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ISOLATION AND CHARACTERIZATION OF COLLAGENASE-ACTIVE PREPARATION FROM *RAPANA VENOSA* SALIVARY GLANDS

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Collagenases have found practical applications in both medicine and the food industry, but the search for novel collagenase sources remains an active area of studies. Rapana, a predatory mollusk that primarily feeds on bivalves rich in connective tissue, has emerged as a potential source of collagenolytic enzymes. This study aimed to isolate collagenase-active preparation from Rapana venosa salivary glands and characterize its properties. The salivary gland extract was purified by acetone precipitation followed by ammonium sulfate treatment. Electrophoresis was performed by the Laemmli protocol under both reducing and non-reducing conditions. Proteolytic activity was determined spectrophotometrically using collagen or gelatin as a substrate. The preparation consisted of five protein fractions and exhibited enzymatic polymorphism. A 13.8-fold purification of collagenase activity was achieved, at least 22% of total proteins displayed collagenolytic activity, while 88% showed gelatinolytic activity. The optimum of preparation activity was found in acidic (pH 4.5) and alkaline (-9.5) ranges, with thermal optimum at 46°C. At room temperature, about 90% of activity was maintained for 8 h. Serine protease inhibitors did not affect enzyme activity, metal ion chelators completely inhibited it. Reducing agents enhancing SH-groups increased enzyme activity, disulfide bond regeneration or SH-group modification decreased it. The data obtained showed that the collagenase-active enzyme preparation from Rapana venosa salivary glands consists mainly of metalloproteinases and cysteine proteases, exhibiting high stability.

K e y w o r d s: proteolytic enzymes, collagenase, purification, Rapana venosa, salivary glands.

Among the diverse range of proteolytic enzymes, collagenases hold a distinct position due to their unique ability to cleave the triple-helical structure of collagen protein chains. This capability is essential for normal animal development [1-7], whereas dysfunction of collagenases is associated with the onset of various pathological conditions [8-13].

Collagenases were discovered relatively recently – in the mid-20th century [11, 14]. Since then, they have become widely used in contemporary scientific research [15] and have found practical applications in both medicine [16-18] and the food industry [19]. Despite extensive studies, the search for novel collagenase sources and the investigation of their specific properties remains an active area of research.

Rapana, a predatory mollusk that primarily feeds on bivalves rich in connective tissue, has emerged as a potential source of collagenolytic enzymes. Our previous studies demonstrated the presence of enzymes with significant gelatinase activity in the salivary glands of rapana [20].

However, it remains unresolved whether this is a reflection of the presence of collagenase activity, since it is known that not all gelatinases are capable of degrading native collagen effectively [2, 11, 21]. Therefore, the objective of this study was to isolate a protein with collagenase activity from rapana salivary gland extracts and to characterize the enzymatic and physicochemical properties of the purified enzyme.

Materials and Methods

Sample preparation and enzyme extraction. The dissection of mollusks and the extraction of enzymes were performed as described in [20]. Homogenates were prepared from 15–30 *Rapana venosa* specimens using an extraction buffer containing

Triton X-100 (Sigma, USA) at a tissue-to-buffer ratio of 1:5 (w/v). The homogenates were centrifuged at 2500 g for 30 min at 4°C using a Laborfuge 400 R centrifuge (Thermo Scientific, Germany). The resulting supernatant was used for further purification.

Protein purification. The salivary gland extract was purified following a multistep scheme (Fig. 1), designed to isolate the fraction with collagenase activity.

Determination of proteolytic activity. The general proteolytic activity was determined using casein (2%) from cow's milk (Sigma) according to the method described in [22], with certain modifications. Gelatinolytic activity was evaluated using a 0.5% solution of gelatin (Serva). Collagenolytic activity was determined using collagen isolated from bovine Achilles tendon (Sigma-Aldrich) at a concentration of 2mg per incubation mixture, based on the procedure described in [23], with some modifications. All proteolytic activity assays were modified and performed under unified conditions. To a substrate solution (the concentration of each substrate is indicated above) prepared in 0.05 M Tris-HCl buffer, pH 7.5 with 20 mmol $CaCl_2$, 0.1 ml of the tested enzyme preparation in the same buffer was added. The final volume of the reaction mixture was 2.5 ml. Incubation was carried out at 37°C for 1 h. The reaction was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 5%. The extent of protein hydrolysis was determined spectrophotometrically using a Cary 60 spectrophotometer (Agilent Technologies) based on leucine, and the activity was calculated in μmol leucine/mg protein/min.

Protein concentration determination. Protein concentration was determined by the Lowry method as modified by Hartree [24], using bovine serum albumin (BSA) (Sigma, USA) for the calibration curve.

Effect of pH and temperature on proteolytic activity. The influence of pH and incubation temperature on proteolytic activity was assessed based on gelatin hydrolysis, as described above. The desired pH values were adjusted by adding appropriate volumes of 0.1 N NaOH or HCl to the Tris-HCl buffer. The following pH values were tested: 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, and 10.5. Temperature effects were examined by incubating the enzyme in 0.05 M Tris-HCl buffer (pH 7.5) containing 20 mM $CaCl_2$ at the following temperatures: 10, 20, 30, 40, 50, 60, 70, and 80°C.

