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Invertebrate proteinase inhibitors

Rose-Anne Boigegrain*, Helene Mattras*, Michel Brehelin¶ and Maria-Antonia Coletti-Previero*

**INSERM U 58, 60 rue de Navacelles, 34090 Montpellier and ¶Lab. de Pathologie Comparée, INRA-CNRS, URA 1184, Pl. E. Bataillon, 34095 Montpellier, France*

Abstract

Peptides with inhibiting activity toward proteinases were isolated from marine invertebrates, insect plasma and nematodes. Some of them were characterized and studied in relation to their physiological function.

Protease inhibitors are extremely widespread. In the plant kingdom (ref. 1) they are mainly single chain polypeptides with molecular weights from 3,000 to about 13,000, and showing strong inhibitory power (k_d from 10^{-9} to 10^{-12} M). Kinetically, they behave as no-turnover substrates of the enzyme and two molecular forms of each inhibitor are known, the virgin form (V, whole peptide chain) and the modified form (M, cleaved peptide chain), kept in shape by the disulfide bridges. They show similar inhibitory power (ref. 2) toward their target enzyme (k_d V/k_d M $\sim$ 1). In the animal kingdom they can be both of high and low molecular weight and show different kinetic behaviour.

Proteins with enzyme inhibitory activity have been actively studied as such and in connection with their target protease(s) (ref. 3). Proteinase inhibitors have also been screened, identified and studied to interact with selected target proteases, involved in a pathological condition. They are powerful tools for understanding at a molecular level insect immunity. Insects react to invasion of parasites by capsule formation: this reaction, brought about by their hemocytes, ends with the enzymatic oxidation of Tyr to melanine (ref. 4). The enzyme responsible is a phenoloxidase*, which arises by proteolysis from the pro-enzyme, under the control of a cascade of proteases. Thus, the corresponding inhibitors can be regarded as potential bio-insecticides.

The division between the fundamental studies on inhibitors and their use as tools for other studies is, of course, an intellectual outlook, since the molecular basis is the starting point of any other approach and a research with different aims can, in return, enhance the molecular knowledge.

In this paper we present microproteins with inhibitory power against proteases, isolated from invertebrates. These peptides are short enough (around 35 amino acids) to allow a subsequent chemical synthesis without major difficulties.

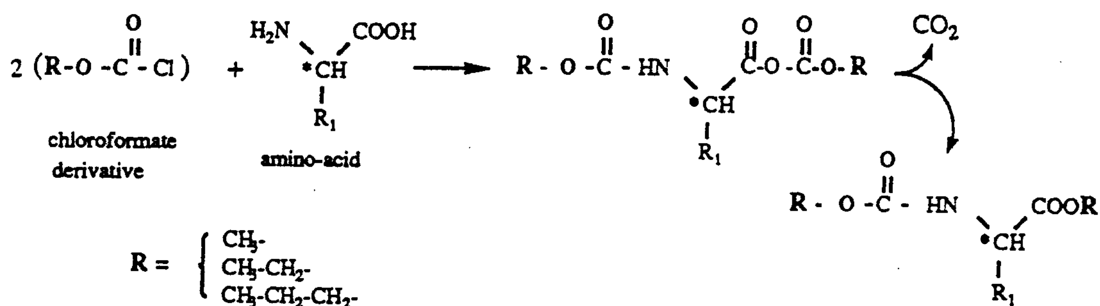
EXPERIMENTAL

Bovine trypsin from Cooper Biomedical, bovine α-chymotrypsin, Carlsberg subtilisin type VIII, human thrombin, porcine elastase, HLE and L-Dopa from Sigma CC were used in the present study. The substrates Bz-Arg-ethyl ester and Bz-Arg *p*NA were from Fluka; Suc-Ala-Ala-PheMCA, Suc-Ala-Ala-Val MCA, Boc-Val-Pro-Arg MCA and Suc-Ala-Ala-Ala *p*NA from Bachem. Initial rates of enzyme activity were measured as a function of time on a Kontron spectrophotometer or an Hitachi spectrofluorimeter, depending on the substrate leaving group.

*Abbreviations. PO, phenoloxidase; pro-PO, pro-phenoloxidase; pro-PO-AS, pro-phenoloxidase activating system; HLE: human leukocyte elastase; LPS, lipopolysaccharide; PCF, propyl chloroformate; ECF, ethyl chloroformate; LMCI I, Locusta migratoria chymotrypsin inhibitor I; LMCI II, Locusta migratoria chymotrypsin inhibitor II.

