a sample of purified enzymes for 15 min at 37 °C, and then the activity was performed as described above using only two chromogenic substrates: Phe-Pip-Arg-*p*NA (S_{2238}) and pyroGlu-Pro-Arg-*p*NA (S_{2366}). Control activity was performed in the absence of inhibitors. The results were expressed as percentage of the control activity.

Statistical analysis: 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. The data from three replicated experiments are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA). followed by a Bonferroni's test. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

The raw materials of natural origin are recognized to be valuable sources of collagenolytic enzymes. Regarding this, the development of innovative technologies to produce and modify natural collagen-containing raw material which would be further applied in various technologies is as relevant as essential as ever.

Amphibians' dermal secretions and their components are widely implemented in medical and pharmaceutical industries [19]; the specific properties of biologically active compounds from skin secretions continue to be studied, and their scope is expanding. Our previous reports have been focused on the results of the preliminary study of amphibian general skin secretions as the potential source of proteolytic enzymes including collagen-degrading enzymes. In this paper, the purification procedure of the collagenolytic enzymes from the skin secretions of *B. variegata* is described. In addition, some properties of the enzymes were studied, and subsequently compared with those of other known collagenases.

The general skin secretions of *B. variegata* were fractionated by ion-exchange chromatography on a DEAE-Sepharose column. In the process of optimization of the set chromatographic conditions, it is important to take into account the properties of the stationary phase used.

It is well known that the pH of the mobile phase needs to be carefully controlled during routine analysis to maintain robust separation conditions. On the other hand, it can be an extremely powerful tool to increase the purification resolution during chromatographic method development. In this study, three mobile phases with the same composition but different pH values (pH 8, 9, and 10) were tested to select the optimal pH. A stepwise NaCl gradient elution was carried out between buffer A (20 mM Tris-HCl, 0.36 mM $CaCl_2$) and buffer B (20 mM Tris HCl, 0.36 mM $CaCl_2$, with 1M NaCl), at a flow rate of 2.0 ml min. The gradient profile was: 0-20 mins-100% A; 20-35 mins-95% A to 5% B; 35-50 mins-90% A to 10% B; 50-65 mins-85% A to 15% B; 65-80 mins-80% A to 20% B; 80-95 mins-75% A to 25% B; 95-110 mins-70% A to 30% B; 110-125 mins-65% A to 35% B; 125-145 mins-100% B. The appearance of peaks was monitored, and the corresponding fractions were collected in the separate tubes (Figure 1).

Zymography with collagen as a substrate protein was conducted to identify the target enzymes in the flow-through fractions. Resultant zymograms illustrate the appearance of light hydrolysis zones due to the manifestation of collagenolytic activity, which is the evidence of the presence of proteolytic enzymes with collagen specificity in the corresponding flow-through fractions of *B. variegata* skin secretions.

According to our results, in case of using mobile phases with pH 8 and 9 (Figure 2, A and B), the appearance of hydrolysis zones was observed in several lanes of zymograms (substrate SDS-PAGE) showing limited separation and poor resolution at these conditions. Significant improvement was observed at pH 10 (Figure 2, C). As could be seen, in this case all hydrolysis zones were concentrated within a single lane #3 which corresponds to the protein fraction yield at 0.15 M NaCl in the working buffer. Our results indicate that mobile phase with pH 10 is the optimal variant for the working buffer, as the concentration of the target proteins within a single peak simplifies further chromatographic separation.