Thermal and pH stability. Thermal and pH stability were evaluated over an 8-hour period, with measurements taken at 0, 1, 2, 3, 4, 6, and 8 hours. The enzyme preparation (1 ml) was incubated under the specified conditions, and aliquots (0.1 ml) were withdrawn and added to 0.5 ml of 0.5 % gelatin solution in 0.05 M Tris-HCl buffer (pH 7.5) containing 20 mM $CaCl_2$ to assess proteolytic activity as described previously. For thermostability analysis, enzyme activity was compared between samples incubated at room temperature (20-22°C) and at 60°C, both at pH 7.5. For pH stability evaluation, the enzyme was incubated at 40 °C in buffers with pH values of 4.5, 7.5, and 10.8.

Changes in enzymatic activity under different conditions were expressed as a percentage of the initial activity. A formal assessment of stability was made using the coefficient obtained by linearizing the data in the time/ln residual activity coordinates [25].

Effect of chemical agents on collagenolytic activity. To assess the effect of various chemical agents on collagenolytic activity, the enzyme preparation was preincubated for 20 min in 0.05 M Tris-HCl buffer (pH 7.5) containing 20 mM $CaCl_2$ and the test compound at room temperature. The enzymatic activity was then measured as described above. The following effectors were tested at the indicated final concentrations: phenylmethylsulfonyl fluoride (PMSF) – 1 mM, soybean trypsin inhibitor – 20 mg/ml, ethylenediaminetetraacetic acid (EDTA) – 1 mM, 1,10-phenanthroline – 1 mM, lectin from Canavalia ensiformis (ConA) – 1 mg/ml, guanidine hydrochloride (Gu) – 5 mM, dithiothreitol (DTT) – 10 mM, sodium salt of iodoacetic acid (IA-Na) – 5 mM, Acetic anhydride (Ac2O) – 10 mM, Dimethyl sulfate – 10 mM, Mercuric chloride ($HgCl_2$) – 1 mM, p-Chloromercuribenzoate (p-CMB) – 1 mM.

Sulfhydryl group oxidation to disulfide bonds was performed using hydrogen peroxide (1 mM), either alone or in the presence of 0.01 mM tetrabutylammonium iodide (TBAI) as a catalyst, following the procedure described in [26].

Molecular weight determination and electrophoretic forms of proteins. Molecular weight determination and electrophoretic forms of proteins with proteolytic (gelatinase) activity were carried out by electrophoresis using the Laemmli buffer system [27] in the presence of sodium dodecyl sulfate (SDS). The concentration and resolving gels were 5 and 10% polyacrylamide (PAA) gels, respectively. Experimental samples were prepared under non-reducing conditions (without the addition of mer-

