Characterization of a novel elastase inhibitor from a fan coral

Caractérisation d’un nouvel inhibiteur d’élastase isolé d’une gorgone

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RÉSUMÉ
L’extrait méthanol-acide acétique de la gorgone Melithaea cf. stormii présente une activité anti-élastase. A partir du mélange de peptides, nous avons isolé et purifié jusqu’à homogénéité une protéine dont la masse moléculaire, 21 159 daltons, a été déterminée par spectrométrie de masse Maldi/Tof. Cette nouvelle protéine d’origine marine, nommée iela melst, inhibe fortement l’amidolyse du Suc(Ala)\_pNA par l’élastase pancréatique porcine. La séquence des 39 résidus de la partie N-terminale présente les caractéristiques d’un domaine de type Kazal non classique. Iela melst se comporte comme un inhibiteur réversible de l’élastase pancréatique porcine de type liaison forte. L’inhibition compétitive suit le mécanisme A proposé par Cha avec une constante de dissociation à l’équilibre \( K_a \) de 1,5 \( \times 10^{-9} \) M. ▲

Mots clés: protéine inhibitrice de l’élastase pancréatique porcine, inhibiteur de type liaison forte, gorgone, Melithaea cf. stormii.

KEY WORDS: porcine pancreatic elastase inhibitor, tight-binding inhibitor, gorgonian, Melithaea cf. stormii.

ABSTRACT
An acidic hydromethanolic extract of the tropical gorgonian Melithaea cf. stormii exhibited anti- elastase activity. From the polymeric mixture we isolated and purified to homogeneity a protein with a molecular mass determined at 21,159 Da by Maldi/Tof mass spectrometric analysis. The novel protein of marine invertebrate origin strongly inhibited amidolysis of Suc(Ala)\_pNA by porcine pancreatic elastase (PPE) and was labeled iella melst. The N-terminal aminocacid sequence of its 39-first residues revealed the characteristics of a non-classical Kazal-type domain. Iella melst behaved as a reversible tight-binding inhibitor of PPE. The competitive inhibition followed Cha mechanism A with an equilibrium dissociation constant, \( K_a \), calculated at 1.5 \( \times 10^{-9} \) M. ▲

Key words: porcine pancreatic elastase inhibitor, tight-binding inhibitor, gorgonian, Melithaea cf. stormii.

VERSION ABRÉGÉE
La gorgone d’origine tropicale Melithaea cf. stormii se caractérise par une forte activité anti-élastase de l’extrait protéique. De celui-ci nous avons isolé, après plusieurs chromatographies d’exclusion (Sephadex G-75 et Superdex 75 HR) et en phase inverse (Nucleosil C18 et Inertil ODS2), une protéine dont la pureté a été estimée > 95% par sequencing des acides aminés N-terminaux. La masse moléculaire a été établie à 21 159 Da par spectrométrie de masse Maldi/Tof. Une solution de cette protéine (14,3 nM), à 25°C, dans un milieu tamponné à pH 8, inhibe à 50% la vitesse d’amidolyse du Suc(Ala)\_pNA (1 500 nM) par l’élastase pancréatique porcine (EPP) (7,5 nM).

La séquence en acides aminés des 39 premiers résidus N-terminaux a été réalisée de façon automatique sur la protéine entière. La comparaison de structures primaires protéiques à l’aide de l’algorithme FASTA à travers les banques de données PIR et SWISS-PROT fait ressortir une forte homologie de séquence (65% pour les 20 premiers résidus) avec iella anesu. Cette dernière, extraite d’une anémone de mer Anemonia sulcata, présente une activité inhibitrice d’élastase et possède un domaine structural de type Kazal non classique. L’inhibiteur produit par Melithaea cf. stormii, dorénavant dénommé iella melst, renferme 3 cystéines aux positions correspondantes des demi-cystines I, II et III de iella anesu. La longueur du segment Cys II-Cys III (7 résidus) est caractéristique de celle des inhibiteurs de type Kazal alors que l’on sait que le segment Cys I-Cys II peut varier de 1 à 16 résidus. Les résidus correspondant aux positions Cys IV, V et VI n’ont pour l’instant pas été identifiés, ce qui empêche toute hypothèse sur l’arrangement des 3 ponts disulfures prévisibles.