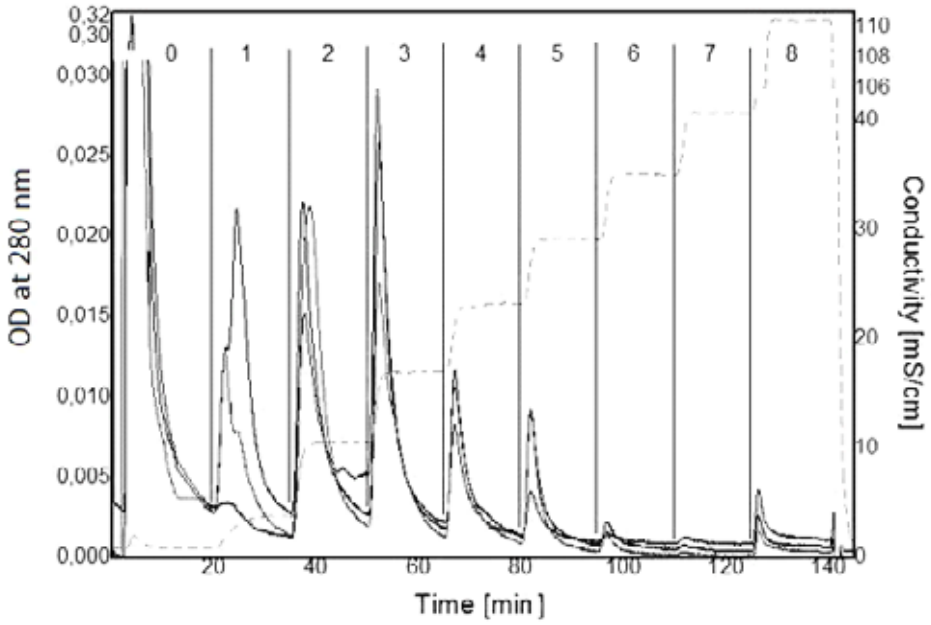

Figure 1. Fractionation of *B. variegata* general skin secretions by ion-exchange chromatography on a DEAE-Sepharose column. Conditions for chromatography shown in the figure are in the text. Three mobile phases with the same composition but different pH values (pH 8, 9, and 10) were tested. Elution was performed by a stepwise NaCl gradient (------). The chromatograms depicted show the absorbance of the eluted material at 280 nm (——). Numbers from 0 to 8 indicate the protein fractions that were collected.

The amount of total protein applied to column was the same in each experiment and set at 3.8 mg. In case of using mobile phase with pH 10, the target activity was shown for the components of protein fraction #3, and the total protein content in this fraction was found to be 0.783 mg.

An important part of chromatographic separation process optimization is the reduction of time that is required to obtain the target protein. For this purpose, the number of elution gradient steps was shortened to three. The gradient profile was: 0-15 mins-90% A to 10% B; 15-30 mins-85% A to 15% B; 30-40 mins-100% B. As demonstrated by the Figure 3A, the time required for chromatographic separation of the target fraction was considerably reduced. The substrate SDS-PAGE with collagen as a substrate protein was conducted to evaluate the proteolytic potential of the flow-through fractions. As can be seen from Figure 3B, the target activity was revealed in the fraction #2 that corresponds to the proteins yielded at 0.15 M NaCl in the working buffer, and in the fraction #3, which corresponds to the proteins eluted by 1 M NaCl.

In order to minimize the potential loss of protein and to ensure its maximum concentration within one fraction, the salt concentration within the second gradient step was increased from 0.15 to 0.18 M NaCl in the initial buffer (Figure 4, A). To estimate the presence of proteolytic activities in the obtained fractions substrate gel electrophoresis with collagen was applied. No enzyme activity was detected in fraction #1 and #3 and two clear areas of hydrolysis were found at the electrophoregram of fraction #2 (Figure 4, B), suggesting the presence of active enzymes. Our results indicate that the increase in the elution gradient to 0.18 M NaCl allowed to effectively concentrate the target protein within a single fraction. To characterize the protein composition of the flow-through fractions SDS-PAGE was carried out. The SDS-PAGE analysis revealed that the target fraction #2 was not homogeneous and consisted of several protein bands with different molecular weights (Figure 4, C).

The obtained data clarify the relationship between the 3% increment of the NaCl gradient for elution of the target fraction and the increase in the

