Screening of serine proteinase inhibitors from marine organisms

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ABSTRACT

Marine organisms are an important source of bioactive polypeptides with hemolytic or cytolytic activity, potassium channel-modulators and proteinase inhibitors. In this work we carried out the screening of serine proteinase inhibitors from twenty three species of marine organisms. Marine animals belonging to *Phyla Coelenterata (Subphylum Cnidaria)*, *Annelida*, *Mollusca*, *Echinodermata*, Chordata *(Subphylum Tunicata Class Ascideaceae)*, and marine plants were collected on the northern coast of Havana, Cuba. Aqueous extracts were prepared by homogenization of whole animal bodies or the marine plants and freeze-dried. Trypsin, human plasma kallikrein and chymotrypsin inhibitory activities were determined by monitoring the hydrolysis of specific chromogenic substrates. Trypsin inhibitory activity was found in four species: *Plexaura sp.*, *Aplysia sp.*, *Actinopyga sp*. and *Galaxaura sp*. Antichymotrypsin activity was found in *Galaxaura sp*. and *Gorgonia sp*. Human plasma kallikrein inhibitory activity was found in *Plexaura sp*. The action of the extracts from marine organisms on enzymes that participate in the blood clotting was observed by the prolongation of prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT), used as clotting time assay. Five species (*Plexaura sp.*, Gorgonia sp., *Galaxaura sp.*, *Aplysia sp*. and *Actinopyga sp.*) increased aPTT. *Gorgonia sp., Actinopyga sp*. and *Aplysia sp*. extracts prolonged aPTT but not PT. *Actinopyga sp*., *Aplysia sp*., *Gorgonia sp*., *Galaxaura sp*. and *Plexaura sp* extracts increased TT, however, only *Aplysia* sp. did not prolong TT after heat treatment at 60 °C for 15 min.

Keywords: inhibitors, invertebrates, marine organisms, serine proteinases, tunicate

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RESUMEN

Tamizaje de inhibidores de serino proteinasas procedentes de organismos marinos. Los organismos marinos son una fuente importante de polipéptidos bioactivos con actividad hemolítica y citolítica, moduladores de canales de potasio e inhibidores de proteasas. En el presente trabajo realizamos el tamizaje de inhibidores de serino proteinasas en 23 especies de organismos marinos así como su acción sobre el tiempo de protrombina (TP), tiempo de tromboplastina parcial activada (TTPa) y tiempo de trombina (TT). Los organismos marinos fueron colectados en la costa norte de la Habana, Cuba y pertenecen a *Phyla Coelenterata (Subphylum Cnidaria)*, *Annelida*, *Mollusca*, *Echinodermata*, *Chordata (Subphylum Tunicata, Clase Ascideacea)* y plantas marinas. Se encontró actividad inhibidora frente a tripsina en 4 especies: *Plexaura sp.*, *Aplysia sp.*, *Actinopyga sp*. y *Galaxaura sp*. y actividad antiquimotripsina en *Galaxaura sp*. y *Gorgonia sp*. Se detectó actividad inhibidora frente a calicreína plasmática humana en *Plexaura sp*. El TTPa se incrementó con 5 especies (*Plexaura sp.*, Gorgonia sp., *Galaxaura sp.*, *Aplysia sp*. y *Actinopyga sp.*). Los extractos de *Gorgonia sp., Actinopyga sp*. y *Aplysia sp*. incrementaron el TTPa pero no PT. El TT fue incrementado por *Actinopyga sp*., *Aplysia sp*., *Gorgonia sp*., *Galaxaura sp*. y *Plexaura sp*. Después del calentamiento de los extractos a 60 °C, por 15 min sólo el extracto de *Aplysia* sp. no prolongó el TT.

Palabras claves: inhibidores, organismos marinos, serino proteinasas, Tunicata

*I*ntroduction

Proteinases have been firmly established as critical regulatory components in a number of physiological and pathophysiological processes such as fertilization, cell migration, hormone processing, digestion, fibrinolysis, tumor invasion and blood coagulation [1].

Trypsin is the best-studied serine endopeptidase. Other proteinases such as kallikreins and chymotrypsin belong to the trypsin family [2] and although they are structurally and catalytically very similar to trypsin, they differ in some aspects such as their substrate preference [3].