Extraction and isolation of the peptides were achieved with classical chromatographic methods. Their activity against proteinases was tested as follows: 5 to 20 μl of pure commercial enzyme and suitable samples of the inhibitor were incubated in 50 mM Tris buffer, 20 mM $CaCl_2$, pH 8, 25°C. The total volume of the mixture was 1 ml. The specific substrate was added after a suitable time and the changes in O.D. or in fluorescence emission recorded. Kd values were determined at high dilution by the method of Green & Work (ref. 5) as modified by Laskowski (ref. 6) and were evaluated by a computerized fitting of the experimental values of Vrel, as already described (ref. 7).

For the determination of PO activity (ref. 8), the *Locusta migratoria* hemocyte pellet was rinsed, homogenized and centrifuged 10 min at 12,000 g. The supernatant (10 μl), which contains all the enzymes of the pro-PO-AS, was diluted with 100 μl of cacodylate buffer (10 mM, pH 6.9, 10 mM $CaCl_2$) and 10μl of trypsin (3.10^{-6} M in water) or LPS were added. After incubation (2 hours, 25°C) the PO activity was tested with L-DOPA as substrate. The inhibition was tested with the same method, only adding 1 to 10 μl of the inhibiting solution to the mixture before starting the activity. The amino acid analysis were performed with the method of Husek (ref. 9), modified as follows. After acid hydrolysis, the amino acid residues (5 μl) were treated with PCF in water/ethanol/pyridine, 60:32:8 (5 μl), 5 min, room temperature.



Chloroform (100 μl) was added and, after 10s strong agitation followed by a 5s centrifugation, an aliquot of the chloroform layer was injected in a Shimadzu gas-chromatograph, model GC 14 APFSC, with a FID detector. PCF was prefered to EDF (Scheme) because it allowed a better separation between Ser and Glu and the response of the detector was higher.

Fig. 1 shows a typical elution profile of an equimolecular mixture (25 picomoles) of amino acids.

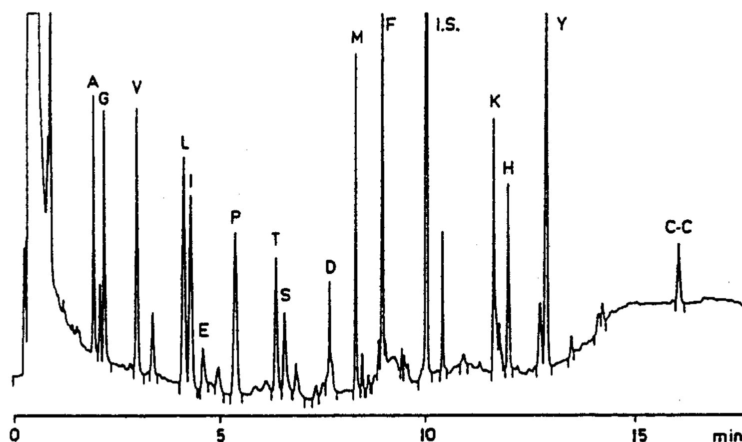


Fig. 1. GC-FID analysis of an equimolecular mixture of the specified amino acids on OV-1701 capillary.

MARINE INVERTEBRATE THROMBIN INHIBITOR (*Elisella*)

Thrombin is a target protease for the development of drugs aimed to avoid blood clots. The recently discovered (refs. 10,11) action of thrombin on neuronal cells by activation of a specific receptor, initiating a still unknown cascade, has revived the interest for specific inhibitors.

Table I : Purification of thrombin inhibitor from *Elisella* powder

step	protein (mg)	total activity*	specific activity	yield %
crude powder	138	87.5	0.616	-
G 25 Sephadex	0.515	63	122	72
HPLC	0.4	60	150	68.5

* IC 50 against 0.15 nanomole of human thrombin.

While studying proteinase inhibitors from storage organs of plants (refs. 12,13), no thrombin inhibitors were found, which was hardly surprising since a plant inducing bleeding would not have escaped attention, even in ancient cultures. We started a screening of marine invertebrates from the New Caledonian lagoon and found this activity in Gorgonidae (*Cnidaria, Gorgonacea*) also called sea fans and horny corals. Two invertebrates were studied, *Elisella sp.* and, to a lesser extent, *Subergorgia mollis.* This choice was partly due to chance and partly due to the observation, known to all submarine divers, that coral wounds bleed more strongly than normal.

The *Elisella* extract was adsorbed on Sephadex G 25 and can only be eluted by enhancing the acetic acid concentration. In the process it purifies more than 200 times (Table I, Fig. 2). An HPLC chromatography completed the isolation, which was therefore surprisingly simple.