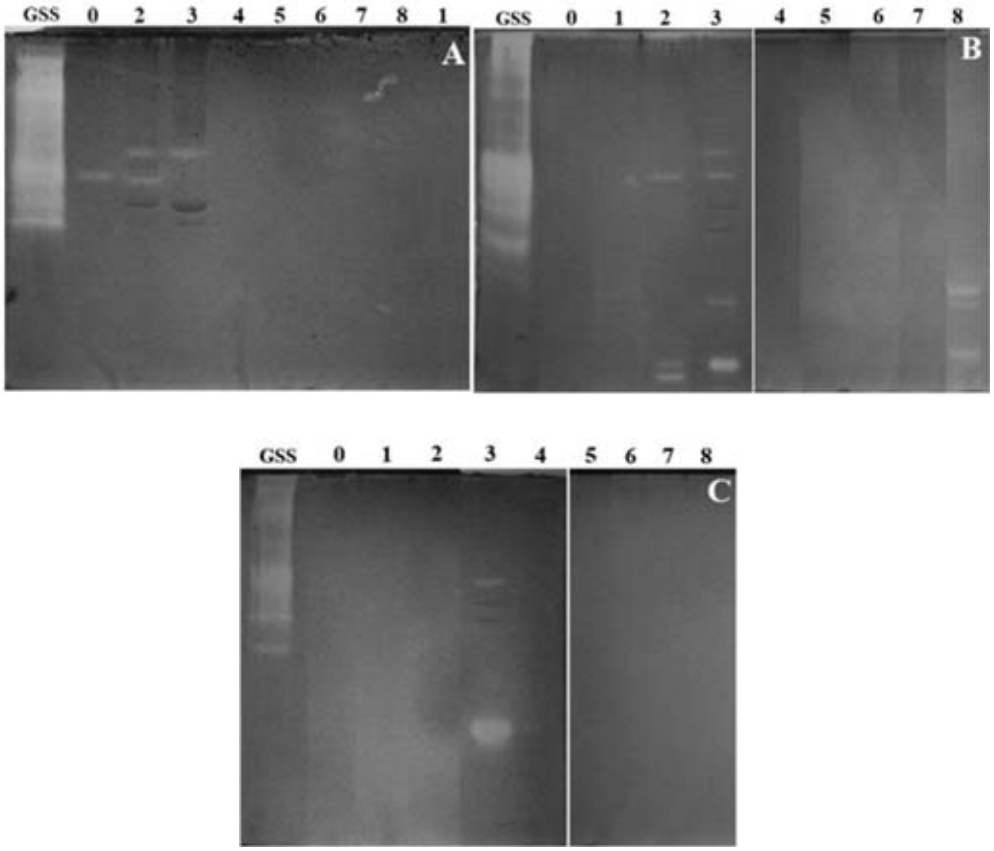

Figure 2. Zymograms (substrate-SDS-PAGE) of the fractions obtained in DEAE ion-exchange chromatography applied to general skin secretion of *B. variegate.* Lane: GSS – general skin secretion; lanes 0-8 – fractions washed out by stepwise NaCl gradient in buffer A (20 mM Tris-HCl, 0.36 mM $CaCl_2$) at pH 8 (A), 9 (B), and 10 (C).

amount of protein in the target fraction from 0.8 mg to 1.8 mg. Furthermore, these results conform with the data of electrophoretic studies and indicate that the target fraction, with the increased NaCl concentration in the elution buffer, contained not only the target proteins, the enzymes with collagenolytic activity, but also the other ballast proteins. Such results indicate the need for further purification, which can be realized, for example, by using other chromatographic approaches. In our opinion, the complete purification of target enzymes may be accomplished by a combination of ion-exchange chromatography followed be size exclusion chromatography.

Except the optimization of the fractionation conditions of the target enzyme, an equally important stage of research is the characterization of its activity. Therefore, the next step of our research was to study the specificity of enzymes in the flow-through fractions, and to identify their possible inhibitors.

To evaluate the substrate specificity of the active enzymes of the examined fraction #2 substrate-containing gel electrophoresis with collagen, gelatin, or fibrinogen was conducted. Figure 5 illustrates the proteolytic potential of the flow-through fraction #2 collected at 0.18 M NaCl in the working buffer. According to our results, the most pronounced zones of hydrolysis were observed while performing zymography using collagen as a substrate: the data indicate the presence of two hydrolysis zones. Three bands of slight intensity were identified at the electrophoregram with gelatin, but no hydrolysis zones were detected at the electrophoregram with fibrinogen. Such findings indicate the presence of the enzymes with collagenase and gelatinase

