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Collagenolytic enzymes from Antarctic hydrobionts: a promising biotechnological approach for accelerating purulent-necrotic wound healing

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Abstract. The objective of this study was to isolate the fractions of proteolytic enzymes from Antarctic hydrobionts, specifically *Sterechinus neumayeri* and *Odontaster validus*, and assess their wound-healing potential on purulent-necrotic wounds in rats. The purification procedure included ammonium sulfate precipitation and ion-exchange chromatography on Q-Sepharose. The enzymes derived from both hydrobionts exhibited significant collagenolytic activity, averaging around 14 rel. units per mg of total proteins, and were different in molecular weight and substrate specificity. Enzyme-electrophoresis with gelatin as a substrate revealed that enzymes from *S. neumayeri* were mostly above 40 kDa, while those from *O. validus* ranged between 15–35 kDa. The inhibitory analysis demonstrated that *O. validus* enzymes were predominantly metal-dependent proteases (67%), whereas *S. neumayeri* contained both serine proteinases (44%) and metal-dependent enzymes (34%). This enzymatic heterogeneity suggests broad substrate specificity, which is advantageous for cleansing various wound types. The enzymes exhibited optimal activity at an alkaline pH (8.0–9.0), which aligns with the pH conditions of chronic and infected wounds, making them particularly effective in the early stages of wound healing. To evaluate the therapeutic potential, a composition based on the mixture of collagenolytic enzymes from both hydrobionts was applied to purulent-necrotic wounds in rats. The group of rats treated with the composition showed faster wound reduction than the control group. By the 12th day, wound size in the treated group had reduced to 24.4 ± 6.7 mm^2, significantly smaller than in the control group – 49.8 ± 6.9 mm^2. Complete epithelialization occurred by day 27 in the treated group, while the control group achieved full healing by day 30. The results confirm that collagenolytic enzymes from Antarctic hydrobionts are effective in enzymatic debridement, promoting faster wound healing. Combining serine and metal-dependent proteases enhances the range of protein breakdown, improving the efficiency of wound cleansing. The enzymes' high activity at alkaline pH is beneficial for treating both acute and chronic wounds, with enzyme activity decreasing as healing progresses, minimizing potential damage to healthy tissues.

Keywords: complicated wounds, marine organisms, proteinases

1 Introduction

The marine biodiversity allows hydrobionts to be considered an enormous and under-explored resource of biomolecules for various purposes (Anjum et al., 2017; Carroll et al., 2022). In recent years, bioactive compounds derived from marine organisms have attracted considerable interest due to their potential use as effective pharmaceuticals for treating a wide range of diseases (Mayer

et al., 2013; Martins et al., 2014; Huang et al., 2021). Among the compounds with promising therapeutic potential are proteins and peptides, especially those derived from organisms living in extreme degrees of salinity, temperature, pressure, and light availability.

Cold-adapted enzymes derived from polar marine species possess high catalytic efficiency at low temperatures, making them especially suitable for biomedical and biotechnological applications that require mild reaction conditions (Cavicchioli et al., 2011; Sarmiento et al., 2015). Their activity at physiological temperatures and rapid thermal inactivation also offer precise control of enzymatic action, reducing off-target effects and increasing safety. For instance, recombinant proteases derived from *Pseudoalteromonas haloplanktis* (ZoBell and Upham 1944), a psychrophilic bacterium isolated from Antarctic waters, have demonstrated efficient activity in molecular biology and pharmaceutical processing, particularly in low-temperature catalysis and protein digestion protocols (Hoyoux et al., 2001). Additionally, the full genome sequencing of various Antarctic species, including invertebrates and fish, has facilitated the heterologous expression and functional characterization of cold-adapted enzymes, such as serine proteases, lipases, and collagenases, expanding their utility in drug formulation, tissue engineering, and wound debridement. These developments underscore the untapped therapeutic potential of Antarctic biodiversity in generating novel biocatalysts for clinical use, espesially in the context of wound treatment.

In our work, we focused on searching for natural molecules that could be effective for wound treatment, as wounds represent a global public health issue that affects patients' quality of life and requires significant efforts and resources from patients, their families, and the healthcare system. Purulent-necrotic wounds are among the most difficult to treat. They are often contaminated with antibiotic-resistant bacteria, complicating treatment (Han & Ceilley, 2017). Impaired blood circulation, tissue necrosis, and abundant purulent exudate significantly slow down regeneration. Also, necrotic tissue and purulent exudate worsen the state of the surrounding healthy tissues.

A promising approach to wound treatment is the use of proteolytic enzymes, which are particularly effective for the treatment of necrotic wounds, chronic, or actively inflamed wounds. Removal of devitalized, necrotic, infected tissue, purulent exudate, and fibrin deposits from the wound surface has a positive effect enhancing granulation and ultimately promoting healing (Thomas et al., 2021). Medical preparations containing collagenases can significantly increase treatment effectiveness, reduce healing time and improve the patient's quality of life. The use of collagenases allows for gentle cleansing of wounds from necrotic tissue, which is a key step in creating a favorable environment for regeneration. As a result, such drugs will help reduce the economic burden on patients while lowering the development of complications associated with the wound process. Therefore, it is relevant to find new sources of collagenolytic enzymes and develop new preparations for accelerating wound healing. Our previous studies have shown that the tissues of certain hydrobiont species from the Antarctic region contain enzymes capable of effectively degrading fibrinogen and collagen (Raksha et al., 2020). Additionally, some of them exhibit atypical characteristics, such as an optimal pH in strongly alkaline conditions or a temperature optimum of 65 °C (Raksha et al., 2023).