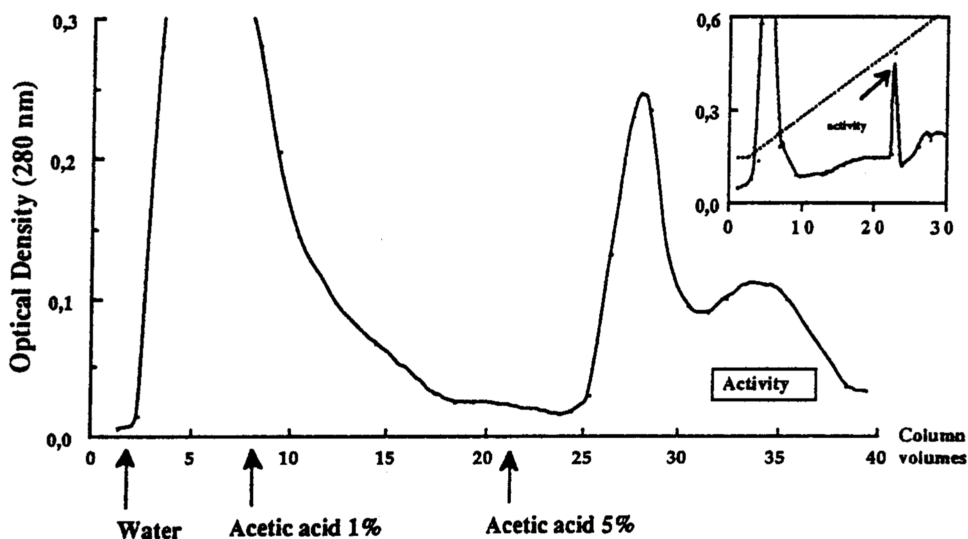


Fig. 2. Sephadex G 25 pseudo-affinity chromatography of *Elisella* extract. Thrombin inhibitory activity is indicated.

INSECT PROTEASE INHIBITORS (*Locusta migratoria*)

Phenoloxidase, a key enzyme in the defense mechanism of several arthropods (crustaceans and insects), is present as a proenzyme in their hemolymph and cuticle (ref. 4). In hemolymph it leads to the formation of antifungal quinones and to melanine around foreign bodies such as potential parasites of insects. Activation of pro-PO is the final step of a complex cascade of proteolytic enzymes and can be triggered by a number of events including molecules from microorganism walls. Some of the proPO-AS components also act as "opsonin-like molecules" in the recognition of the non-self in arthropods (ref. 14). The pro-PO-AS from the hemocytes of *Locusta migratoria*, is very convenient since it is practically devoided of auto-activation and the enzymes of the cascade are located in a different hemolymph compartment from their inhibitors.

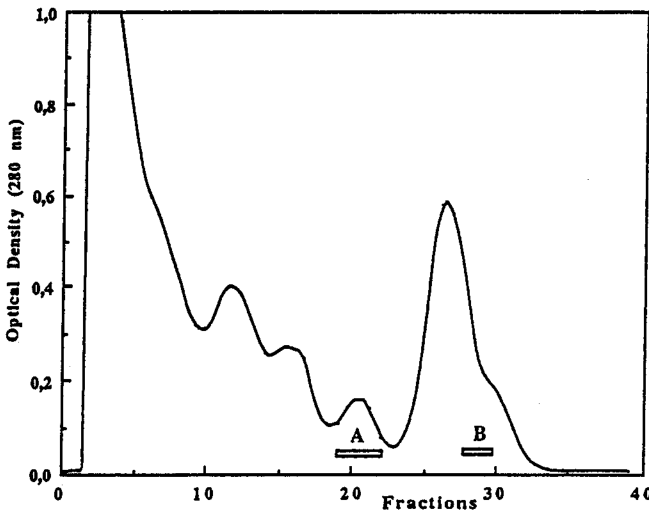


Fig. 3.
Sephadex G75 chromatography of *Locusta* plasma. Zone A inhibits the pro-PO-AS, when triggered by trypsin, zone B inhibits the pro-PO-AS, when triggered by LPS.

When *Locusta* plasma was applied on a Sephadex G 75 column, two zones of inhibition could be detected, not only different in position (Fig. 3) but also for their effect on PO-AS. Zone A inhibits the trypsin induced PO formation and zone B the LPS induced PO formation. Beside this physiological activity, they both proved to be inhibitors of α-chymotrypsin.

This result suggests that the cascade leading to PO formation is branched, with one branch stimulated by trypsin or a trypsin-like enzyme, the other by LPS (Fig. 4).