En manifestant son activité anti-élastase à des concentrations proches de celle de l’enzyme, iella melst se comporte comme un
inhibiteur de type liaison forte. L'étude cinétique du mécanisme d'inhibition a été réalisée suivant la méthode analytique de Cha. L'amidolyse du Suc(Ala)pNA (0,5; 0,75; 1,0 ou 1,25 nM) par l'EPP (5 nM) est suivie à 410 nm et à différentes concentrations d'ela mélée (1; 5; 10; 15; 20 et 25 nM). La vitesse d'hydrolyse suit une loi exponentielle et tend vers une limite dont la valeur dépend de la concentration en inhibiteur. Celui-ci agit donc d'une façon réversible. Le traitement informatique des données expérimentales a été réalisé à l'aide du logiciel ENZFITTER, selon l'algorithme \( P = \frac{v_f}{1 - e^{-(v_f/v)k + Pi}} \), où \( P \) et \( Pi \) représentent respectivement l'absorbance aux temps t et zéro. On calcule ainsi la constante de vitesse apparente \( k \) d'ordre 1 pour la décroissance exponentielle de la concentration en substrat, ainsi que les vitesses à l'état stationnaire \( v_s \) et à l'état initial \( v_i \) de l'hydrolyse du substrat. Pour une concentration en substrat donnée on observe une relation linéaire entre la constante \( k \) et la concentration en inhibiteur. Cela démontrerait que l'inhibition suit le mécanisme A défini par Cha où l'enzyme et l'inhibiteur s'associent pour former un complexe inactif d'une façon réversible et compétitive sans accumulation d'intermédiaire initial. L'hypothèse est confortée par le fait que la vitesse initiale \( v_i \) est indépendante de la concentration d'inhibiteur. Les constantes d'association \( k_1 = (3,6 \pm 0,3) \times 10^3 \text{M}^{-1}\text{s}^{-1} \) et de dissociation \( k_2 = (5,4 \pm 0,4) \times 10^{-4} \text{s}^{-1} \) pour l'équilibre EPP-ela mélée sont déduites des relations \( k_{1} = \frac{(v_f - v_s)}{v_i} + k_i1/1 + \frac{v_f}{(v_s)} \) où \( S \) et \( K \) sont respectivement la concentration et la constante de Michaelis-Menten du substrat. Finalement on en déduit la constante de dissociation \( k_1 \) à l'état initial \( k_{i1} \) du complexe EPP-ela mélée géale à 1,5 \( \times 10^{-9} \text{M} \). L'inhibition de l'EPP par la mélée est comparable à celle d'une protéine antiléucoprotéasine d'origine humaine nommée elfin \( (K_1 = 1 \times 10^{-9} \text{M}) \) isolée de squames chez des patients présentant des lésions de type psoriasis.

**Materials and methods**

**Specimen collection**

*M. cf. stornii* specimens were collected from the outer reef slopes of Ulitoe Pass, Southern Province, New Caledonia, at approximately 20 m depth. The batches were immersed into a methanol/1M acetic acid mixture (70/30) prior to deep-freezing. Voucher specimens were housed at ORSTOM, BP A5 Noumea Cedex, New Caledonia, under the code HG163, together with photographic records.

**Protein isolation and purification**

The acidic hydromethanolic extract from 0.5 kg of original material yielded 4 g of crude extract after freeze-drying.

Samples (500 mg) were fractionated by size-exclusion chromatography on a 2.8 \( \times \) 50 cm G75 Sephadex gel column (Pharmacia) running at 30 ml/h after equilibration with 50 mm ammonium bicarbonate.

Fractions containing the elastase inhibitor were pooled and separated using an HPLC system (LKB) equipped with a 10 mm Nucleosil C18, 1 \( \times \) 25 cm column (SFCO) through a 50-min 0.1% trifluoroacetic acid aqueous solution to acetonic gradient (0 to 67%). Fractions with anti-elastase activity were pumped and submitted again to reverse-phase chromatography using a 5 mm Inertisil ODS2, 0.46 \( \times \) 25 cm Interchrom column (Interchrom) and a 40-min, 0-90% acetonic gradient. Active fractions were then submitted to size-exclusion HPLC on a Superdex 75 HR 10/30 column (Pharmacia) eluted with 50 mM ammonium bicarbonate. Inhibitor-containing fractions were dialyzed against distilled water then lyophilized. The preparation was checked for purity by N-terminal sequence analysis (see Protein sequencing).

**Protein assay**

Protein contents were assayed using the bicinchoninic acid procedure (BCA kit by Pierce).