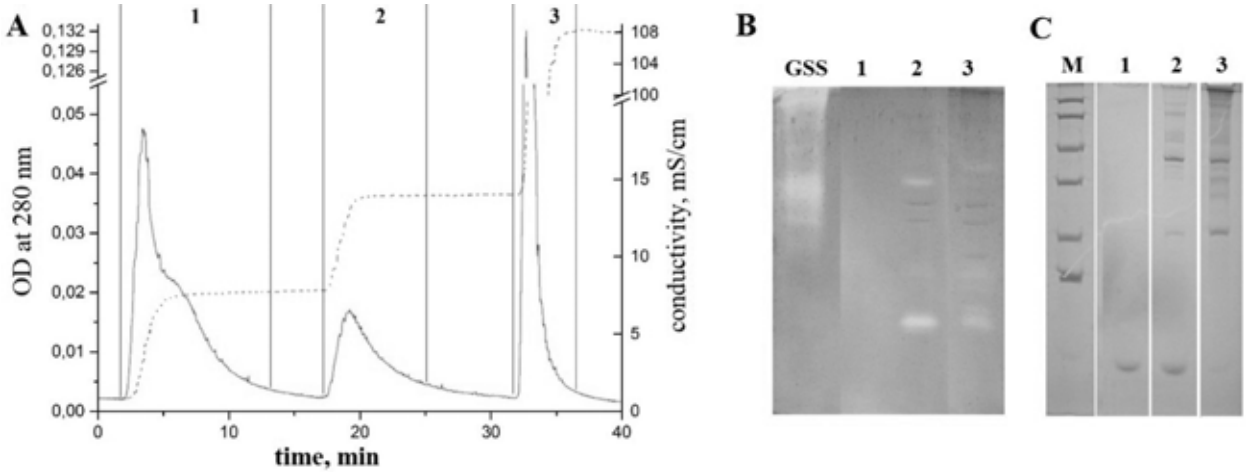

Figure 3. Fractionation of *B. variegata* general skin secretions (GSS) by ion-exchange chromatography on a DEAE-Sepharose column in 20 mM Tris-HCl buffer, pH 10, containing 0.36 мМ $CaCl_2$ (A); zymogram (substrate-SDS-PAGE) of the flow-through fractions (B); coomassie-stained SDS-PAGE gel of the flow-through fractions (C). Elution was performed by a stepwise NaCl gradient (------). The gradient profile was: 0.10, 0.15, and 1.00 M of NaCl in the initial buffer. The chromatograms depicted show the absorbance of the eluted material at 280 nm (——). Numbers from 1 to 3 indicate the protein fractions that were collected. Lane M – molecular weight markers (97, 66, 45, 31, 21, and 14 kDa).

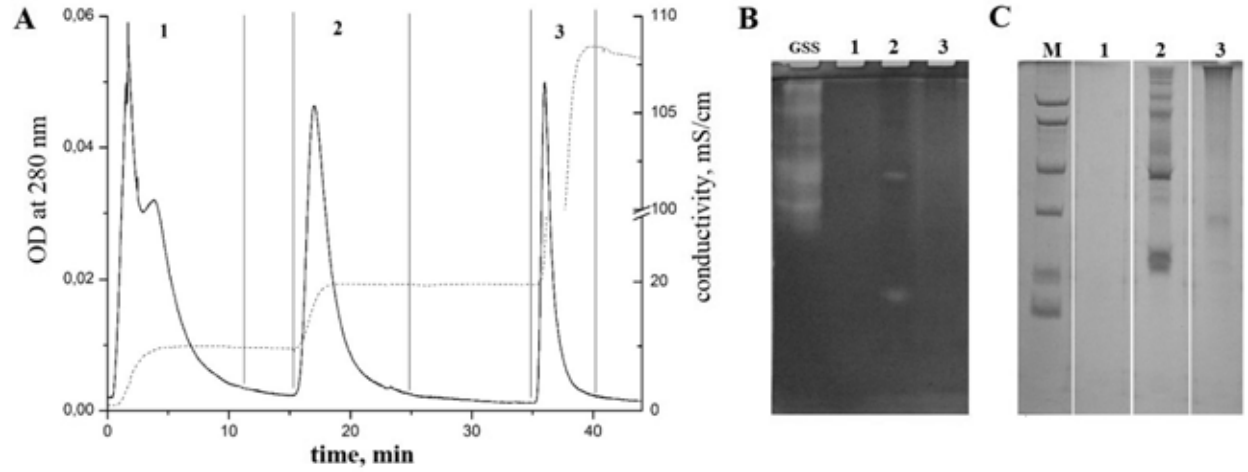

Figure 4. Fractionation of *B. variegata* general skin secretions (GSS) by ion-exchange chromatography on a DEAE-Sepharose column in 20 mM Tris-HCl buffer, pH 10, containing 0.36 мМ $CaCl_2$ (A); zymogram (substrate-SDS-PAGE) of the flow-through fractions (B); coomassie-stained SDS-PAGE gel of the flow-through fractions (C). Elution was performed by a stepwise NaCl gradient (------). The gradient profile was: 0.10, 0.18, and 1.00 M of NaCl in initial buffer. The chromatograms depicted show the absorbance of the eluted material at 280 nm (——). Numbers from 1 to 3 indicate the protein fractions that were collected. Lane M – molecular weight markers (97, 66, 45, 31, 21, and 14 kDa).

activities in the *B. variegata* skin secretions and suggest the feasibility of further studies aimed at purification and characterization of individual enzymes.

