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Biochimie/*Biochemistry*

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 *Melithea cf. stormii* présente une activité antiélastase. 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 $Suc(Ala)_3pNA$ 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_i de $1,5 \times 10^{-9}$ M. ▲

Mots clés : *protéine inhibitrice de l'élastase pancréatique porcine, inhibiteur de type liaison forte, gorgone,* Melithea cf. stormii.

ABSTRACT

An acidic hydromethanolic extract of the tropical gorgonian Melithea cf. stormii *exhibited antielastase activity. From the polypeptidic 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 $Suc(Ala)_3pNA$ by porcine pancreatic elastase (PPE) and was labelled iela melst. The N-terminal aminoacid sequence of its 39-first residues revealed the characteristics of a non-classical Kazal-type domain. Iela melst behaved as a reversible tight-binding inhibitor of PPE. The competitive inhibition followed Cha's mechanism A with an equilibrium dissociation constant, K_i, calculated as 1.5×10^{-9} M.* ▲

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

VERSION ABRÉGÉE

La gorgone d'origine tropicale *Melithea cf. stormii* se caractérise par une forte activité antiélastase 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 inversée (Nucléosil C18 et Inertsil ODS2), une protéine dont la pureté a été estimée > 95 % par séquençage 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 $Suc(Ala)_3pNA$ (1 500 µM) 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 iela 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 *Melithea cf. stormii*, dorénavant dénommé iela melst, renferme 3 cystéines aux positions correspondantes des demi-cystines I, II et III de iela 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 antiélastase à des concentrations proches de celle de l'enzyme, iela melst se comporte comme un

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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)$_3$pNA (0,5 ; 0,75 ; 1,0 ou 1,25 mM) par l'EPP (5 nM) est suivie à 410 nm et à différentes concentrations d'iela melst (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 $P = v_s t - (v_s - v_i)(1 - e^{-kt})/k + Pi$, 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 réversibl 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 en inhibiteur. Les constantes d'association $k_1 = (3,6 \pm 0,3) \times 10^5\ M^{-1}s^{-1}$ et de dissociation $k_{-1} = (5,4 \pm 0,4) \times 10^{-4}\ s^{-1}$ pour l'équilibre EPP – iela melst sont déduites des relations $k_{-1} = (v_s/v_i)$ et $k = k_{-1} + k_1\,[I]/(1 + [S]/K_m)$, où [S] et K_m sont respectivement la concentration et la constante de Michaelis-Menten du substrat. Finalement on en déduit la constante de dissociation à l'équilibre $K_i = k_{-1}/k_1$ du complexe EPP-iela melst égale à $1,5 \times 10^{-9}$ M. L'inhibition de l'EPP par iela melst est comparable à celle d'une protéine antileucoprotéinase d'origine humaine nommée elafin ($K_i = 1 \times 10^{-9}$ M) isolée de squames chez des patients présentant des lésions de type psoriasis. ▲

Known bioactive proteins from marine invertebrates remain rather scarce, in contrast to secondary metabolites where 25 years of extensive investigations have led to structural elucidation and pharmacological evaluation of thousands of molecules of all classes, as part of extensive taxonomy-oriented screening programs ([1] and references therein).

Published work on marine bioactive proteins most often deal with venoms obtained from secretory glands of toxic species of few taxa [2], which often display enzyme activity [3] or being classified as lectins, proteins for the detection of lectins or vitamin B_{12}-binding proteins [4].

Moreover biochemical studies on benthic invertebrate proteins include serine proteinase inhibitors, *i.e.* serpins, described as classical types or non-classical variants [5] of the terrestrial models. Such inhibitors may provide inspiration for new modulators of enzyme dysfunctions in important pathologies, *e.g.* rheumatoid polyarthritis or lung emphysema [6].

A preliminary screening program for detection of antielastolytic peptides enabled us to delineate taxonomic groups of promise across common benthic invertebrates from coral reef environments [7]. We now report the isolation of a polypeptide purified from the tropical gorgonian *Melithea cf. stormii* (Studer, 1895) [8], as a novel inhibitor of porcine pancreatic elastase. The mechanism of the inhibition is discussed and a putative reactive site is deduced from the partial N-terminal amino acid sequence.

Materials and methods

Specimen collection

M. cf. stormii specimens were collected from the outer reef slopes of Uitoe Pass, Southern Province, New Caledonia, at approximately 20 m depth. The batches were immersed into a methanol/1 M acetic acid mixture (70/30) prior to deep-freezing. Voucher specimens are 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 x 50 cm G75 Sephadex gel column (Pharmacia) running at 30 ml/h after equilibration with 50 µm ammonium bicarbonate.