In this regard, the study aims to isolate a fraction of collagenolytic enzymes from the hydrobionts of the Antarctic region (*Sterechinus neumayeri* (Meissner, 1900), *Odontaster validus* Koehler, 1906) and evaluate the wound-healing effect of the enzyme-based composition on purulent-necrotic wounds in rats.

2 Materials and methods

2.1 Reagents

Tris(hydroxymethyl)aminomethane, Triton X-100, sucrose, trypsin from porcine pancreas, casein, soy-

bean trypsin inhibitor, ethylenediamine tetraacetic acid (EDTA), collagen type I, ninhydrin, and reagents for electrophoresis were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals, such as $HClO_4$, NaCl, $(NH_4)_2SO_4$, ethanol, and trichloroacetic acid, were of analytical grade quality and were purchased from Khimlaborreaktyv, LLC (Ukraine).

2.2 Preparation of homogenates of Antarctic hydrobionts

Hydrobionts *S. neumayeri* and *O. validus* were used to obtain the fractions of collagenolytic enzymes. The specimens of hydrobionts were collected near Galindez Island (65°15′ S, 64°15′ W) of the Argentine Islands during the Ukrainian Antarctic Expedition of 2023–2024. The hydrobionts were frozen and delivered to the Department of Biochemistry of the Taras Shevchenko National University of Kyiv. To obtain tissue homogenates, the frozen hydrobionts were washed with running water, and then, without removing the calcified exoskeletons, were ground using a hand blender (Braun MultiQuick 5 MQ50236M, De'Longhi Braun Household GmbH, Germany). Then, an equal volume of cold 0.05 M Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl and 0.5% Triton X-100 was added to the ground mass of hydrobionts, and the samples were continuously stirred at 4 °C for 30 min using an orbital shaker PSU-10i (BioSan, Latvia). The samples were first centrifuged at 1 000 × g for 30 min (4 °C), followed by 10 000 × g for 40 min (4 °C). The supernatant was used for enzyme isolation.

2.3 Purification of collagenolytic enzymes

The homogenates were mixed with saturated ammonium sulfate solution, and the mixture was left at 4 °C for 12 hours. The precipitates were centrifuged at 10 000 × g for 15 min, and the pellets were dissolved in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl. To obtain a fraction of collagenolytic enzymes, the sample was put on a column of Q-Sepharose. About 20 to 50 mg of protein was applied per 1 mL of the sorbent, which corresponded to the binding capacity of the column. The column was washed with 0.01 M Tris-HCl buffer (pH 9.0), and the bound proteins were eluted with 0.01 M Tris-HCl buffer (pH 9.0) containing 1 M NaCl. The sample was loaded onto the column, and the bound fraction was eluted at a flow rate of 2 mL · min^{-1}. The obtained fractions were further purified by size-exclusion chromatography on a G-25 column and concentrated using an ultrafiltration membrane system with a molecular weight cut-off of 10 kDa.

2.4 Collagenolytic activity

Collagenolytic activity was measured after Moore & Stein (1954) with a slight modification. The samples containing 0.1% type I collagen, 50 mM Tris-HCl (pH 7.8) with 2 mM $CaCl_2$ and the enzyme fraction (0.05 mg of total protein) were incubated at 37 °C for 5 h. The reaction was stopped by heating the samples at 95 °C for 5 min. After centrifugation at 10 000 × g for 20 min, the supernatants were mixed with an equal volume of 4% ninhydrin solution in 0.1 M citrate buffer (pH 5.0). The samples were placed in a boiling water bath for 15–20 min until color development. After cooling, the optical density of the samples was measured at 570 nm. The concentration of hydrolyzed amino acids was determined using a standard curve prepared using an L-leucine solution.

2.5 Total proteolytic activity and activity of serine proteinases and metal-dependent enzymes

Total proteolytic activity was measured using casein as a substrate according to Munilla-Moran & Stark (1989). Casein (2 %) in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl was incubated in the presence of the enzyme fraction (0.05 mg of total protein) at 37 °C for 30 min. The reaction was stopped with trichloroacetic acid (10 %), and the sample was left to stand for 15 min

at 4 °C. After centrifugation at 15 000 × g for 30 min, the absorbance of the supernatants was measured spectrophotometrically (SmartSpecPlus, Bio-Rad, USA) at 280 nm and 320 nm against the blank in which the enzyme fraction was substituted by the buffer. Total proteolytic activity was expressed as rel. units per mg of proteins as described by Robbins & Summaria (1970).

To determine the activity provided by serine proteinases and metal-dependent enzymes, the appropriate inhibitors were added to the samples – soybean trypsin inhibitor (1 mg per mL) and metal chelator EDTA (10 mM). In this case, blank samples also contained soy trypsin inhibitor or EDTA. The activity of metal-dependent or serine proteinases was expressed as a percentage of the total proteolytic activity.

2.6 Effects of pH

To test the influence of pH on the activity of the collagenolytic enzyme, 0.1 M glycine-HCl buffers (pH 2.0 and 3.0); 0.1 M glycine-NaOH buffers (pH 9.0, 10.0, and 11.0) and 0.1 M phosphate buffers (pH 6.0, 7.0, and 8.0) were used. Enzyme activity was assessed using a 2 % casein solution according to the method described above.

