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SOME PROPERTIES OF THE PEPSIN NATURAL INHIBITOR EXTRACTED FROM THE MARINE GASTROPODE *Rapana thomassiana* GROSSE

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ABSTRACT:

A natural inhibitor of pepsin, developing an intensive antipeptic activity, was separated and purified from the marine gastropode Rapana thomasiana Grosse; the value of the inhibition constant, k_i, is only 0,009 mg/ml. This inhibitor is of peptidic nature, reacts almost instantaneously with the enzyme and causes an inhibition of the noncompetitive type as related to the hemoglobin used as a substrate. The inhibition developed is a function of the inhibitor concentration in the reaction medium and does not depend on the purity of the preparation used.

Within the frame of a wider research program with a view to turning the biological resources of the Black Sea to better account, during the latest years we have approached research work concerning the evidencing, isolation and purification of some natural inhibitors of pepsin, trypsin and chymotrypsin in several species of invertebrates and fish. Through the investigations

carried out, some contributions were brought to evidencing several natural inhibitors of proteolytic enzyms. Comparative data were obtained which show that the manifest inhibitory activity has some characteristics depending on the species, and within this, on the organ or tissue of extraction (3, 4).

We have found only few data in the available literature referring to a natural inhibitor of pepsin, isolated from a mixture of pepsinogen and activated pepsinogen, which has the property of inhibiting the peptic activity only within the pH range of 4.o to 6.o (1). We have no information about any such investigation in marine organisms.

We are presenting in this paper some properties of the pepsin natural inhibitor which was separated and purified from the marine gastropode Rapana thomasiana GROSSE.

MATERIAL AND METHODS

The meat (total body) of the gastropode Rapana thomasiana GROSSE was thoroughly washed with running water, then with distilled water. After having been dried by tamponing between filter paper sheets, the biological material was weighed exactly and triturated by the aid of a Potter homogenizer, the extraction ratio being 1 g tissue/lo ml distilled water. The resulted homogenate was preserved at +4°C for an hour for completion of protein extraction, then was centrifuged at 4,ooo x g for 15 minutes; the supernatant thus obtained was considered as total protein extract.

Purification of the pepsin natural inhibitor from the total protein extract was conducted following the process described in the romanian licence (5).

The antipeptic activity was expressed in percentage of pepsin lost in presence of the inhibitor. The inhibitor solution (the total protein extract dilluted as the case stood or the solution of purified inhibitor) and the pepsin solution Merck, were mixed and left to react by incubation in a thermostat at 37°C for an hour, then the residual activity of pepsin was determined.

In order to exclude any source of error, two series of determinations were carried out as follows:

- 1.o ml of Merck pepsin solution + 1.o ml of distilled water were incubated in a thermostat at 37°C for an hour, after which enzyme activity was determined;

- 1.o ml of Merck pepsin solution + 1.o ml of total protein extract (or purified inhibitor solution) were incubated in a thermostat at 37°C for an hour, after which residual enzyme activity was determined.

The percents of pepsin inhibition (antipeptic activity) by the raw or purified inhibitors are found out by calculation.

Determination of protein concentration in the total protein extract and the purified inhibitor solution was performed by the method of LOWRY and collabs. (2) and the burette method.

The pepsin activity was determined by the ANSON method (1), using hemoglobin at pH 2.o as a substrate and was expressed in nmoles tyrosine/mg protein/minute at 37°C.The antipeptic activity was determined by the same method, with the difference that the work was done on the solution of pepsin Merck, previously incubated with the inhibitor solution.

For determinations of enzymatic reaction kinetics, the inhibitor solution was introduced directly into the reaction mixture, without having been previously incubated with pepsin Merck solution.

RESULTS AND DISCUSSION

The data obtained in a previous work (4) show that the antipeptic activity presents a number of peculiarities in the marine organisms, depending on the species, and within it, on the organ or tissue. Thus, among the species under investigation, namely, the coelenterate Actinia aequina, the gastropode Rapana thomasiana and the fishes Squalus acanthias, Odontogadus merlangus euxinus and Engraulis encrassicholus ponticus, the strongest inhibition activity was made evident in Rapana thomasiana.