Proteinase inhibitors play a crucial role in the regulation of proteolytic processes [4, 5] and have been identified as important factors in the physiology of several human disorders [6]. Blood serine proteinase inhibitors have been explored in recent years and increasingly applied in sepsis, therapy of cancer and other human diseases [7]. Serine proteinase inhibitors make up the major group of proteinase inhibitors known and characterized so far. They are found in animal and vegetal tissues as well as in microorganisms [4, 8]. Serine proteinase inhibitors are classified in several families like Kunitz-type, Kazal-type or serpins, among others. The Kunitz family is one of the best-characterized serine proteinase inhibitors [9] that can block enzymes involved in platelet aggregation, blood coagulation, fibrinolysis and inflammation [10, 11]. Kazal-type inhibitors are found in all studied vertebrates and their main physiologi-

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cal role is to inhibit trypsin in the pancreas or pancreatic duct [12]. Kazal-type inhibitors do not block plasma kallikrein activity, but they have been appearing as important controllers of blood clotting and fibrinolytic factors [13].

Marine organisms are interesting sources of proteinases and their inhibitors. The physiological role of proteinase inhibitors in such organisms is not yet clear. It has been suggested that they could play a defensive role against preys or predators. *Phylum Coelenterata* is the most widely studied in marine animals. In this *Phylum*, serine proteinase inhibitors have already been isolated or partially purified from eight species such as *Anemonia sulcata* [14-17], *Radianthus macrodactylus* [18, 19], *Radianthus koirensis* [20], *Rhodactis rhodostoma* [20], *Stoichactis* sp [21], *Stichodactyla helianthus* [22, 23], *Antholeura aff. xanthogrammica* [24] and *Condylactis gigantea* [25]. All described inhibitors belong to the Kunitz family, except the *A. sulcata* elastase inhibitor that is a Kazal-type inhibitor. Regarding other *Phyla*, an inhibitor has been described against trypsin, chymotrypsin and plasmin in the *Sabellastarte indica* [26] and a trypsin inhibitor from *Lymnaea* mollusc belonging to the Kunitz family [27]. In tunicates, a Kazal-type inhibitor in ascidian *Halocynthia roretzi* [28, 29] has been isolated and characterized.

In the present work we screened serine proteinase inhibitors from the aqueous extracts of twenty-three species of marine organisms against three serine proteinases: trypsin, chymotrypsin and human plasma kallikrein. Moreover, we tested their activity on prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin time (TT) using normal human plasma.

Material and Methods

Twenty species of marine animals and three species of marine plants (Table 1) were collected on the northern coast of Havana, Cuba.

The marine animals have sea water inside their bodies, which is expelled when they are captured. This solution was used to prepare the aqueous extracts by homogenization of the whole animal bodies (w/v:1/2); the extracts were centrifuged and freeze-dried. Protein concentration was evaluated by the Lowry method [30].

Soybean trypsin inhibitor (SBTI) was purchased from Serva and p-nitrophenyl-p-guanidinobenzoate (NPGB) from the Merck Chemical Company; p-Nitroanilide (p-Nan) substrates DL-Bz-Arg-pNan and Suc-Phe-pNan were purchased from the Sigma Chemical Company, and Ac-Phe-Arg-pNan was a gift from Dr. Luiz Juliano, Department of Biophysics, UNIFESP, São Paulo, Brazil.

Bovine pancreatic trypsin and chymotrypsin were purchased from Boehringer Mannheim; homogenous human plasma kallikrein (HuPK) was purified by a previously described procedure [31]. Human thrombin was purchased from Roche.

The molar concentration of the trypsin solution was determined by active-site titration with NPGB [32]. HuPK concentration was determined on the hydrolysis of Ac-Phe-Arg-pNan in the presence of SBTI assuming a slow tight-binding mechanism, where enzyme and inhibitor form a stoichiometric complex [33].

Table 1. Marine organisms collected on the northern coast of Havana, Cuba.