2.7 Enzyme electrophoresis (zymography)

Enzyme-electrophoresis is a specific variant of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that enables simultaneous detection of active proteolytic enzymes in the samples and estimation of their molecular weight. Enzyme electrophoresis was performed according to the method of Ostapchenko and co-authors (2011). Substrate proteins (gelatin or collagen, 2 mg per mL of gel solution) were polymerized in the separating polyacrylamide gel. The separating gel (12 % (w/v) solution was polymerized in the presence of gelatin or fibrinogen (2 mg per mL of gel solution). Samples for electrophoresis were prepared by mixing the purified fractions with sample buffer (5 mM Tris-HCl, pH 8.8, 2 % SDS, 5 % sucrose, and 0.02 % bromophenol blue) at a 1:1 (v/v) ratio. The total amount of proteins applied per well of gel was 20 μg. After electrophoresis, the gels were soaked in 2.5% Triton X-100 solution with gentle shaking (60 min) to remove SDS and renature proteins. The gels were washed with distilled water for 5 min to remove Triton X-100 and then incubated in 50 mM Tris-HCl pH 7.5 at 37 °C for 12 h. The gels were stained with 2.5 % Coomassie Brilliant Blue R-250 in 10 % (v/v) ethanol, 10 % (v/v) acetic acid, and 15 % (v/v) isopropanol. Transparent areas on a dark blue background of the gel indicate the presence of active enzymes. The TotalLab 2.04 program was used to analyze the electrophoregrams. Trypsin and plasmin and its degraded forms were used to estimate the molecular weight of enzymes.

2.8 Protein concentration

The protein concentration was determined by the method proposed in Bradford (1976). Bovine serum albumin was used as the standard. During the purification of collagenolytic enzymes, the protein elution profile was additionally monitored spectrophotometrically at 280 nm.

2.9 Animal models and experimental design

All experiments involving animals were carried out in full compliance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, as well as the National Institute of Health Guidelines (NIH, USA) for the care and use of laboratory animals. By Ukrainian legislation, animal studies were conducted in line with the Law of Ukraine "On the Protection of Animals from Cruelty" (2006, with amendments) and the Order of the Ministry of Education and Science of Ukraine No. 249 (01.03.2012) "On Approval of the Procedure for Conducting Scientific Experiments, Using Animals". All procedures were reviewed and approved by the Bioethical Commission of Taras Shevchen-

ko National University of Kyiv (Protocol No. 3 of 30.07.2024).

The study was conducted on non-linear male rats weighing 200–250 g; the number of animals included in the experiment was 16 (8 in the control and 8 in the treated groups). The rats were kept in a controlled environment (temperature 20 °C – 24 °C, humidity 30%–70 %, 12-h day-night cycle) and fed standard rodent food and water *ad libitum*. Before the start of the experiment, the rats had been quarantined for two weeks. The wounds were created under anesthesia with xylazine hydrochloride at a dosage of 20 mg · kg^{-1} of animal weight (Interchemie, Netherlands). The analgesic was administered intraperitoneally. To simulate purulent-necrotic wounds, animals were subcutaneously injected with 0.1 mL of 10% $CaCl_2$. On the 4th–5th day, necrotomy of the affected areas was performed, after which the wounds were treated with a composition containing collagenolytic enzymes from Antarctic hydrobionts until the wound was completely epithelialized.

2.10 Statistical analysis

Data entry and analysis were performed using MS Excel (MS Office) and Statistica 8.0 software for Windows. The data were reported as mean ± ± standard error of the mean (SEM) for each group (n = 8). After testing for normality (by Kolmogorov-Smirnov test), a one-way analysis of variance (ANOVA) was used to compare the means among different groups. Differences were statistically significant when $p < 0.05$.

3 Results

3.1 Purification of collagenolytic enzymes

Collagenolytic enzymes were isolated from the crude homogenates of *O. validus* and *S. neumayeri*. The purification procedure consisted of two steps – precipitation with ammonium sulfate and ion-exchange chromatography on Q-Sepharose. A gel filtration step was performed using a Sephadex G-25 column to remove NaCl from the obtained fractions. Figure 1 presents the chromatograms of the purification of proteolytic enzyme fractions from the hydrobionts *S. neumayeri* and *O. validus*.

The second peaks on the chromatograms correspond to the fractions that potentially contain the target enzymes. They vary depending on the hydrobiont from which the enzyme fractions were obtained. Despite the homogenization procedure being standardized for all hydrobionts and the same amount of material being applied to the chromatographic column, the amounts of proteins in the fractions purified from the homogenates of *O. validus* and *S. neumayeri* were different and amounted to 12.50 mg and 6.75 mg, respectively.

3.2 Total proteolytic and collagenolytic activities

The comparison of total proteolytic and target activity in the unbound fractions (the first peaks) and the fractions eluted from the Q-Sepharose column (the second peaks) confirmed the effectiveness of our approach for obtaining collagenolytic enzymes. As shown in Table 1, the main activity was detected in the bound fractions, whereas the unbound fractions contained only trace enzyme activity. The total proteolytic activity in the fraction purified from *O. validus* was 2.89 times higher than that of the enzymes from *S. neumayeri* (Table 1). At the same time, the collagenolytic activity values in both fractions were 14.2 ± 0.65 and 14.6 ± 0.57 relative units per mg of total proteins, respectively.