Studying the organ and tissue distribution of the antipeptic activity in Rapana thomasiana, an intensive activity of pepsin inhibition was made evident in all the investigated organs and tissues. Thus, the standard pepsin inhibition percentage took values varying from 79.8% (foot)to 100% (salivary glands,vitelline

gland,stomach,kidney and testis); in total body it was 81.3% ($\frac{4}{m}$).

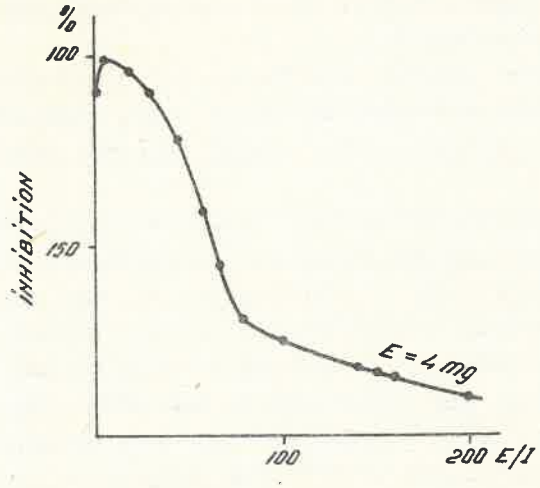

Fig.1 - Antipeptic activity as a function of the ratio of enzyme/inhibitor (E/I) in conditions of constant enzyme concentration

Incubating a constant quantity (4mg/ml) of pepsin Merck for an hour at 37°C with varying quantities of raw inhibitor (protein concentration o.o2-3.4o mg/ml), pepsin inhibition percentage was determined as a function of the Enzyme /Inhibitor (E/I) ratio (Fig.1). From the data (Fig.2),it is to be noticed that up to a certain value of E/I, the inhibition percent rises gradually up to 100%,then a short level follows and after that, a gradual decrease. Thus, within the E/I range of 1.18 - 3.28, the pepsin inhibition percent rises from 89.6% to 100%; it keeps constant (loo%) until E/I=2o, then follows a gradual sloping down to 9.8%, which corresponds to E/I=2oo (that is, o.o2 mg of unpurified inhibitor inhibits 4 mg of pepsin Merck by 9.8%).

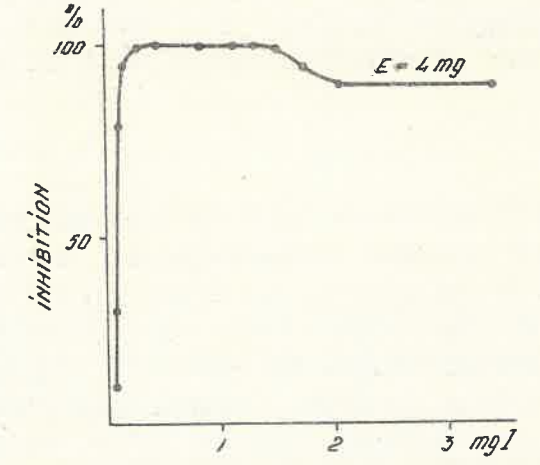

Fig.2 - Antipeptic activity as a function of inhibitor concentration in the reaction mixture

The pepsin inhibition capacity of the effector maintains itself at values within 9o-loo% for inhibitor concentrations between 3.4o and o.2o mg/ml, then falling gradually down to 9.8% for inhibitor concentrations comprised between o.2o and o.o2 mg/ml (Fig.2). At excessively high concentrations of the inhibitor in the reaction me-

dium, a slight decrease in the antipeptic activity is observed, probably due to the intermolecular interactions which are established between the peptidic chains of the effector; thus this becomes partially unable to interact with the enzyme.

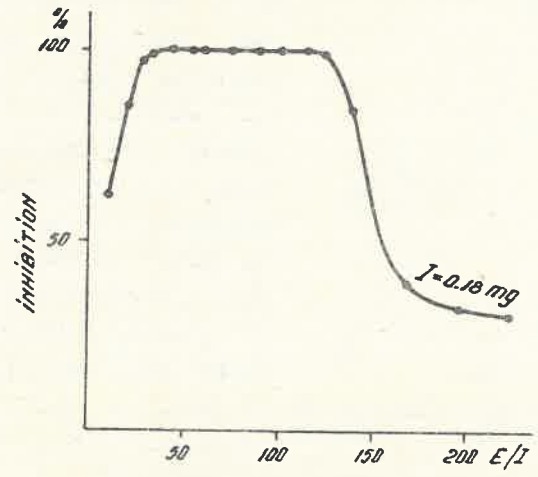

Fig.3 - Antipeptic activity as a function of the ratio of enzyme/inhibitor (E/I) in conditions of constant inhibitor concentration in the reaction mixture