Marine ANIMALS	
A) *Phylum Coelenterata*	C) *Phylum* Annelida
Subphylum Cnidaria	1. *Hermodyce sp.* (Her)
1. *Cassiopeia sp.* (Cas)	2. *Sabellatarte sp.* (Sab)
2. *Bartolomea sp.* (Bar)	D) *Phylum Mollusca*
3. *Condylactis sp.* (Con)	1. *Aplysia sp.* (Apl)
4. *Budonosoma sp.* (Bud)	2. *Lima sp.* (Lim)
5. *Gorgonia sp.* (Gor)	E) *Phylum Chordata*
6. *Plexaura sp.* (Ple)	*Subphylum Tunicata*
7. *Palitoa sp.* (Pal)	Class Ascideacea
8. *Zoanthus sp.* (Zoa)	1. *Molgula sp.* (Mol)
B) *Phylum Echinodermata*	2. One specie of *Microcosmus* (Micr)
1. *Isostichopus sp,* (Iso)	3. Another specie of *Microcosmus* (Mic)
2. *Holoturia sp.* (Hol)	4. *Pyura sp.* (Pyu)
3. *Actinopyga sp.* (Act)	5. *Phallusia sp.* (Pha)
Marine PLANTS *(Algae)*	
1. *Galaxaura sp.* (Gal)	
2. *Caulerpa sp.* (Cau)	
3. *Opuntia sp.* (Opu)	

Proteinase inhibitory activity and dissociation constant determination

The inhibitory activities on trypsin, chymotrypsin or human plasma kallikrein were determined by measuring the remaining hydrolytic activity towards synthetic specific substrates DL-Bz-Arg-pNan, Suc-Phe-pNan and Ac-Phe-Arg-pNan, respectively, after pre-incubation of the enzyme with different marine organisms extracts for 10 min at 37 ºC. Enzyme-inhibitor mixtures (120 mL) were added to a solution containing substrate (0.8 mM) in 0.05 M Tris buffer, pH 8.0, 37 ºC, in 290 mL final volume, the reaction being followed for 10 min and stopped by 50 mL of 30% (v/v) acetic acid. The substrate hydrolysis was followed by absorbance at 405 nm and the molar extinction for *p*-nitroaniline used for concentration calculation was 9100 [34].

The equilibrium dissociation constant (Ki) and the inhibitor concentration were evaluated for each proteinase, following preincubation of the enzyme with increasing concentrations of the inhibitor. Residual activity was subsequently measured with convenient substrates. Apparent Ki were determined assuming a slow-tight binding mechanism, using non-linear fitting (Grafit software) [35].

One unit of inhibition activity was defined as the amount of protein needed to inhibit one unit of enzymatic activity. One unit of enzymatic activity was defined as the enzyme activity that hydrolyzed one mole of substrate per min under specified conditions. The specific activity was expressed as inhibitory activity units of each extract related to protein concentration determined by Lowry's method [30].

Coagulation test

Prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time, (aPTT) were measured according to standard procedures [36]. All incubations were performed in the presence of different marine organism extracts to study the inhibitory activity on clotting time. Saline solution was used in the control experiments. PT and aPTT were developed with Test

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Excel Simplastin and Platelin LS, respectively, purchased by Organon Teknika Corporation. TT was assayed with citrate plasma and thrombin [36].

The extracts that prolonged TT were heated at 60 ºC for 15 min and centrifuged. Then, they were tested again.

Results and Discussion

Proteinase inhibitory activity and dissociation constant determination

Inhibitory activity against serine proteinases: bovine pancreatic trypsin, chymotrypsin, and human plasma kallikrein is shown in Table 2. Four species (Ple, Apl, Act and Gal) showed antitrypsin activity, Gal and Gor presented the chymotrypsin inhibitory activity and only the Ple extract showed inhibitory activity against HuPK in the assayed conditions.

Table 3 and Figure 1 show the apparent equilibrium dissociation constant (Ki) values for the inhibitors from these extracts. The best apparent value of Ki (14 nM) for trypsin was found in the Apl extract which did not inhibit either chymotrypsin nor plasma kallikrein in the assayed conditions. 0nly two extracts (Gal and Gor) showed inhibitory activity against chymotrypsin; Gal presented the best value for apparent Ki extract (38 nM). Ple was the only extract that inhibits HuPK (Ki 500 nM).

Coagulation test

Physiological models were also used to investigate the inhibitory activity of the marine extracts. Figure 2 shows the action of extracts on aPTT, PT and TT. These extracts should interfere with contact phase clotting enzymes, because pre-incubation of Ple, Apl, Act, Gal and Gor extracts with fresh human plasma causes a prolongation of aPTT; as Ple is the only extract that inhibits HuPK in the assayed conditions, probably Apl, Act, Gal and Gor extracts could be acting in other contact system proteins.

These extracts were heated at 60 ºC for 15 min and their effects on thrombin time were subsequently determined. Under these conditions Apl extract did not prolong TT, but this time was excessively increased for Ple, Apl, Act, Gal and Gor extracts; it is an evidence that these extracts could have some thrombin inhibitor in the coagulation cascade.