3.3 Molecular weight of the enzymes

Electrophoretic analysis of bound fractions revealed differences in the composition of enzymes by molecular weight and specificity to various substrates. Thus, the molecular weight of enzymes from *S. neumayeri* capable of cleaving gelatin was 20, 23, 60, and 65 kDa, while the majority of enzymes from *O. validus* with activity against this substrate had a molecular mass below 40 kDa – 35, 30, 18, and 15 kDa (Fig. 2a). However, the

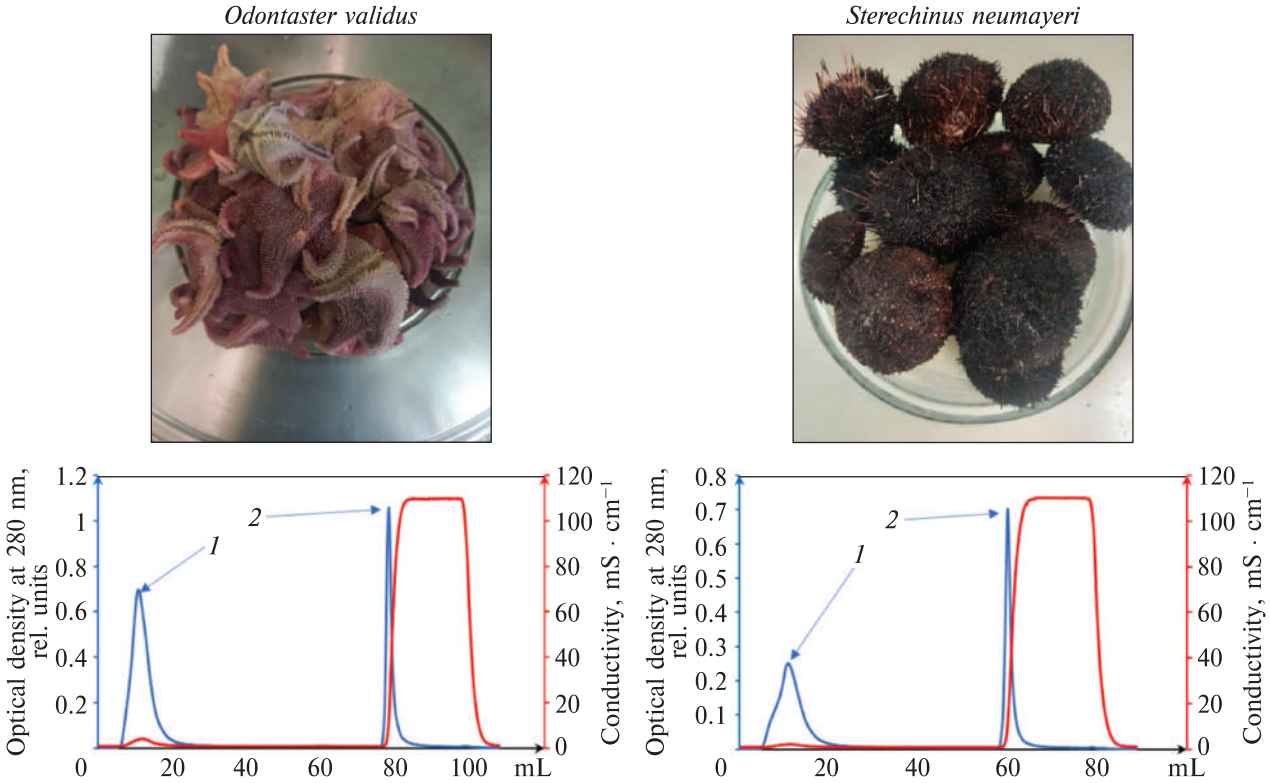

Figure 1. Chromatograms of the purification of the fractions enriched in collagenolytic enzymes by ion exchange chromatography on Q-Sepharose: *1* – the unbound fraction; *2* – the bound fraction

molecular weight of collagen-degrading enzymes was higher than 35 kDa for both hydrobionts (Fig. 2b).

3.4 Inhibitory analysis

To determine the contribution of proteinases with different types of active centers, specific enzyme inhibitors were used. The data obtained after incubation of the fractions enriched with collagenolytic enzymes with soybean trypsin inhibitor and EDTA are presented in Figure 3. EDTA caused a significant inhibition of activity in the sample obtained from *O. validus*, confirming the presence of metal-dependent enzymes as the dominant proteinases in the tissues of this marine organism (67%). On the other hand, the enzyme fraction from *S. neumayeri* consisted of nearly equal amounts of serine proteinases and metal-dependent enzymes – 44% and 34%, respectively.

Table 1. Enzymatic activity in the fractions obtained during the chromatographic separation of tissue homogenates of hydrobionts of the Antarctic region (mean ± SEM, n = 8)

Hydrobiont	Total proteolytic activity, rel. units per mg of total proteins		Collagenolytic activity, rel. units per mg of total proteins	
	Unbound fraction	Bound fraction	Unbound fraction	Bound fraction
O. validus	0.8 ± 0.04	12.50 ± 0.52	1.7 ± 0.07	14.2 ± 0.65
S. neumayeri	0.2 ± 0.01	4.32 ± 0.20	2.5 ± 0.08	14.6 ± 0.57