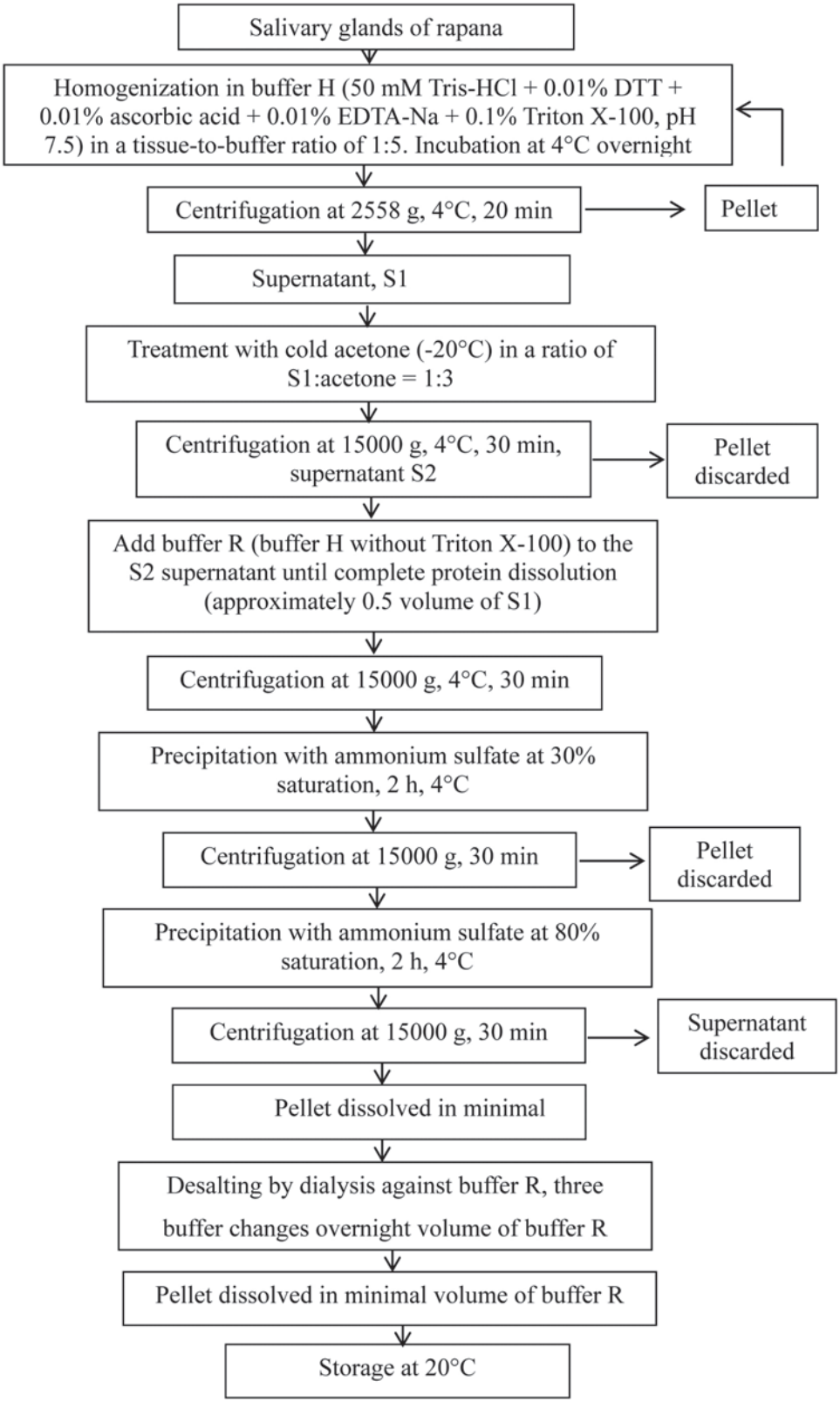

Fig. 1. Scheme for obtaining the enzyme preparation from the salivary glands of the rapana and its partial purification

captoethanol) or reducing conditions (with 10 mM DTT). To detect protease activity during gel preparation, gelatin solution was added to the gel at a final concentration of 0.125%.

Electrophoresis was conducted in a Cleaver Scientific Ltd apparatus using 15×15 cm gels with a thickness of 1 mm. Molecular weight markers (CSL-PPL Prestained Protein Ladder, UK) were run in a separate lane. After electrophoresis, to visualize protein forms, the gels were rinsed with water and stained using the following solution: 25 ml isopropanol, 10 ml glacial acetic acid, 5 g trichloroacetic acid (TCA), 250 mg brilliant blue R-260, and distilled water to a final volume of 100 ml. Gels were washed with a mixture of 7% acetic acid and 2% isopropanol after staining.

For the gelatinase spectrum, after electrophoresis, gels were washed from SDS using 0.5% Triton X-100 solution in distilled water (5 changes, 10–15 min each), then incubated in 0.01 M Tris-HCl buffer (pH 7.5) with 2 mM $CaCl_2$ for 16-18 h at 37°C. After incubation, the gels were rinsed with water and stained with the solution described above. The gels were washed until clear colorless bands appeared in the areas of proteolytic enzyme action.

Electrophoregrams were documented using a scanning device connected to a computer, and quantitative analysis of the densitograms was performed using the AnaIS software. The number of electrophoretic forms, their relative electrophoretic mobility, and the proportion in the total spectrum of each sample were determined.

Statistical analysis. Statistical analysis, construction of diagrams and graphs, and function approximation were performed using Microsoft Excel. The arithmetic mean and its standard error (±SE) were calculated. The significance of the results was assessed using Student's t-test and analysis of variance. A critical level of significance was accepted at $P < 0.05$. Proteolytic activity and protein concentration were measured in 3-7 independent replicates, with control samples containing additional components (for studies on the influence of added compounds).

Results

It is well established that many collagenases exhibit broad specificity and are capable of hydrolyzing other proteins. The investigated enzymes exhibited broad substrate specificity, and casein proved to be a more "accessible" substrate, as it was hydrolyzed more efficiently than gelatin and collagen. Therefore, for the purpose of "standardizing" hydrolytic activity, the degree of hydrolysis was determined based on a single amino acid-leucine – for all proteins, and the activities toward gelatin and collagen (as specific substrates) were calculated relative to the degree of hydrolysis of casein (taken as nonspecific or general activity), which was set at 100%. The results of the comparison of collagenase activity in the studied glands of the rapana digestive system are presented in Table 1.

The highest collagenase activity – and more importantly, its relative contribution to the total proteolytic activity – was observed in the salivary glands. Consequently, these glands were selected for further investigation.

Table 2 summarizes the results of the purification of proteins from the salivary glands of Rapana. It is important to note that the degree of purification varied depending on the substrate used to assess proteolytic activity. The highest purification level was achieved when collagen was used as the substrate, which is likely due to the high proportion of collagenases present in the studied glands.