Curious results were shown by the Apl extracts: the best antitrypsin activity (Ki 14 nM) and prolonged aPTT and TT. When this extract was preheated for 15 min at 60 ºC, it did not prolong TT. Glycosaminoglycans as condroitin sulfate and heparan sulfate forms have been identified in Aplysia [37]. These substances inhibit coagulation and this

Table 3. Apparent dissociation constant (Ki) for different marine species.

Species	Ki (nM)		
	Trypsin	Chymotrypsin	HuPK
Act	430	ni	ni
Apl	14	ni	ni
Heated Apl	5.0	ni	1.0
Ple	320	ni	500
Gal	720	38	ni
Gor	ni	350	ni

ni: inhibition not detected.

Ki and the inhibitor concentration were evaluated for each proteinase, following the preincubation of the enzyme with increasing concentrations of the inhibitor using specific substrates for each enzyme: DL-Bz-Arg-pNan (trypsin), Suc-Phe-pNan (chymotrypsin) and Ac-Phe-Arg-pNan (HuPK).

Table 2. Effect of marine organism extracts on serine proteinase activity.

Species	Protein (mg/mL)	Inhibition activity (U/mL)			Specific inhibitory activity (U/mg)		
		Try*	Chymo**	HupK	Try*	Chymo**	HuPK
Gor	0.9	ni	222	ni	ni	247	ni
Ple	1.2	157	ni	146	131	ni	122
Pal	1.4	ni	ni	ni	ni	ni	ni
Cas	0.2	ni	ni	ni	ni	ni	ni
Bar	0.8	ni	ni	ni	ni	ni	ni
Con	9.2	ni	ni	ni	ni	ni	ni
Bud	1.0	ni	ni	ni	ni	ni	ni
Zoa	0.4	ni	ni	ni	ni	ni	ni
Her	1.0	ni	ni	ni	ni	ni	ni
Sab	0.6	ni	ni	ni	ni	ni	ni
Apl	0.6	32	ni	ni	50	ni	ni
Heated Apl	0.5	54	ni	66	100	ni	122
Lim	0.3	ni	ni	ni	ni	ni	ni
Act	0.2	42	ni	ni	175	ni	ni
Iso	0.6	ni	ni	ni	ni	ni	ni
Hol	0.2	ni	ni	ni	ni	ni	ni
Mol	0.5	ni	ni	ni	ni	ni	ni
Micr	1.0	ni	ni	ni	ni	ni	ni
Mic	0.9	ni	ni	ni	ni	ni	ni
Pyu	0.7	ni	ni	ni	ni	ni	ni
Pha	0.7	ni	ni	ni	ni	ni	ni
Gal	0.3	23	29	ni	78	97	ni
Cau	0.1	ni	ni	ni	ni	ni	ni
Opu	0.3	ni	ni	ni	ni	ni	ni

*Trypsin, ** Chymotrypsin, ni – inhibition not detected. Protein concentration was evaluated by the Lowry method. One unit of inhibition activity was defined as the amount of protein needed to inhibit one unit of enzymatic activity. The specific activity was expressed as inhibitory activity units of each extract related to protein concentration determined by Lowry method.

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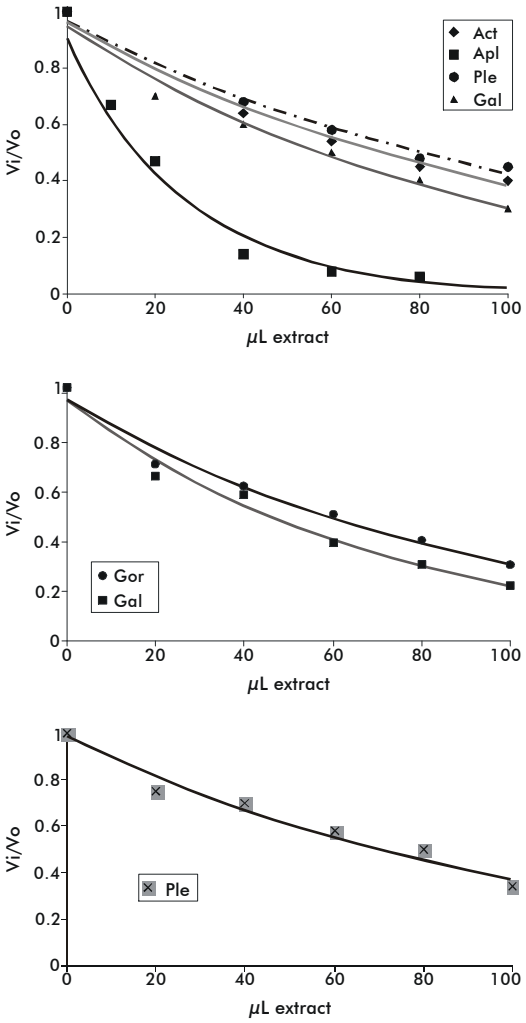