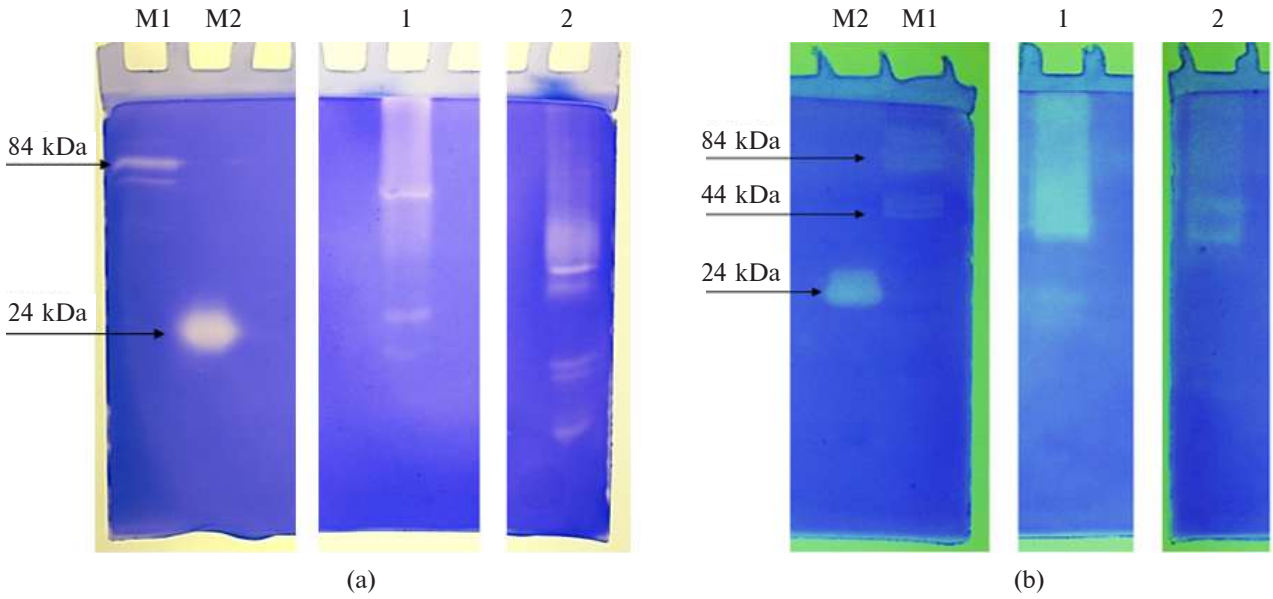

Figure 2. Enzyme-electrophoregram of the bound fractions using gelatin (a) and collagen (b) as a substrate: M1, M2 – molecular weights markers (trypsin, 24 kDa; mini-plasmin, 44 kDa; plasmin, 84 kDa); 1 – fraction of proteolytic enzymes from *S. neumayeri*; 2 – fraction of proteolytic enzymes from *O. validus*

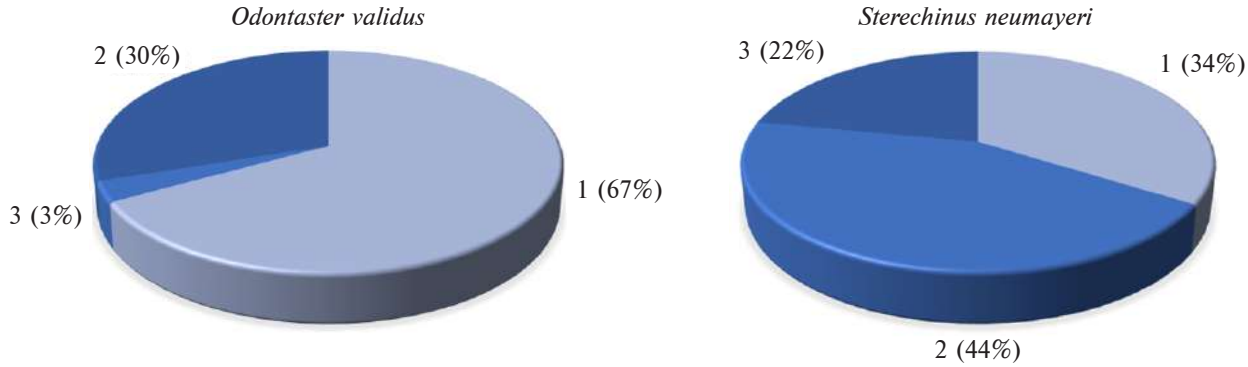

Figure 3. Contribution of the metal-dependent enzymes (1), serine proteinases (2), and aspartyl and cysteine proteinases (3) to total proteolytic activity

The main difference in the enzymatic composition of the tissues of aquatic organisms is the contribution of aspartyl proteinases and cysteine proteinases to the total proteolytic activity, which was 22% for *S. neumayeri* and only 3% for *O. validus*.

3.5 pH optimum of collagenolytic enzymes

The dependence of the activity of proteolytic enzymes purified from hydrobionts on the pH value is presented in Figure 4. To find the optimal pH value, the activity was measured in the pH range from 2.0 to 11.0. As can be seen from Figure 4, the pH dependence was described by a bell-shaped curve. The activity of enzymes in both fractions was quite low at pH below 4.0; it gradually increased with increasing pH values and peaked at pH 9.0 for enzymes from *O. validus* and at pH 8.0–9.0 for enzymes from *S. neumayeri*.

3.6 Study of the wound-healing effect of the composition based on collagenolytic enzymes from the hydrobionts on the purulent-necrotic wounds in rats

According to our results, at the beginning of the experiment, the average area of purulent-necrotic wounds was 136.4 ± 5.3 mm² (Table 2). On the third day of healing, the wound area in the group of animals whose wounds healed without the application of any compositions was 108.0 ± 6.4 mm², while in the group of animals treated with a composition based on a mixture of collagenolytic enzymes from hydrobionts of the Antarctic region, it was 114.4 ± 9.3 mm². The positive effect of the composition on wound healing was observed throughout the entire experimental period. By the twelfth day, the wound area in the control group was 49.8 ± 6.9 mm², whereas in the group of animals whose wounds were treated with the enzyme-based composition, it was reduced to 24.4 ± 6.7 mm².

Photos in Figure 5 show the purulent-necrotic wounds at the beginning and at the end of the experiment confirming the wound-healing effect of the composition based on collagenolytic enzymes from the hydrobionts of the Antarctic region.