Electrophoretic analysis demonstrated the polymorphism of the enzyme preparation (Fig. 3). Under non-reducing conditions, five protein forms were

Table 1. Comparison of proteolytic activity toward various substrates in the studied glands of the rapana digestive system

Glands	Specific activity, μmol leucine/mg protein/min ± SE (share of specific activity in total activity determined using casein, %)	
	Gelatinolytic activity	Collagenolytic activity
Gland of Leiblein	2.46 ± 0.11 (40.9)	0.59 ± 0.02 (9.8)
Hepatopancreas	2.73 ± 0.17 (49.0)	0.52 ± 0.02 (9.3)
Salivary gland	4.48 ± 0.19 (86.1)	1.13 ± 0.07 (21.7)

Note. SE is the standard error of the arithmetic mean

Table 2. Purification of proteolytic enzymes from the salivary glands of Rapana

Stages	Volume, cm^3	Protein, mg	Yield, %	Total activity with different substrates, μmol of leucine			Specific activity with different substrates, μmol of leucine/mg protein/min			Purification level with different substrates, (times)		
				casein	gelatin	collagen	casein	gelatin	collagen	casein	gelatin	collagen
1	24	367.5	100	2103.0	1411.3	640.7	5.7	3.8	1.7	–	–	–
2	41	114.7	31	1184.6	849.2	537.1	7.4	5.3	3.4	1.3	1.4	1.9
3	30	51.7	14	894.4	609.7	417.5	17.3	11.8	8.1	3.0	3.1	4.6
4	13	14.2	4	711.4	447.6	343.0	50.0	31.4	24.1	8.7	8.2	13.8

Note: 1 – crude extract, 2 – after treatment with acetone, 3 – after treatment with ammonium sulfate at 30% saturation, 4 – after ammonium sulfate salting-out at 80% saturation followed by desalting using dialysis

identified with molecular masses of approximately 210, 110-115, 75, 62, and 48 kDa, with two major forms at 210 and 75 kDa. To assess the enzymatic activity among the proteins, gelatin zymography was compared with the electrophoretic profiles obtained after SDS-PAGE of the proteins (Fig. 2). Excluding the possibility of overlapping molecular masses of inactive proteins and enzymes with proteolytic activity, it was calculated that gelatinases in the preparation account for at least 88±3% (Table 3).

To investigate the quaternary structure of the gelatinases and the role of disulfide bonds in their formation, electrophoresis was performed on samples with and without the addition of reducing agents (DTT), and the relative proportions of the electrophoretic fractions in each spectrum were analyzed (Fig. 2, Table 4). As shown, the presence of free SH-groups in the experimental samples significantly increased the proportion of molecules with a lower molecular mass, and a 48 kDa form of the hydrolytic enzyme, which was not detected under non-reducing conditions, became evident.

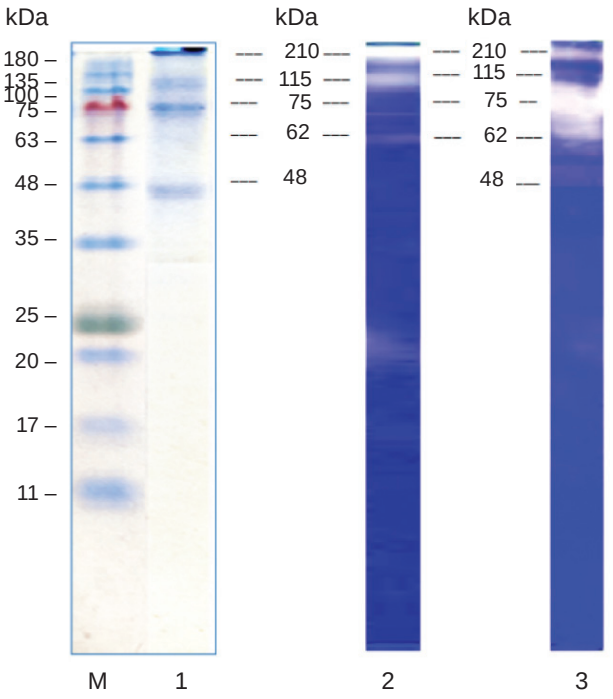

Fig. 2. Electrophoretic spectra of the partially purified enzyme preparation from rapana salivary glands: M – molecular weight markers, 1 – total protein spectrum (Coomassie staining), 2 – gelatinase spectrum; 1,2 – non-reducing conditions, 3 – reducing conditions (10 mM DTT added to the sample)

Table 3. Comparative analysis of total protein and gelatinase spectra

Molecular weight of the electrophoretic fraction, kDa	Relative fraction content in the total protein spectrum, %	Presence of the fraction in the gelatinase spectrum
210 ± 6	29.70	present
110 ± 3	19.40	present
75 ± 2	37.72	present
62 ± 3	1.28	present
48 ± 2	11.90	not detected

Note: electrophoretic spectra obtained under unreduced conditions were compared

Table 4. Comparison of gelatinase spectra under non-reducing and reducing conditions

Molecular weight of the electrophoretic fraction, kDa	Relative fraction content in the total spectrum of samples without reducing agent, %	Relative fraction content in the total spectrum of samples after DTT addition, %
210÷230	53.80	10.10
111÷115	33.50	5.56
72÷77	4.03	61.60
62÷64	8.67	11.70
47÷49	0.00	11.00

Since gelatin is a derivative of collagen, it was used in place of insoluble collagen to assess certain physicochemical properties of the enzyme preparation.