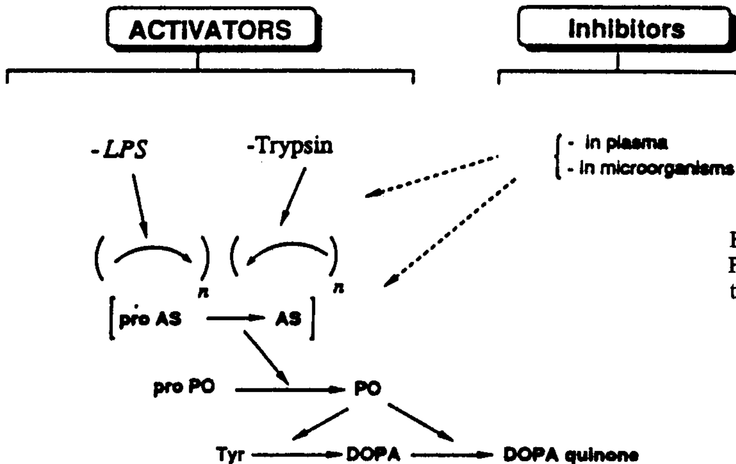


Fig. 4.
Proteolytic cascade leading to PO formation

Two protease inhibitors from zone A, LMCI I and LMCI II (refs. 15,16) were isolated and sequenced.

	1 10 20 30
LMCI I	E E K C T P G Q V K Q Q D C N T C T C T P T G V W G C T R K G C Q P A
	1 10 20 30
LMCI II	E I S C E P G K T F K D K C N T C R C G A D G K S A A C T L K A C P N Q

* identical residues underlined

In spite of 42 % identity with identical cysteines position, the two peptides showed a different specificity. They both inhibited α-chymotrypsin: LMCI I was also active on HLE while LMCI II inhibited pancreatic elastase. Both peptides lack methionine, which is usually the target of α-chymotrypsin cleavage in other natural inhibitors.

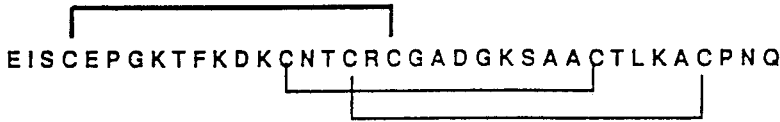


Fig. 5. Disulfide bridges assembly.

LMCI II was digested with trypsin and subjected to HPLC. The aminoacid composition of the tryptic peptides clearly indicated a 1st-4th, 2nd-5th and 3rd-6th cysteines linkage (Fig. 5). The LMCI II disulfide bridge pattern and size resemble the Squash family (ref. 17).

The Chou & Fasman (ref. 18) probability plot (Fig. 6) showed similar β-turn forming section for the two peptides, with a consistent probability value near the N-terminal.

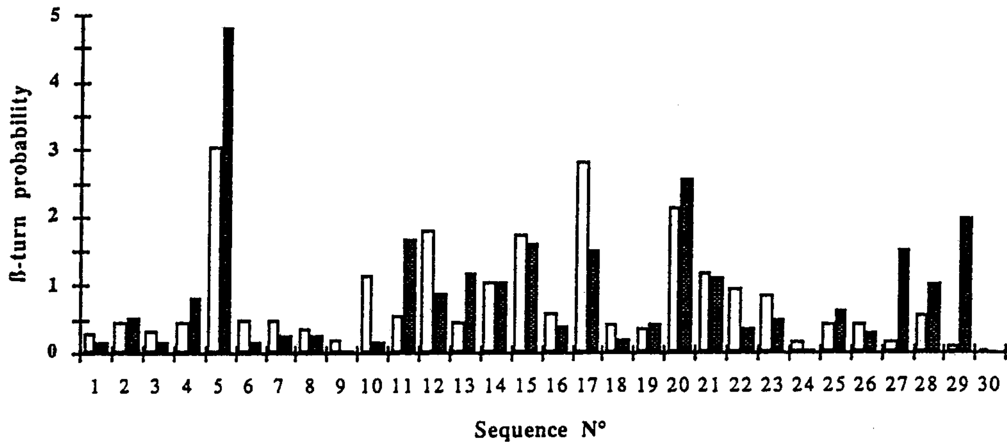


Fig. 6. Chou & Fasman probability plot (x 10 000) of LMCI I (white), and LMCI II (gray).

Peptides nearly identical to LMCI I and LMCI II were isolated from *Locusta* brain and given as putative neuropeptides (ref. 19) of unknown activity. If they are not contaminants from the hemolymph, they could be involved in the brain as safegard components to prevent proteolytic damage or have additional control functions, important for the development and the plasticity of the nervous system.