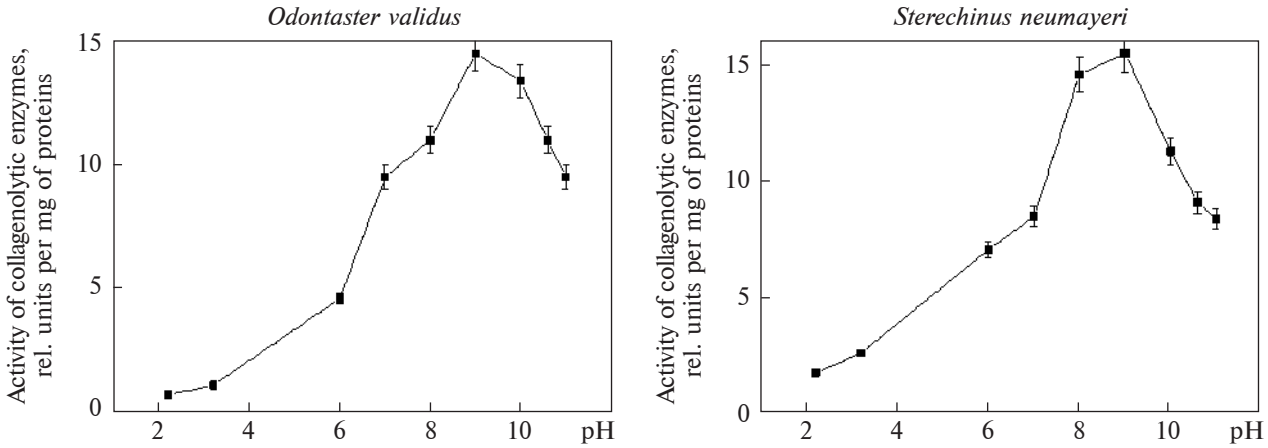

Figure 4. Effect of pH on the activity of collagenolytic enzymes derived from the hydrobionts of the Antarctic region

Table 2. The influence of a composition based on a mixture of proteolytic enzymes from hydrobionts of the Antarctic region on the area (mm²) of purulent-necrotic wounds (mean ± SEM, n = 8)

Group	Day of experiment				
	0	3	6	9	12
Control	136.4 ± 5.3	108.0 ± 6.4	106.0 ± 7.2	102.0 ± 6.4	49.8 ± 6.9
Treated	130.2 ± 16.2	114.4 ± 9.3	87.0 ± 16.0	60.4 ± 19.7*	24.4 ± 6.7*
Group	Day of experiment				
	18	21	24	27	30
Control	26.6 ± 8.9	15.6 ± 8.5	3.2 ± 0.06	0.2 ± 0.01	Complete epithelialization
Treated	5.8 ± 0.14*	2.0 ± 0.08*	0.4 ± 0.02*	Complete epithelialization	Complete epithelialization

* $p < 0.05$ vs. control group

4 Discussion

In this work, a purification procedure with ammonium sulfate precipitation and ion exchange chromatography on Q-sepharose was used to obtain the fractions enriched in collagenolytic enzymes from hydrobionts of the Antarctic region. The methodological approaches used by different authors for obtaining proteolytic enzymes are quite diverse and may include multiple stages, combining various types of chromatography (Park et al., 2002; Kusampudi et al., 2023; Saggu et al., 2023). At each stage, some material is inevitably lost. Therefore, we employed an approach with a minimal number of purification steps. Since our goal was not to obtain a pure enzyme but rather a fraction enriched with target enzymes, the fraction obtained after ion-exchange chromatography on Q-Sepharose was not subjected to further purification.

The fact that collagenolytic activity in the bound fraction was 8.35 times higher for *O. validus* and 5.84 times higher for *S. neumayeri* than in the corresponding unbound fractions confirms the effectiveness of our approach for obtaining collagenolytic enzymes. Both hydrobionts' enzymes exhibited similar activity against collagen, approximately 14.2–14.6 rel. units per mg of total protein. The enzymes were also capable of breaking down casein and gelatin but did not act on fibrin or fibrinogen (data not shown).

Electrophoretic analysis of the fractions of collagenolytic enzymes revealed the presence of enzymes of various molecular weights. Moreover, enzymes from *O. validus* and *S. neumayeri* differed in both molecular weight and specificity for gelatin and collagen. With gelatin as a substrate, the most active enzymes from *S. neumayeri* had a molecular weight above 40 kDa, while enzymes from *O. validus*, on the contrary, were in the molecular weight range of 15–35 kDa. The discovery of enzymes with a molecular weight of approximately 15 kDa is rather intriguing and requires further research aimed at purifying these specific enzymes from the proteolytic fraction and conducting a detailed investigation of their biochemical properties. Typi-