**PPE inhibitor assays**

The purification procedure was monitored by measuring the inhibition of the amidolysis of N-succinyl-alanyl-alanyl- alanyl p-nitroanilide (Suc(Ala)pNA) (Sigma) by porcine pancreatic elastase (PPE) (BIOSYS) at 410 nm. Assays were performed in 0.1 M Tris buffer, pH 8, containing 0.1 M NaCl, 0.1 ml/ml Triton X-100 and 0.1 ml/ml dimethyl sulfoxide, using a Ceres 500 kinetic microplate reader (Bio-tec instruments) in 96-well microplates. Equal 95 μl aliquots of 16 mM PPE and of different concentrations of gorgonian extracts in pH 8 buffer were mixed at 25°C for 20 min prior to addition of 10 μl of substrate solution (30 μmol of Suc(Ala)pNA in 1 ml of dimethyl sulfoxide) in order to assay the remaining elastase activity. Final concentrations of PPE and substrate were 7.5 nM and 1.5 mM, respectively. In absence of inhibition the increase in product concentration, assuming \( t_0 = 10,000 \text{M}^{-1}\text{cm}^{-1} \), was 2 μM min⁻¹. With a concentration of the inhibitor causing ≤ 60% inhibition, the assay was virtually linear for at least 10 min. One
Elastase inhibitor from a fan coral

Figure 1. Comparisons of amino acid sequences between iela melst and other protease inhibitors presenting best homology. Alignments are tentative and do not include gaps in order to place the half-cystines in corresponding positions. Iela, Ipst and Iovo are inhibitors of porcine pancreatic elastase, bovine pancreatic secretory trypsin and avian ovomucoid, respectively. The star pair indicates PI-PI' residues around putative reactive site. Roman numbers indicate half-cystines of the non-classical Kazal-type inhibitor iela anesu. Arabic numerals indicate residue numbers from N-terminal (left) and total residues of proteins (right). Residues with strict identity to iela melst sequence are boxed.

The kinetic of the inhibitory activity of the purified gorgonian protein

The kinetic of inhibition of PPE activity by purified protein from gorgonian extract was studied by an analytical procedure described for tight-binding inhibitors [10, 11]. In one set of experiments the inhibitor and the substrate Suc(Ala)pNA were mixed at 25°C in 990 μl of pH 8 buffer (as defined above) and the progressive inhibition of elastase activity was monitored after the reaction was started by addition of 10 μl of PPE. In a second set of experiments the inhibitor and elastase were incubated at 25°C in pH 8 buffer for 20 min. Then the addition of 30 μl of solution of Suc(Ala)pNA in dimethyl sulfoxide enabled to follow the progressive dissociation of the enzyme-inhibitor complex. For both types of experiments, final concentrations of PPE, substrate and inhibitor were 5 nM, 0.5–0.75–1.0–1.25 μM and 0–1–5–10–15–20–25 nM, respectively. The amidolysis of Suc(Ala)pNA was monitored at 410 nm using a UVikon 930 spectrophotometer (Kontron). The 180 absorbance values collected at 20 seconds intervals were fitted to equation (1) (see Results) by nonlinear regression using Enzfitter software (Biosoft, Cambridge, UK).
Results

Purification and molecular mass determination

The novel inhibitor of porcine pancreatic elastase was purified from crude extracts of the tropical gorgonian Melithaea cf. Stormii by classical size-exclusion liquid chromatography then by reverse-phase and size-exclusion high performance liquid chromatography (HPLC) analyses. The protocol used for purification is summarized in Table I. The purity of the protein which eluted as a single sharp peak from Superdex 75 HPLC was estimated >95% from automated N-terminal aminoacid sequence determination. Analysis by matrix assisted laser desorption ionization/time of flight (MALDI/TOF) determined a molecular weight of 21,159 Da.

N-terminal amino acid sequence

The partial sequence determination encompassing 39 residues from the amino end of the molecule, is displayed in Figure 1. This sequence carries a non-classical Kazal-type domain, presenting characteristics typical of all natural elastase inhibitors [12], and for convenience we hereafter refer to our protein as iela melst, in accordance with major database nomenclature. Examples of variants from homologous sequences, including residue types at P1-P1' positions are listed for comparison. They include vertebrate and invertebrate sequences presenting highest identity scores through available protein databanks. Strict sequence identity score between homologous protein zones of Melithaea cf. stormii and another coelenterate, A. sulcata is high (65% for the first 20 residues), and both display significant antielastase activity [6]. The putative iela melst reactive site P1-P1' was located at positions 7-8, very close to the N-terminus of the 39-residues sequence, and identified by sequence homology as P1 = leucine and P1' = isoleucine. P1 as Leu and Met are typical of elastase inhibitors of the Kazal family while P1' identity requirement are much broader for site reactivity [12]. Within the region surrounding the reactive site of the aligned sequences, the P1 and P1' positions are the least conserved. Such hypervariability is well documented in vertebrate Kazal domains [12]. Common to all (but one) sequences are Cys I at position 7 at the N-terminus. Moreover, position 24 of the gorgonian protein is a Gly residue whereas all other serine protease inhibitors hold Cys IV in this position.