Fig. 3 shows the dependence of gelatinolytic activity on pH and incubation temperature.

Two distinct pH ranges exhibited the highest proteolytic activity (Fig. 3, *A*). One of these was at pH 4.5. The alkaline range proved to be more favorable for enzymatic activity: a high level of activity was sustained over a broad pH interval from 7.5 to 10.5, with a peak around pH 9.5.

The thermal profile of the enzyme activity indicated that the maximal gelatinolytic activity of the salivary gland enzymes occurred at temperatures between 35°C and 45°C (Fig. 3, *B*).

To determine the thermal optimum, Arrhenius plots were constructed for the ascending and descending branches of the enzyme activity vs. temperature curve (Fig. 4). The intersection point of the two lines corresponded to 46°C.

The results of the study on the pH and thermal stability of the partially purified enzyme preparation are shown in Fig. 5. The stability coefficients of the enzymes at pH 4.5 and 7.5 are nearly identical, amounting to 0.027±0.002, which is significantly lower than the value at pH 10.8, which was 0.049±0.004. This indicates that a slightly acidic or neutral environment is more favorable for maintaining the optimal state of the enzyme molecules in the studied preparation. The thermal stability coefficient at high temperature is almost five times higher than that at room temperature – 0.123±0.014 and 0.025±0.002, respectively.

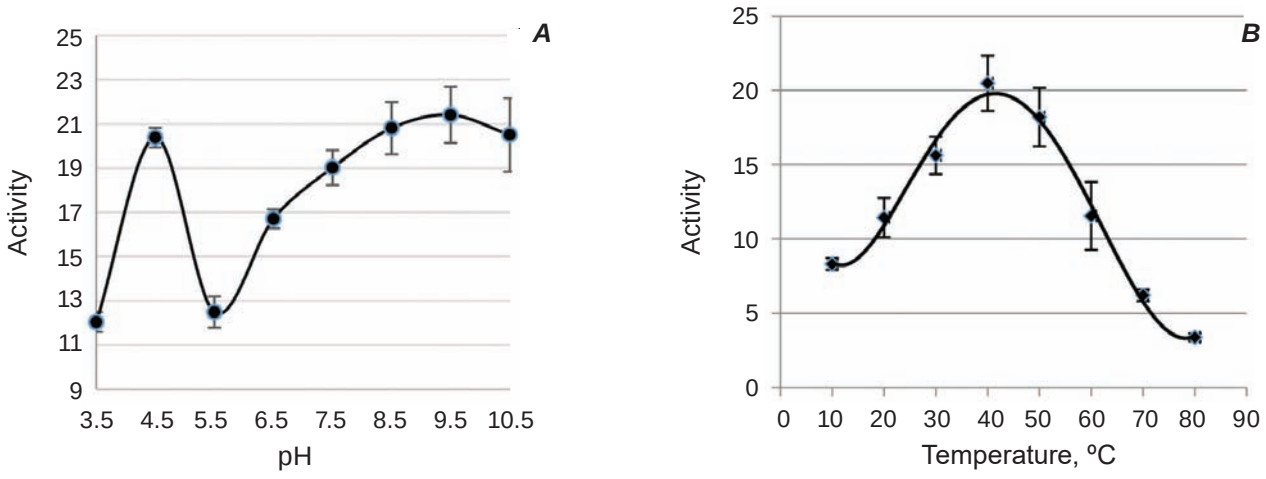

*Fig. 3. Enzyme activity at different pH values (**A**) and temperatures (**B**): the ordinate axis shows activity (μmol leucine/mg protein/min), and the abscissa axis indicates pH values (**A**) and temperature in °C (**B**)*

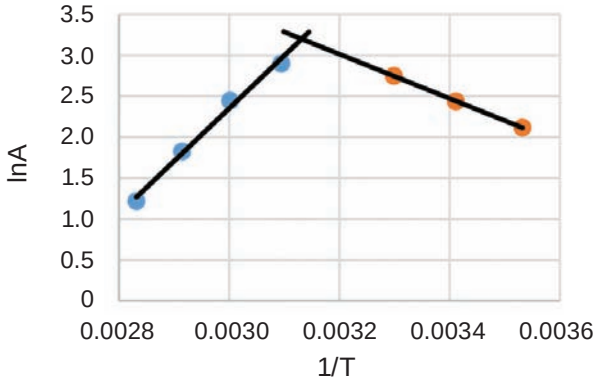

Fig. 4. Arrhenius plot for optimal activity temperature. lnA – natural logarithm of enzyme activity is plotted on the y-axis; reciprocal absolute temperature 1/T is shown on the x-axis

The effect of various tested substances on the collagenolytic activity of the obtained preparation was investigated. Enzyme activity in the presence of effectors was expressed as a percentage relative to the activity under standard conditions. The obtained results are presented in Table 5.