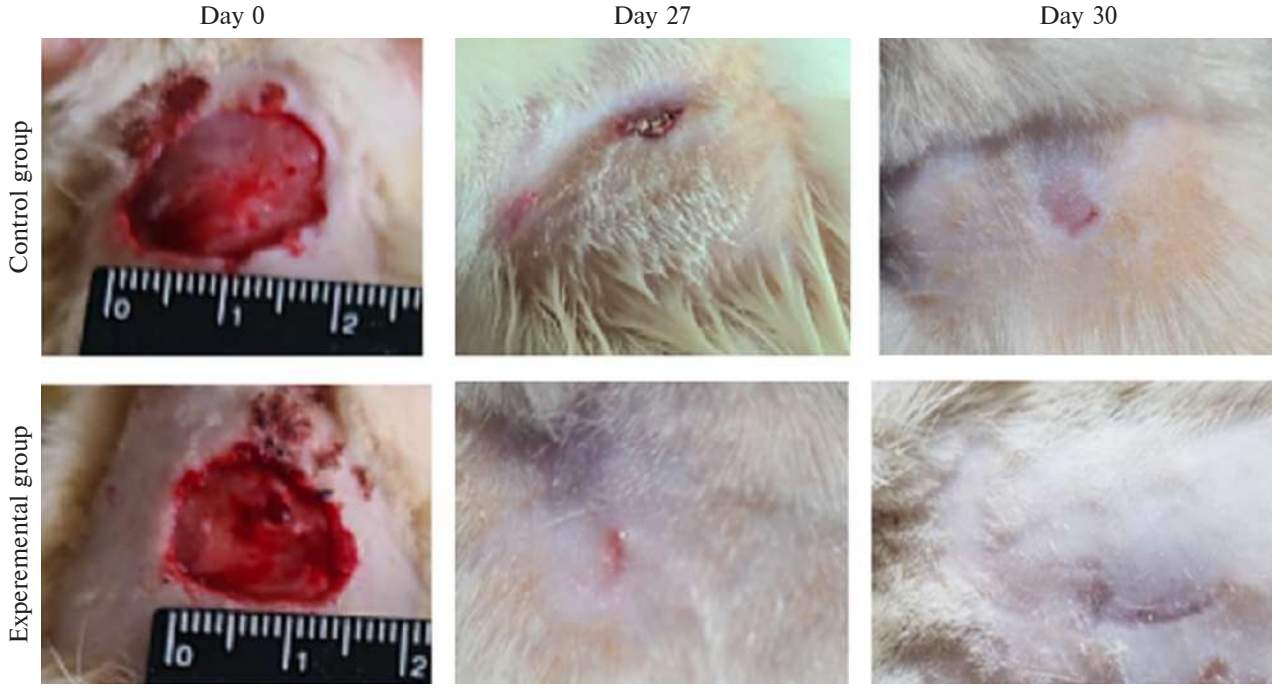

Figure 5. Photographs of purulent-necrotic wounds in the healing process

cally, the molecular weight of proteolytic enzymes is greater than 15 kDa. At the same time, when collagen was used as a substrate, the molecular weight of *O. validus* enzymes was in the same range as enzymes from *S. neumayeri*. Overall, such results indirectly indicate that in the fraction obtained from *S. neumayeri*, the majority of enzymes are collagenolytic, and the fraction purified from the tissues of *O. validus* appears to be a mixture of various proteases.

According to the literature, the molecular weight of collagenolytic enzymes varies significantly depending on whether they are serine proteinases or metal-dependent proteinases. Park et al. (2002) purified a collagenase from mackerel with a molecular weight of 14.8 kDa, which is quite atypical, whereas enzymes from mammalian tissues have molecular weights ranging from 30 to 150 kDa. The results of inhibitor analysis using specific proteinase inhibitors confirmed that the fractions of collagenolytic enzymes purified from hydrobionts contained various proteinases. The contribution of serine and metal-dependent proteases differed: the *O. validus* enzyme fraction consisted predominantly of metal-dependent proteases, which accounted for 67% of the total proteolytic activity. In contrast, the dominant enzymes in the tissue of *S. neumayeri* were serine proteinases. Such heterogeneity in the enzyme composition of the obtained fractions ensures broad substrate specificity. It may be a beneficial characteristic when developing enzyme-containing compositions for wound bed cleansing, particularly in treating purulent-necrotic wounds.

The optimal pH of the collagenolytic enzymes we identified falls within the mildly alkaline range, indicating that most enzymes in the purified fractions are alkaline proteases. These results are not unexpected, as the literature suggests that enzymes from invertebrates often exhibit pH optima in the alkaline range. Collagenases with a pH optimum higher than 8.0 have been isolated from microorganisms (Vijay Kumar et al., 2011), fungi (Wanderley et al., 2017), and yeasts (Ni et al., 2008), whereas the pH optimum of mammalian collagenases is usually about 7.0 (Stricklin & Hibbs, 1988).

Considering the significant biotechnological potential of alkaline proteases, identifying new sources of such enzymes is very promising not only for their use in medicine but also for possible implementation in technological processes, which can help reduce the cost of production while improving quality.

Since the obtained fractions were supposed to be used to create a wound-healing composition, the pH optimum of enzymes precisely at alkaline pH can be one of the mechanisms regulating enzyme activity and, as a consequence, the intensity of wound cleansing from purulent exudates depending on the stage and intensity of the pathological process. pH of chronic or infected wounds varies depending on the stage of the wound process (Gethin, 2007; Bennison et al., 2017). At the initial stage during an acute inflammatory process, the pH is usually more alkaline, which, on the one hand, is a consequence of infection with pathogenic microorganisms and, on the other, creates a favorable environment for further bacterial growth. Furthermore, applying compositions or ointments based on collagenolytic enzymes from *S. neumayeri* and *O. validus* to the wound surface can help regulate pH, creating optimal conditions for tissue repair while simultaneously providing a cleansing effect through enzymatic activity. Therefore, enzymes that work effectively in the alkaline pH range can be extremely effective, and their use is advisable precisely at the initial stages of the pathological process or in the case of chronic wounds.