The intensive pepsin inhibition capacity of the protein extracts from total body of Rapana was made still more evident in experiments during which the quantity of inhibitor was kept constant (protein conc.=o.18mg/ml) and the quantity of enzyme varied between 2 and 4o mg/ml (Fig.3). Thus, from E/I = 11.11 to E/I= 33.33 the pepsin inhibition percent rises gradually from 62.4% to 99% and beginning from a ratio 44.44 parts of enzyme to 1 part of inhibitor to a ratio of 122.22 parts of enzyme to 1 part of inhibitor the pepsin inhibition percent recorded was 100%. At higher values of the E/I ratio - from 138.9 to 222.2 - pepsin inhibition percent begins to decrease, at first gradually, then abruptly,down to 32.9%, holding afterwards at this level. In this case too, a fall in the antipeptic activity is observed, as a consequence of the excess of inhibitor in the reaction medium.

The high pepsin inhibition capacity of the protein extracts from total body of Rapana thomasiana is once more evidently pointed out (Fig.4). Thus, an amount of only o.18 mg of protein in the unpurified extract inhibits by 100% a quantity of 22 mg of Merck pepsin (the residual enzymatic activity of the pepsin after preincubation with the inhibitor being null).

Testing the time action of the purified natural inhibitor upon pepsin by incubating the standard enzyme solution with the inhibitor solution for o, 5, 1o ..... 6o minutes, 100%

pepsin inhibition was found in all the variants of the experiment, which shows that the effector reacts almost instantaneously with the enzyme and does not need any time for completing the inhibition reaction. At the same time, an high pepsin inhibition capacity is found in the unpurified preparation if taking into account that one part of purified natural inhibitor inhibits by

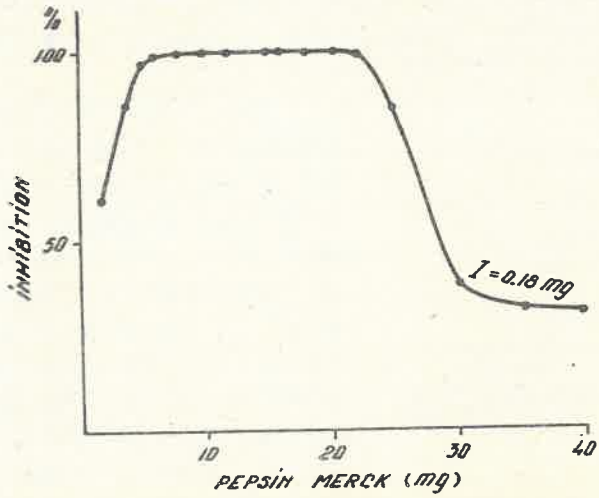

Fig.4 - Antipeptic activity as a function of enzyme concentration in the reaction mixture

100% 20 parts of enzyme almost instantaneously (Table 1).

Drawing up the Lineaweaver-Burk diagram (1/v as a function of 1/(S) for reaction kinetics of the enzyme in the absence and in the presence of the natural inhibitor isolated and purified from Rapana thomasiana, we obtained results indicating an inhibition of the non-competitive type exerted by the effector, which allow determination of the Michaelis constants (k_M and k'_M) and inhibition constant (k_i) (Fig.5).