Figure 1. Serine proteinase inhibition by different extracts from marine organisms. The enzymes were pre-incubated (10 min at 37 °C in 0.05 M Tris buffer, pH 8.0) with increasing amounts of the extracts. The residual activities were assayed on specific substrates (0.8 mM), as described in Materials and Methods. A) trypsin (0.2 μM) activity was tested on DL-Bz-Phe-pNan. B) chymotrypsin (0.1 μM) activity was tested on Suc-Phe-pNan. C) HuPK (0.1 μM) activity was tested on Ac-Phe-Arg-pNan. Vi rate was determined in the presence of the inhibitor, Vo rate in the absence of the inhibitor.

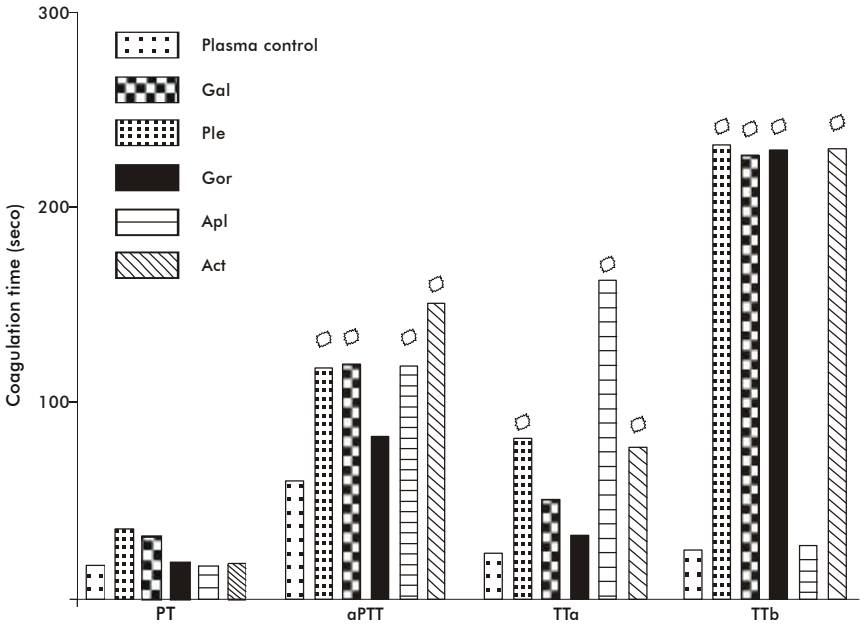

Figure 2. Effect of the marine organism extracts on blood clotting. Prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TTa) were used for the clotting time test. Extracts from Ple, Gal, Gor, Apl and Act were used in the range of 10 - 20 mg (Lowry's method). Thrombin time was also determined for heated extracts at 60 °C for 15 min (TTb). Saline solution was used in the plasma control. The bar diagram represents the ±S.E. values taken from three independent experiments, ⬡ denotes significant difference at levels of $p < 0.05$.

mechanism has been extensively studied. As the glycosaminoglycans are stable during this heat treatment, the lost of prolonged TT, is probably due to the existence of unstable proteins at 60 ºC, which could be involved in the inhibition of the intrinsic coagulation pathway. On the other hand, the heated extract inhibited plasma kallikrein and prolonged aPTT (results not shown), suggest the presence of a stable kallikrein inhibitor.

Ple extract showed inhibitory activity against trypsin and kallikrein; furthermore it also prolonged aPTT and TT. When this extract was pre-heated for 15 min at 60 ºC, TT was also increased. As prostaglandin inhibits platelet aggregation, it is possible that the anticoagulant action of the Ple extract can be due to these compounds. It is known that prostaglandin intermediaries were found in *Plexaura homomalla*, until now the only non-mammalian source of these compounds [38].

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