Considering the differences in the composition of enzyme fractions purified from hydrobionts, as well as to maximize the wound-healing effect, we used a composition containing a mixture of enzymes from *O. validus* and *S. neumayeri* in our study. The results indicate a significant wound-healing effect of the composition containing a mixture of collagenolytic enzymes from hydrobionts of the Antarctic region. In the purulent-necrotic wound model, the topical application of

this composition led to an accelerated wound reduction rate. Complete epithelialization of the wounds was observed by day 27, whereas in the control group, wound healing occurred on day 30 of the experiment.

Wound healing is a complex physiological process that involves several stages: coagulation, inflammation, proliferation, and remodeling. All these stages interact in an orderly manner, meaning that any disruption in the precisely regulated reactions at one stage will affect the overall dynamics and rate of wound healing. It can be assumed that the observed effect is multifaceted, involving several mechanisms. Applying collagenases enables the gentle cleansing of wounds from necrotic tissues, which is a key step in creating a favorable environment for tissue regeneration. Moreover, collagen breakdown by collagenolytic enzymes leads to the release of growth factors, stimulating the migration of fibroblasts and keratinocytes to the wound bed.

5 Conclusion

Despite the significant potential of proteolytic enzyme-based treatments, certain challenges exist in their development. These include ensuring enzyme stability within the formulation, minimizing the risk of adverse reactions such as excessive degradation of healthy tissues, and optimizing the delivery method (creams, gels, dressings). Considering our results, using collagenolytic enzymes from Antarctic hydrobionts *Sterechinus neumayeri* and *Odontaster validus* to develop wound-healing agents may help overcome or mitigate these challenges. The observed differential sensitivity of proteolytic enzymes to specific inhibitors suggests the presence of multiple classes of proteases with distinct catalytic mechanisms (e.g., serine-, cysteine-, and metalloproteases), which significantly enhances the overall hydrolysis efficiency and broadens the range of target substrate proteins. The presence of both serine proteinases and metal-dependent enzymes in the purified fractions, along with the well-defined pH-dependent enzymatic activity, provides the ability to regulate the activity of collagenolytic enzymes within wound-healing formulations.

In vivo experiments on rats confirmed the wound-healing potential of collagenolytic enzymes derived from hydrobionts, demonstrating a significant reduction in wound size and accelerated epithelialization compared to the control group. Given the high collagenolytic activity and broad substrate specificity, the purified enzymes may be considered promising candidates for incorporation into topical formulations such as fibrin-based or collagen-containing gels, ointments, or powders for wound treatment, subject to further preclinical validation. From a practical application perspective, enzyme-based preparations derived from hydrobionts of the Antarctic region can be used not only for the treatment of purulent-necrotic wounds but also for chronic wounds (ulcers, pressure sores), burns, traumatic skin injuries, and post-surgical wounds.

Thus, the findings highlight the biotechnological potential of Antarctic hydrobionts as a promising source of collagenolytic enzymes for medical applications. Their ability to efficiently remove necrotic tissue while minimizing damage to healthy cells presents a valuable opportunity for developing advanced therapeutic agents for hard-to-heal wounds. Further research into these enzymes' detailed biochemical properties and clinical applications could lead to innovative treatments in wound management and regenerative medicine.

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Колагенолітичні ферменти антарктичних гідробіонтів: перспективний біотехнологічний підхід для прискорення загоєння гнійно-некротичних ран

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Реферат. Метою роботи було очистити з гідробіонтів Антарктичного регіону *Sterechinus neumayeri* та *Odontaster validus* фракції, збагачені на колагенолітичні ферменти, та оцінити їх потенційний ранозагоювальний ефект на моделі гнійно-некротичних ран у щурів. Процедура очищення включала осадження сульфатом амонію та іонообмінну хроматографію на Q-Sepharose. Ферменти з обох гідробіонтів виявляли схожу колагенолітичну активність (14 ум.од/мг білка), проте відрізнялися за молекулярною масою та специфічністю щодо окремих субстратів. Електрофоретичний аналіз з використанням желатину як субстрату виявив, що ферменти з *S. neumayeri* мали молекулярну масу понад 40 кДа, тоді як молекулярна маса ферментів з *O. validus* складала 15–35 кДа. Інгібіторний аналіз показав, що більшість ферментів з *O. validus* (67%) є металозалежними протеазами, тоді як у *S. neumayeri* було виявлено як серинові протеази (44%), так і металозалежні (34%). Така гетерогенність свідчить про широку субстратну специфічність, що є важливою перевагою для ефективного очищення ран. Максимальна ферментативна активність спостерігалася за лужних значень pH (pH 8,0–9,0), що відповідає умовам ранніх етапів хронічних ран. Для оцінки ранозагоювального ефекту було створено композицію, яка містила суміш колагенолітичних ферментів з *S. neumayeri* та *O. validus*. Аналіз площі ранової поверхні у динаміці гоєння підтвердив, що досліджувана композиція пришвидшувала процес загоєння ран. На 12-ий день площа ран у дослідній групі зменшилася до 24,4 ± 6,7 мм^2, що було суттєво менше, ніж у контрольній групі (49,8 ± 6,9 мм^2). Повна епітелізація ран спостерігалася на 27 добу у порівнянні з результатом у групі тварин без нанесення композиції, для яких повне загоєння мало місце на 30 добу. Отримані результати свідчать про певний терапевтичний потенціал колагенолітичних ферментів з гідробіонтів Антарктичного регіону. Поєднання серинових та металозалежних протеаз розширює спектр деградації білкових субстратів, підвищуючи ефективність очищення ран. Висока активність ферментів у лужного pH є перевагою при лікуванні гострих та хронічних ран, при цьому їхня активність поступово знижується в міру загоєння, що мінімізує потенційне ушкодження здорових тканин.

Ключові слова: морські організми, протеїнази, ускладнені рани