Next part of our study was to analyze some biochemical characteristics of the isolated enzymes. Firstly, we investigated the specificity of the enzymes towards some chromogenic substrates

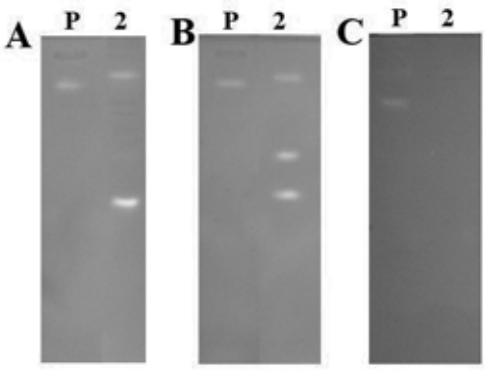

Figure 5. Zymograms (substrate-SDS-PAGEs) of the fraction #2 obtained in DEAE ion-exchange chromatography applied to general skin secretion of *B. variegate.* Polymerized proteins – collagen (A), gelatin (B), and fibrinogen (C). Lane P – plasmin (85 kDa); lane 2 – fraction #2.

that have a unique amino acid sequence, Phe-Pip-Arg-*p*NA, pyroGlu-Pro-Arg-*p*NA, Ile–Glu–Gly–Arg–*p*NA, and Val-Leu-Lys-*p*NA, which differ from each other by the presence of Arg and Lys residues at the P1-position of the substrate. This method is based on the measurement of free *p*-nitroaniline that is cleaved from a colorless synthetic substrate. Table 1 represents the results of this experiment. The data have shown a strong activity towards all the substrates containing Arg residue. The target enzymes from fraction #2 cleaved the substrate Phe–Pip–Arg–*p*NA with the highest efficiency. A relatively lower cleavage efficiency was observed while pyroGlu–Pro–Arg–*p*NA and Ile–Glu–Gly–Arg–*p*NA substrates were used. The lowest activity was observed for Val–Leu–Lys–*p*NA specific substrate. Based on these

Table 1. Proteolytic activity of the fraction #2 obtained in DEAE ion-exchange chromatography applied to general skin secretion of *B. variegate*.

Substrate	**Proteolytic activity (μmol *p*NA·min^{-1}·mg protein^{-1})**
Phe–Pip–Arg–*p*NA (S_{2238})	61.87 ± 2.59
pyroGlu–Pro–Arg–*p*NA (S_{2366})	45.00 ± 1.53
Bz-IIe-Glu-Gly-Arg-*p*NA (S_{2222})	36.87 ± 1.36
Val–Leu–Lys–*p*NA (S_{2251})	10.94 ± 0.58

Table 2. Effect of inhibitors on enzymatic activity of the fraction #2 obtained in DEAE ion-exchange chromatography applied to general skin secretion of *B. variegate*.

	Proteolytic activity (μmol *p*NA·min^{-1}·mg protein^{-1})	**% of inhibition[1]**
Basal activity	61.87 ± 2.59	-
+ SBTI (1 mg/mL)	12.43 ± 3.54*	80%
+ benzamidine (2 mM)	9.38 ± 2.07*	85%
+ PMSF (2 mM)	43.35 ± 3.21*	30%
+ EDTA (5 mM)	58.63 ± 3.14	*no inhibitory effect*
+ iodoacetamide (1 mM)	60.07 ± 1.87	*no inhibitory effect*

Values are expressed as Mean ± SD (n = 5); *p<0.05 significantly different from the basal activity.
Note: [1]The enzyme activity in the sample without inhibitor (basal activity) was taken as 100% (that corresponds the 0% of inhibition).

results the optimal chromogenic substrate for the next experiment was selected.

To determine the effect of inhibitors on the activity of the target fraction enzymes the substrate Phe–Pip–Arg–*p*NA was used. In this experiment the percentage of inhibition by various class-specific inhibitors (SBTI, PMSF, benzamidine, iodoacetamide, and EDTA) was determined. Table 2 shows the results of this experiment. The data indicate the highest inhibitory effect under the action of SBTI, benzamidine and PMSF, which are specific inhibitors of serine proteases. Only 15-20% of the remaining activity was revealed after the incubation of enzymes from fraction #2 with benzamidine and SBTI. The enzyme activity was inhibited by almost 30% while applying 2 mM PMSF and was insensitive to EDTA and iodoacetamide. These results indicate that enzymes in fraction #2 belong to serine proteases.

CONCLUSION

Nowadays, environmentally friendly manufacturing along with safe product yield has led to the gradual replacement of chemical methods of production by methodological approaches using biological catalysts – enzymes. Due to a great demand for highly purified active enzymes that will find their application in medicine, pharmacy and various other industries, the isolation of such enzymes and the development of optimal methods for their screening and purification are important areas of modern researches. In the current study the purification procedure as well as some biochemical characteristics of the collagenolytic enzymes from *Bombina variegata* general skin secretions were elaborated. Although, it was determined that isolated enzymes belong to the class of serine proteases, further studies are required to provide more detailed biochemical characteristics of their action.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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