Fractions containing the elastase inhibitor were pooled and separated using an HPLC system (LKB) equipped with a 10 µm Nucleosil C18, 1 x 25 cm column (SFCC) through a 50-min 0.1% trifluoroacetic acid aqueous solution to acetonitrile gradient (0 to 67%). Fractions with anti-elastase activity were lumped and submitted again to reverse-phase chromatography using a 5 µm Inertsil ODS2, 0.46 x 25 cm Interchrom column (Interchim) and a 40-min, 0-50% acetonitrile 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)$_3$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 900 kinetic microplate reader (Bio-tek Instruments) in 96-well microplates. Equal 95 µl aliquots of 16 nM 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 µmoles of Suc(Ala)$_3$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 $\varepsilon_{410nm} = 10{,}000\ M^{-1}cm^{-1}$, 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

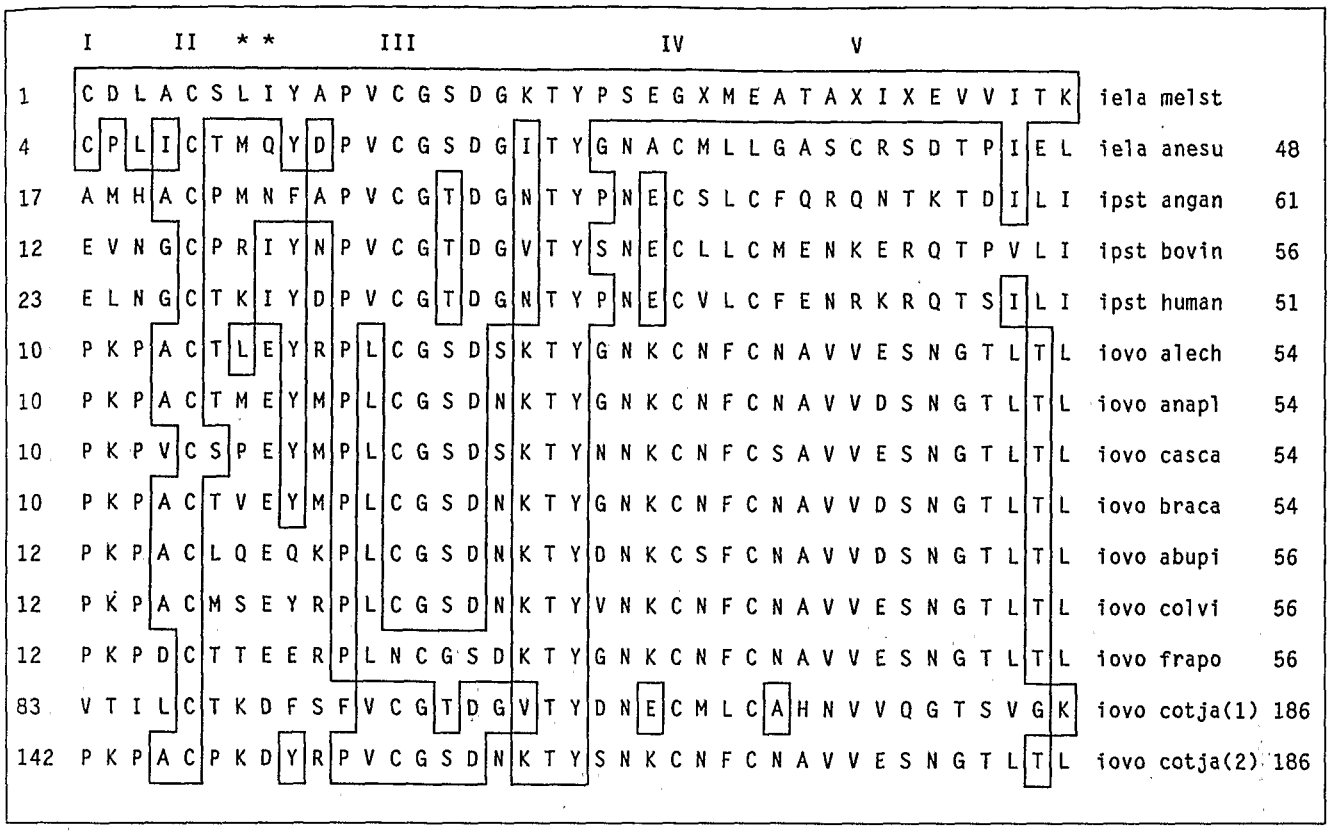

Figure 1. ***Comparisons of amino acid sequences between iela melst and other protease inhibitors presenting best homology.*** *Alignments are tentative and do not include gaps in order to place the half-cystines in corresponding positions. Iela, ipst and iovo are inhibitors of porcine pancreatic elastase, bovine pancreatic secretory trypsin and avian ovomucoid, respectively. The star pair indicates P1-P1' residues around putative reactive site. Roman numbers indicate half-cystines of the non-classical Kazal-type inhibitor iela anesu. Arabic numerals indicate residue numbers form N-terminal (left) and total residues of proteins (right). Residues with strict identity to iela melst sequence are boxed.*

inhibition unit (IU) was defined as the amount of inhibitor which reduced the increase in anilide concentration by 1 µM per min.

Molecular mass determination

The molecular weight of the protein was determined by matrix assisted laser desorption mass spectrometric analysis using a VG analytical Tofspec mass spectrometer equipped with a 337 nm laser. A 25 kV acceleration voltage was used. Samples were prepared by mixing 1 µl of protein solution (10 µM) to 1.5 µl matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid).