Discussion

Despite the relatively small mass of rapana salivary glands, they represent a promising source of enzymes with collagenase activity. In these glands, the relative content of collagenases is several times higher than in the larger glands of rapana, such as the gland of Leiblein and the hepatopancreas.

A comparison of our purification results with those reported by other authors demonstrates that the achieved level of purification under our experimental conditions is reasonably high, despite the use of only acetone and ammonium sulfate precipitation. For example, in studies [28-30], collagenase purification using ammonium sulfate yielded values ranging from 1.3 to 2.0, which are lower than those observed in our work.

In the cited studies, achieving a higher purification level (>40) required the application of more sophisticated techniques, such as chromatographic methods [28-30]. The relatively high purification efficiency observed in our study may be attributed to the substantial content of collagenases (exceeding 20% of total proteases) in the enzyme preparation obtained from rapana salivary glands.

It is well established that proteases can be activated during SDS-PAGE. As a result, even the inactive proenzyme forms of gelatinases or collagenases may become detectable in gelatin-containing gels.

Electrophoretic analysis revealed polymorphism of gelatinolytic enzymes in rapana salivary glands (Fig. 3). The protease spectra under non-reducing conditions differed from those of the samples treated with DTT: under reducing conditions, the gelatinase spectrum shifted toward lower molecular weight molecules (Table 4, Fig. 3). According to [10, 31], high-molecular-weight forms above 200 kDa can be considered polymeric complexes of various gelatinases. These complexes, as suggested

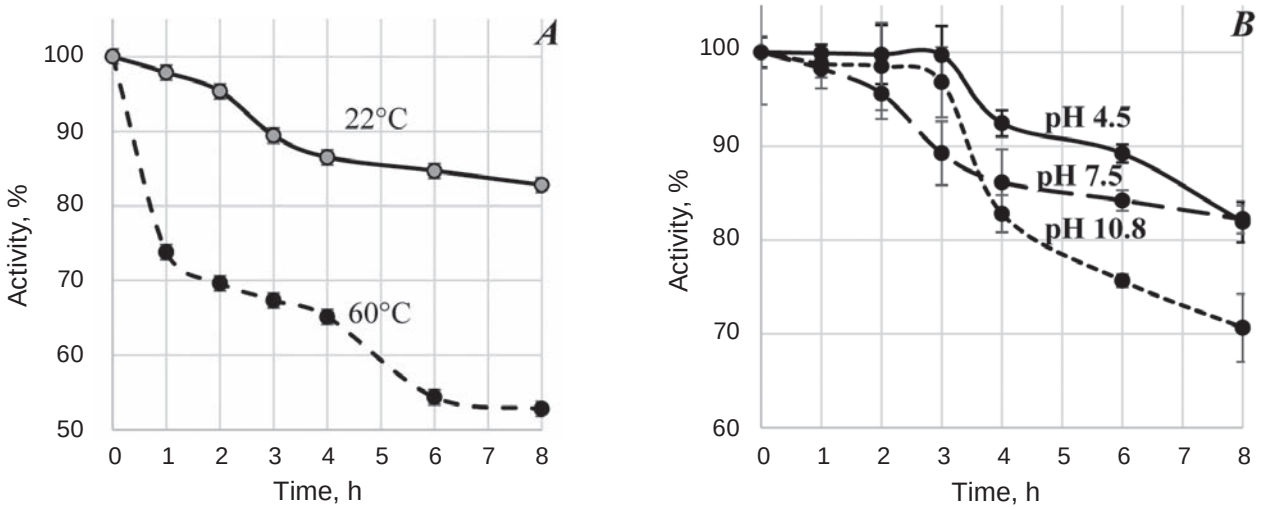

*Fig. 5. Time-dependent stability of the enzyme preparation at different temperatures (**A**) and pH values (**B**). Relative enzyme activity (Activity, %) is plotted on the y-axis; incubation time is shown on the x-axis*

Table 5. Relative collagenolytic activity (%) of the enzyme preparation from rapana salivary glands in the presence of various effectors

Added compound	Relative activity, % ± SE	Statistical significance compared to control, *P*
Control	100	
PMSF (1 mmol)	100.0 ± 3.0	none
Soybean trypsin inhibitor (20 mg/cm^3)	93.5 ± 4.0	none
EDTA (1 mmol)	0.0	significant
1,10-Phen (1 mmol)	6.1 ± 0.8	significant, $P < 0.001$
Con A (1 mg/cm^3)	76.1 ± 8.7	significant, $P = 0.05$
DTT (10 mmol)	165.9 ± 10.1	significant, $P < 0.001$
IA-Na (5 mmol)	50.4 ± 8.4	significant, $P < 0.001$
H_2O_2 (1 mmol)	23.8 ± 1.8	significant, $P < 0.001$
H_2O_2 (1 mmol) + TBAI (0.01 mmol)	11.8 ± 1.2	significant, $P < 0.001$
$HgCl_2$ (1 mmol)	35.0 ± 3.6	significant, $P < 0.001$
p-CMB (1 mmol)	63.8 ± 4.0	significant, $P < 0.001$
Ac_2O (10 mmol)	14.1 ± 0.8	significant, $P < 0.001$
Dimethyl sulfate (10 mmol)	21.4 ± 1.2	significant, $P < 0.001$
Heparin (2 mg/cm^3)	16.7 ± 1.3	significant, $P < 0.001$
Heparin (2 mg/cm^3) + DTT (10 mmol)	60.0 ± 1.4	significant, $P < 0.001$
Gu (5 mmol)	70.9 ± 10.6	significant, $P = 0.02$

by the authors mentioned above, serve to reduce the pool of active enzymes in native tissues.