Mechanism of the inhibition of porcine pancreatic elastase activity by iela melst

Figure 2 shows that the protein purified from gorgonian extracts exerted its inhibitory effect on the amidolysis of Suc(Ala),pNA by PPE at a concentration comparable to that of the elastase. The rate of amidolysis decreased exponentially over a period of several minutes and reached a limit value. At fixed concentrations of enzyme, substrate and inhibitor, that limit value was independent whether the reaction was started by addition of PPE or by addition of the substrate to a previously incubated mixture of enzyme and inhibitor. This is exemplified in Figure 2 with an inhibitor concentration of 20 nM. The limit rates of concave-down and concave-up progress curves were (1.96±0.007) x 10^{-3} μM s^{-1} and (1.91±0.008) x 10^{-3} μM s^{-1}, respectively, while less than 3% of the substrate were consumed. The data indicated that the reaction had reached an overall steady-state [10]. Moreover, the steady-state rate value decreased with increasing concentrations of inhibitor but did not reach zero. Therefore, the association of PPE and iela melst appeared to be reversible [13].

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**Table I**

Purification of elastase inhibitor from 4 g gorgonian tissue homogenate. One inhibitory unit (IU) is defined as the amount of gorgonian extract that reduces the increase in nitroanilide concentration by 1 μM/min under experimental conditions described in Materials and methods

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Activity (IU x 10^{-2})</th>
<th>Specific activity (IU/mg x 10^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>950</td>
<td>480</td>
<td>0.5</td>
</tr>
<tr>
<td>G75-Sephadex</td>
<td>35</td>
<td>52.4</td>
<td>1.5</td>
</tr>
<tr>
<td>C18 HPLC-1</td>
<td>7</td>
<td>10.5</td>
<td>1.5</td>
</tr>
<tr>
<td>C18 HPLC-2</td>
<td>0.2</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Superdex 75 HR</td>
<td>0.18</td>
<td>3</td>
<td>16.6</td>
</tr>
</tbody>
</table>

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**Figure 2. Amidolysis of Suc(Ala),pNA by PPE in presence of different concentrations of iela melst.** Reaction conditions: elastase, 5 nM; substrate, 1,250 μM; inhibitor (from top to bottom) 0, 5, 10, 20, 25 and 200 nM, in pH 8.0 buffer. Open circles: reactions were initiated by addition of elastase to a mixture of substrate and inhibitor. Filled circles: reaction was started by addition of substrate to a mixture of elastase and inhibitor preincubated for 20 min. The circles shown are digitized experimental values. Solid lines are theoretical curves computed by fitting experimental data to equation (1) as indicated in Results.
The reversible inhibition of an enzyme by tight-binding inhibitor may follow several mechanisms for which the rate of increase in product concentration varies with time according to [10]:

\[ P = v_t - (v_i - v) \left(1 - e^{-kt}\right)/k + PI \]  

(1)

where \( P \) and \( PI \) are the absorbance at \( t \) and zero time, \( v_i \) and \( v \) the initial and steady-state rates, respectively. \( k \) is an apparent first-order rate constant depending on the type of mechanism [10]. 

**Figure 2** shows the theoretical curves obtained by fitting into equation (1) the experimental data collected for the amidolysis of Suc(Ala),pNA (1.25 mM), in presence of various concentrations of \( \text{ela melst} \), by addition of PPE (5 nM). The mean value calculated for \( v_i \) with 6 different concentrations of inhibitor (1, 5, 10, 15, 20 and 25 nM) was \((1.8 \pm 0.2) \times 10^{-2} \mu M \cdot s^{-1} \). As \( v \) was determined as \((2.1 \pm 0.08) \times 10^{-2} \mu M \cdot s^{-1} \) in absence of inhibitor we might deduce that the initial velocity of substrate amidolysis was independent of the concentration of inhibitor. Moreover, at each fixed substrate concentration (0.5, 0.75, 1.0 and 1.25 mM) the calculated values for \( k \) presented a linear relationship with inhibitor concentration. As shown in **Figure 3**, for substrate concentrations of 0.75 and 1.25 mM, the plot of \( k \) vs inhibitor concentrations [I] gave straight lines with positive slopes. All the results are consistent with a simple competitive inhibition corresponding to Cha's mechanism A [10, 14]. Therefore, the association rate constant \( k_a \) and the dissociation rate constant \( k_d \) for the complex between \( \text{ela melst} \) and the elastase can be determined from plots shown in **Figure 3**, according to [10]:

\[ k = k_{a} + k_{d}[I]/(1 + [S]/K_c) \]

where \([S]\) is the substrate concentration and \( K_c \) the Michaelis constant of PPE. From the slopes and intercepts values determined with 4 different substrate concentrations (0.5, 0.75, 1.0 and 1.25 mM) we calculated a mean value of \((3.6 \pm 0.3) \times 10^{5} M^{-1} \cdot s^{-1} \) for \( k_a \), assuming a \( K_c \) of 1 mM for PPE, and of \((7.8 \pm 1.5) \times 10^{-4} s^{-1} \) for \( k_d \). However, the relation \( k_{d} = k(\nu/\nu_i) \) allows a more accurate determination of the dissociation rate constant [15]. Data for \( k_a, \nu \) and \( \nu_i \) were collected from 24 progress curves of the amidolysis of the substrate, as described in Materials and methods and shown in **Figure 2** for \([S] = 1.25 \text{ mM}. \) The mean value calculated for \( k_d \), was then \((5.4 \pm 0.4) \times 10^{-4} s^{-1} \).

Finally we deduced for the PPE-\( \text{ela melst} \) complex an equilibrium dissociation constant of \( 1.5 \times 10^{-4} M \) by using the relationship \( K_c = k_{d}/k_a \) [10].

**Discussion**

\( \text{ela melst} \) is a novel elastase inhibitor produced by the gorgonian *Meliithea cf. stormi*. The protein purified by size-exclusion and reverse-phase liquid chromatography presented a purity >95% as judged by N-terminal amino acid sequencing. Analysis by Maldi/Tof mass spectrometry determined a molecular weight of 21,159 Da which is closer to reported values for skin derived antileukoproteinases of human origin [16, 17] than to the antielastolytic product from the sea anemone *Anemone sulcata*, \( \text{ela anesu} \), that encompasses 48 residues [6], a size matching well with small vertebrate Kazal-type proteins, **Figure 1**.

The amino acid sequence of the 20-first N-terminal residues, **Figure 1**, was clearly homologous to that of \( \text{ela anesu} \), a non-classical Kazal-type elastase inhibitor [5]. As for all inhibitors of that type, both proteins presented a CysII-Cys III segment of 7 residues whereas only 3 residues were found between CysI and II. That segment is known to be hypervariable since it can hold from 1 to 16 residues for inhibitors of the Kazal-type [5]. Residues corresponding to the positions of CysV and VI as well as to the position next to CysIV were not identified. This situation does not allow for any prediction as to how the 6 putative gorgonian half cystines could link up.

\( \text{ela melst} \) inhibited the amidolysis of Suc(Ala),pNA by PPE at concentrations very close to those of the elastase. Kinetically studies developed according to the analytical procedure of Cha [10, 11] demonstrated that the protein worked as a slow tight-binding inhibitor. The reversible and competitive formation of the \( \text{ela melst}-\text{PPE} \) complex would proceed via a single step as described by Cha's A mechanism [10, 14] without accumulation of an initial intermediate.

The equilibrium dissociation constant \( K_c (1.5 \times 10^{-9} M) \) of the complex, as deduced from computer assisted determination of the association and dissociation rate constants, was almost identical to that of the equilibrium between PPE and elafin, \( K_c = 1 \times 10^{-9} M \) [18], an antieleukoproteinase isolated from scales of patients with psoriasis [19]. In bulk, that value compared well with most of those published for natural [12, 20] and synthetic [21, 22] inhibitors of mammalian elastases.

The presence of proteinase inhibitors in gorgonian cortical tissues may counterbalance the various necrotic processes observed in colonial cnidarians, either induced by extracoelenteral digestion by other cnidarian predators [23], or resulting from allelopathy [24], or as antinecrotic healing agents when dealing with epibiotic settlers, prior to the production of undifferentiated "scar" secretions [25].
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8. Voucher specimen number HG 163 housed at ORSTOM, see author's address.