There is very little similitude between these molecules and the proteinase inhibitors, isolated from other insects (Table II). To date they are the lowest molecular weight protease inhibitors isolated from the hemolymph of arthropods and they are the lowest molecular weight inhibitors able to inhibit at least two different seryl-proteases. The role of these proteinase inhibitors in hemolymph of arthropods, although extensively studied, still needs investigation.

NEMATODE pro-PO-AS INHIBITORS (*Steinernema*)

Nematodes of the *Steinernematidae* family are entomopathogenic. They are mass reared on a large scale (ref. 35) for use in biological control. When present in insect hemocoel, they are not recognized as non-self and kill insects after action of their own toxins and those of their symbiotic bacteria *Xenorhabdus sp.* When *Xenorhabdus nematophilus* is plated on a solid substrate, two colony forms were observed (ref. 36) which were named phase I (active) and phase II (inactive). Phase I bacteria were able to secrete into the culture medium inhibitor(s) of the proPO-AS, triggered either by trypsin or by LPS. We studied in parallel the inhibiting power of culture medium from the two phases: they were chromatographed on a Sephadex G 75 and the fractions with a PO inhibiting activity from phase I bacteria eluted almost at the end of the column. These fractions and their

Table II : Protease inhibitors from arthropods hemolymph

Hemolymph of	Target Enzymes	Molecular weight (kDa)	Chemical Characterization	Ref.
Pacifastacus leniusculus	PO cascade, trypsin, chymotrypsin, elastase	155	AAA*	20
P. leniusculus	PO cascade	340	AAA	21
Limulus polyphemus	trypsin, chymotrypsin	15.7	partial sequence	22
Bombyx mori	chymotrypsin	43	AAA	23
Bombyx mori	chymotrypsin	7	sequence	24
Bombyx mori	chymotrypsin	5.5, 5.9, 8.5, 39	25	
Bombyx mori	trypsin, chymotrypsin	41 41	cDNA sequence sequence	26,27 28
Antheraea pernyi	trypsin	---	---	29
Manduca sexta	PO cascade, plasmin, chymotrypsin, trypsin	14 (dimer) 8	AAA, partial seq.	30,31
Manduca sexta	chymotrypsin, p elastase	47	cDNA sequence	32
Sarcophaga bullata	chymotrypsin, trypsin plasmin, thrombin	mass spectr. 5000	32,33 sequence	
Sarcophaga peregrina	insect cysteine protease	9.5, 10	AAA	34
Locusta migratoria I	PO cascade chymotrypsin, HLE	3.76	sequence	16
Locusta migratotia II	PO cascade, chymotrypsin pancreatic elastase	3.8	sequence	16

* Amino Acid Analysis

inactive counterpart from phase II bacteria, treated in the same experimental conditions, were reduced in volume and chromatographed on a Sephadex G 25. Only phase I bacterial extract produced a distinct fraction with inhibitory activity toward pro-PO-AS (Fig. 7).

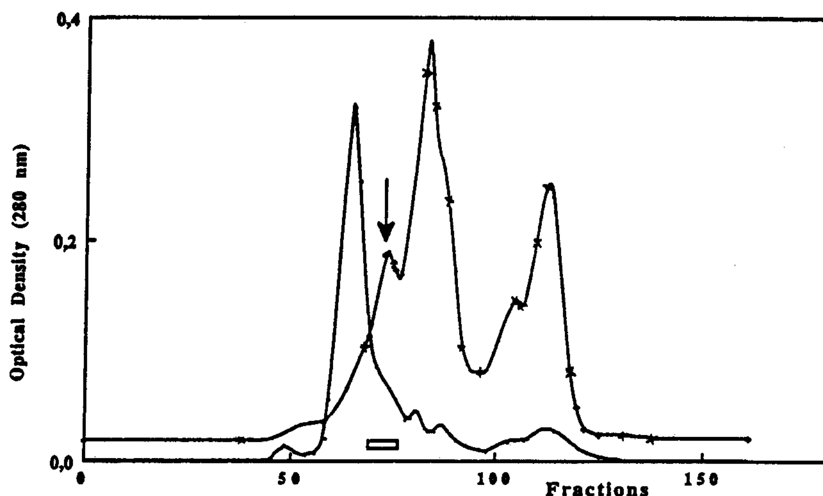


Fig. 7. Sephadex G 25 chromatography of *Xenorhabdus nematophilus* phase 1 (+ – +) and phase 2 culture medium. The arrow indicates the inhibitory activity toward pro-PO-AS.

The final aim of this research is to study the proteolytic cascade controlling the insect immunity and its inhibitors. Hopefully, it will allow to guide the immunological activity of insects either by depressing or by enhancing it, for noxious or useful species, respectively.

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