Based on literature sources [32], proteases with molecular weights ranging from 100–115 kDa and lower may include different matrix metalloproteinases, such as MMP-9 (gelatinase B, ~90 kDa), MMP-8 (collagenase 2), MMP-2 (gelatinase A, 72-75 kDa), MMP-1 (collagenase 1, 62–65 kDa), MMP-13 (collagenase 3), and other members of the extensive matrix metalloproteinase family.

The presence of two distinct optimal pH values for the reaction medium (Fig. 4) indicates the possible existence of two types of proteinases – acidic and alkaline – or the presence of two active ionic forms of the enzyme. For most gelatinases and collagenases, the optimal hydrolytic activity occurs within the neutral to slightly alkaline pH range [33]. Enzymes capable of exhibiting collagenolytic activity under acidic conditions are relatively rare. Among them, certain cysteine cathepsins have been shown to degrade native collagen in acidic environments [34, 35]. Therefore, it is reasonable to hypothesize that the preparation from the salivary glands of the rapana contains enzymes similar to cysteine proteinases.

The stability of the studied enzymes is pH-dependent. At acidic and neutral pH values (4.5 and 7.5), their enzymatic activity remains well preserved over the 8-hour incubation period at 40 °C, with no more than a 15% decrease in activity (Fig. 5). In contrast, under alkaline conditions (pH 10.8), the preparation is less stable, with almost one-third of the enzymatic activity lost after 8 h. This pH sensitivity may be attributed to structural changes in the enzymes caused by alterations in hydrogen bonding, leading to partial destabilization of the secondary structure and active sites. It is known that acidic dicarboxylic acids are integral components of the active site of collagenases [36].

The application of various effectors – chemical compounds that either activate, inhibit, or exert no influence on enzyme activity – can yield valuable information regarding the reaction mechanism, as well as the structural characteristics and active site architecture of the enzyme. It is important to emphasize that the percentage values reflecting the effects of the tested effectors (Table 4) should not be interpreted as precise quantitative indicators of the significance of specific intramolecular structures involved in the catalytic process. To accurately assess these relation-

ships, detailed kinetic studies of enzyme inhibition or activation are required, which fall beyond the scope of this investigation.

The absence of any observable effect from PMSF and soybean trypsin inhibitor, both of which are specific inhibitors of serine proteases, suggests that this enzyme class is not present in the studied preparation. Conversely, the near-complete inhibition of collagenolytic activity by metal ion-chelating agents such as EDTA and 1,10-phenanthroline strongly supports the classification of the enzymes in this preparation as metalloproteinases.

The application of the following reagents can yield insights into both the overall molecular structure and the active sites of collagenolytic enzymes from the salivary glands of rapana. It is essential to recognize, however, that many of the compounds listed below may influence not only the properties of the enzymes but also those of the substrate molecules, which can in turn alter the observed enzymatic activity.

As reported by Nishaz Shaikh and Shirley Agwuocha (2023), Concanavalin A (Con A) specifically binds to α-D-mannose residues on proteins, inducing agglutination and consequently inhibiting their activity [37]. As shown in Table 3, a portion of the collagenases under investigation are glycosylated. For instance, gelatinase B (MMP-9), with a molecular weight of 92-82 kDa, is known to be a glycosylated enzyme (64). It is also possible that Con A hinders enzyme access to glycosylated regions of collagen, thereby reducing the rate of hydrolysis.

An activating effect of dithiothreitol (DTT), a reducing agent that cleaves disulfide bonds, has been demonstrated. Conversely, reoxidation of these disulfide bridges using hydrogen peroxide results in the opposite effect. Several factors may account for these findings. It can be assumed that the salivary gland extracts contain thiol (cysteine) proteases, which are activated by reducing agents. Additionally, it is known that collagenases in tissues can exist in inactive forms as part of complexes to prevent uncontrolled cellular degradation [31, 38]. Some of these complexes are likely stabilized by disulfide bonds between individual molecules. By reducing these bonds, DTT restores free SH-groups, thereby releasing active enzymes and increasing the overall enzymatic activity of the preparation. Moreover, reducing agents may trigger the cysteine switch mechanism, leading to the activation of proenzymes [39]. These assumptions are supported by the altered gelatinase profile observed under reducing conditions (Fig. 3).