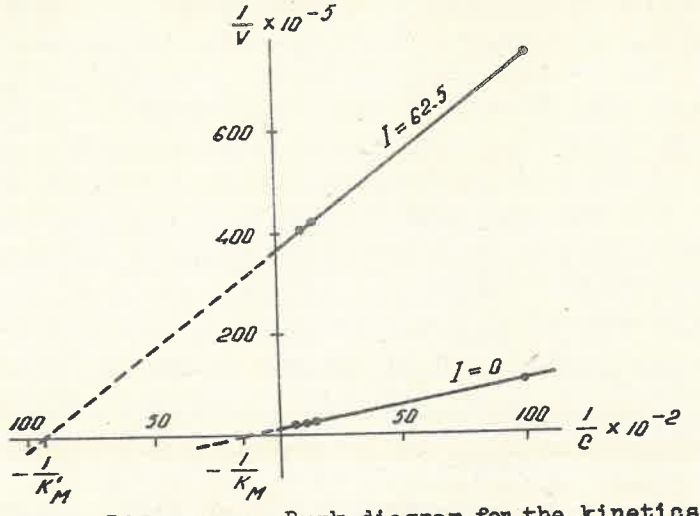

Fig.5 - Lineaweaver-Burk diagram for the kinetics of reaction of pepsin Merck in the absence and in the presence of the natural inhibitor extracted from Rapana thomasiana

Table 1

Time action of the purified natural inhibitor from Rapana thomasiana upon pepsin Merck

Time of preincubation of pepsin Merck solution with inhibitor (minutes)	Dosage of activity in:	nmoles tyr/ mg proteine/ minute, 37°C	Inhibition %
o	Pepsin Merck solution	15,48o	
	Pepsin Merck solution preincub.with inhibitor	o	loo
5	Pepsin Merck solution	15,o66	
	Pepsin Merck solution preincub.with inhibitor	o	loo
1o	Pepsin Merck solution	15,51o	
	Pepsin Merck solution preincub.with inhibitor	o	loo
15	Pepsin Merck solution	15,117	
	Pepsin Merck solution preincub.with inhibitor	o	loo
2o	Pepsin Merck solution	15,7o3	
	Pepsin Merck solution preincub.with inhibitor	o	loo
25	Pepsin Merck solution	15,75o	
	Pepsin Merck solution preincub.with inhibitor	o	loo
3o	Pepsin Merck solution	15,5oo	
	Pepsin Merck solution preincub.with inhibitor	o	loo
45	Pepsin Merck solution	15,545	
	Pepsin Merck solution preincub.with inhibitor	o	loo
6o	Pepsin Merck solution	15,6oo	
	Pepsin Merck solution preincub.with inhibitor	o	loo

Observations:
- inhibitor concentration was o.2o mg/ml;
- Merck pepsin concentration was 4 mg/ml;
- E/I ratio equaled 2o.

The Michaelis constants were calculated from the values of $-1/k_M$ and $-1/k'_M$ determined by intersection of the x axis by the straight lines obtained from the graphic representation of the experimental data in the equation of Lineaweaver-Burk:

$$\frac{1}{v} = f\left[\frac{1}{(S)}\right]$$

Thus: k_M = Michaelis constant of the enzymatic reaction in the absence of inhibitor = 8.33 mg/ml;

k'_M = Michaelis constant of the enzymatic reaction in the presence of inhibitor = 1.05 mg/ml.

The inhibition constant was determined by the equation:

$$k'_M = \frac{k_M}{1 + \frac{i}{k_i}}$$

characterizing the noncompetitive inhibition, where i is the concentration of inhibitor in the reaction medium (in this case i = 0.0625 mg/ml); it resulted that k_i = 0.009 mg/ml.

The value of the inhibition constant (k_i) expresses, as it is known, the inverse of the affinity of an enzyme toward the inhibitor; in other words, the smaller the value of k_i, the stronger the action of the inhibitor on that enzyme. The particulary small value of the inhibition constant (k_i = 0.009) and the fact that a concentration of only 62.5 µg of inhibitor in the reaction medium determines a decrease in the k_M value with 87.4% points out once again a highly pronounced capacity of pepsin inhibition by the natural inhibitor extracted from Rapana thomasiana.

The preliminary data concerning the determination of the chemical character of the inhibitor shows that this is of peptidic nature, with a short chain of aminoacids, among which the following are prevalent: lysine, serine, aspartic acid, glutamic acid and phenylalanine (5).

CONCLUSIONS

The natural inhibitor of pepsin that was separated and purified from Rapana thomasiana is a peptide with the following characteristics:

- it develops an intensive activity of pepsin inhibition;
- is not in competition with hemoglobin for linking the active catalytic situs of the enzyme thus exerting an inhibition of the noncompetitive type;
- reacts almost instantaneously with the enzyme and needs no time for completion of the inhibitive reaction;
- the inhibition developed is a function of concentra-

tion of inhibitor in the reaction medium and does not depend on the purity of the preparation used.

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