Sulfhydryl (SH) groups are likely essential for the hydrolysis of collagen itself [40]. Modification of SH-groups – via alkylation with iodoacetate or the formation of mercaptides using mercury chloride or *p*-CMB – leads to inhibition of collagenase activity. It is known that the secondary and tertiary structures of collagen are stabilized by hydrogen bonds [41] and electrostatic interactions [42-44]. Disulfide bonds are involved only in the assembly of long, triple-helical supramolecular structures [45]. Therefore, these SH-group modifiers do not disrupt the triple-helical structure of collagen but may interfere specifically with the hydrolytic process mediated by the enzyme. In addition, the normal tertiary structure of the enzymes themselves may also be affected.

The importance of electrostatic interactions in the enzymatic reaction is supported by data on the effects of heparin, dimethyl sulfate, and acetic anhydride on collagenolytic activity. These reagents bind to free amino groups, thereby affecting the formation of electrostatic and hydrogen bonds. Dimethyl sulfate also alkylates amino, imino, and thiol groups present in proteins. It is clear that the effects of these reagents are primarily associated with their nonspecific influence on the conformation of protein molecules. However, the role of electrostatic and hydrogen bonds formed by specific amino acids cannot be ruled out [42, 43].

This latter assumption is supported by the effect of guanidine hydrochloride, which can interact with histidine and the carboxyl groups of dicarboxylic amino acids, thereby disrupting the formation of essential interactions [46]. It is important to emphasize that the guanidine concentration used (5 mmol) is too low to cause protein denaturation, as denaturing typically requires much higher concentrations – ranging from 3 to 6 molar.

These findings suggest that the identified amino acids play a crucial role in the enzymatic activity of the preparation derived from *Rapana venosa* salivary glands. The involvement of histidine, along with either glutamic or aspartic acid, in the catalytic core motif of the active site has been demonstrated for several metalloproteinases [36, 43].

Thus, the salivary glands of the rapana contain a high concentration of proteolytic enzymes. Electrophoretic analysis of the obtained preparation revealed that enzymes exhibiting gelatinase activity constitute up to 88% of the total protein content. Of

these, approximately 22% are collagenases. The proteolytic enzymes isolated from the salivary glands display polymorphism: denaturing electrophoresis identified five distinct fractions with different molecular weights.

Complete inhibition of collagenase activity by metal-chelating agents indicates the presence of metalloproteinases in the preparation. Furthermore, the effects of agents that affect disulfide bonds suggest the presence of cysteine proteases and the formation of inactive enzyme complexes through disulfide bridge formation between active polypeptides. It was demonstrated that maintaining specific electrostatic and hydrogen bonding states within the enzyme molecule is essential for collagenolytic activity.

The enzyme polymorphism is further supported by the presence of two distinct pH optima: one in the acidic range (pH 4.5) and another in the alkaline range (pH 7.5-9.5).

The optimal temperature for enzymatic activity was determined to be 46°C.

The enzymatic activity of the rapana venosa salivary gland preparation exhibits considerable stability. Under adverse conditions – pH 10.8 and 60°C – the preparation retains 70 and 50% of its activity, respectively, for up to eight hours.

Conflct of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ВИДІЛЕННЯ ТА ХАРАКТЕРИСТИКА ПРЕПАРАТУ З КОЛАГЕНАЗНОЮ АКТИВНІСТЮ ЗІ СЛИННИХ ЗАЛОЗ *RAPANA VENOSA*

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Колагенази знайшли практичне застосування як у медицині, так і в харчовій промисловості, однак пошук нових джерел колагеназ залишається актуальним напрямом досліджень. *Rapana*, хижий молюск, що переважно живиться двостулковими, багатими на сполучну тканину, привертає увагу як потенційне джерело колагенолітичних ензимів. Метою цього дослідження було виділити препарат із колагеназною активністю зі слинних залоз *Rapana venosa* та охарактеризувати його властивості. Екстракт слинних залоз очищували методом осадження ацетоном із подальшою обробкою сульфатом амонію. Електрофорез здійснювали за протоколом Леммлі в умовах відновлення та без нього. Протеолітичну активність визначали спектрофотометрично з використанням колагену або желатину як субстрату. Показано, що препарат складався з п'яти протеїнових фракцій і проявляв ензиматичний поліморфізм. Було досягнуто очищення у 13,8 раза колагеназної активності, при цьому щонайменше 22% загальних протеїнів виявляли колагенолітичну активність, тоді як 88% – желатинолітичну. Оптимум активності препарату знаходився в кислому (pH 4,5) та лужному (pH ~ 9,5) діапазонах, а температурний оптимум становив 46°C. За кімнатної температури близько 90% активності зберігалося протягом 8 год. Інгібітори серинових протеаз не впливали на активність ензиму, тоді як хелатори іонів металів повністю її пригнічували. Відновлювальні агенти, що активують SH-групи, підвищували активність ензиму, тоді як регенерація дисульфідних зв'язків або модифікація SH-груп знижували її. Отримані дані свідчать, що колагеназно-активний ензимний препарат зі слинних залоз *Rapana venosa* переважно складається з металопротеїназ і цистеїнових протеаз та характеризується високою стабільністю.

Ключові слова: протеолітичні ензими, колагеназа, очищення, *Rapana venosa*, слинні залози.

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