Protein sequencing

The N-terminal amino acid sequence was determined by Edman degradation using a 470A protein sequencer (ABI Perkin Elmer). Phenylthiohydantoin derivatives of amino acids were identified using a 120A PTH analyser. For sequence comparisons the PIR and SWISSPROT databanks were searched using the Pearson and Lipman algorithm FASTA [9].

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 $Suc(Ala)_3pNA$ 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 a solution of $Suc(Ala)_3pNA$ 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 mM and 0 – 1 – 5 – 10 – 15 – 20 – 25 nM, respectively. The amidolysis of $Suc(Ala)_3pNA$ 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 *Melithea 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 *Melithea 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 II and III located at the P3 and P6′ positions from the reactive sites (*i.e.* residues 4 and 12 of iela melst sequence), a preliminary requirement towards being classified as Kazal inhibitors. Unique to iela melst and the protein iela anesu from the other cnidarian *A. sulcata*, is 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.

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 *p*-nitroanilide concentration by 1 µM/min under experimental conditions described in *Materials and methods*

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

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)$_3$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 examplified in *Figure 2* with an inhibitor concentration of 20 nM. The limit rates of concave-down and concave-up progress curves were $(1.96 \pm 0.007) \times 10^{-3}$ µM s^{-1} and $(1.91 \pm 0.008) \times 10^{-3}$ 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].

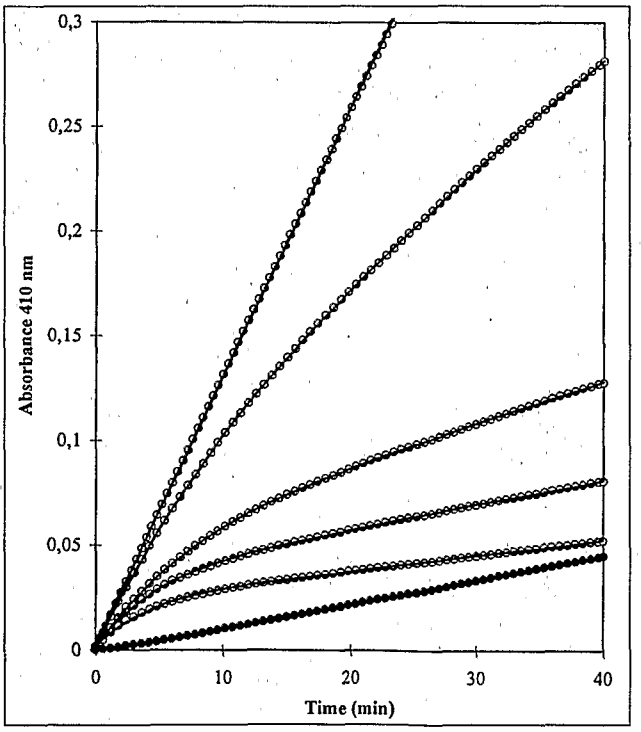

Figure 2. ***Amidolysis of Suc(Ala)$_3$pNA by PPE in presence of different concentrations of iela melst.*** *Reaction conditions: elastase, 5 nM; substrate, 1,250 µM; inhibitor (from top to bottom) 0, 5, 10, 20, 25 and 20 nM, in pH 8.0 buffer. Open circles: reactions were initiated by addition of elastase to a mixture of substrate and inhibitor. Filled circles: reaction was started by addition of substrate to a mixture of elastase and inhibotor prealably incubated for 20 min. The circles shown are digitized experimental values. Solid lines are theorical 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]:

$$P = v_s t - (v_s - v_i)(1 - e^{-kt})/k + Pi \qquad (1)$$

P and Pi are the absorbance at t and zero time, v_i and v_s 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 theorical curves obtained by fitting into equation (1) the experimental data collected for the amidolysis of $Suc(Ala)_3pNA$ (1.25 mM), in presence of various concentrations of iela 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 $(1.8 \pm 0.2) \times 10^{-2}$ µM s^{-1}. As v_i was determined as $(2.1 \pm 0.08) \times 10^{-2}$ µM 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_1 and the dissociation rate constant k_{-1} for the complex between iela melst and the elastase can be determined from plots shown in *Figure 3*, according to [10]:

$$k = k_{-1} + k_1[I]/(1 + [S]/K_m)$$

where [S] is the substrate concentration and K_m 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 $(3.6 \pm 0.3) \times 10^5$ $M^{-1}s^{-1}$ for k_1, assuming a K_m of 1 mM for PPE, and of $(7.8 \pm 1.5) \times 10^{-4}$ s^{-1} for k_{-1}. However, the relation $k_{-1} = k\,(v_s/v_i)$ allows a more accurate determination of the dissociation rate constant [15]. Data for k, v_s and v_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 mM. The mean value calculated for k_{-1} was then $(5.4 \pm 0.4) \times 10^{-4}$ s^{-1}.

Finally we deduced for the PPE-iela melst complex an equilibrium dissociation constant of 1.5×10^{-9} M by using the relationship $K_i = k_{-1}/k_1$ [10].

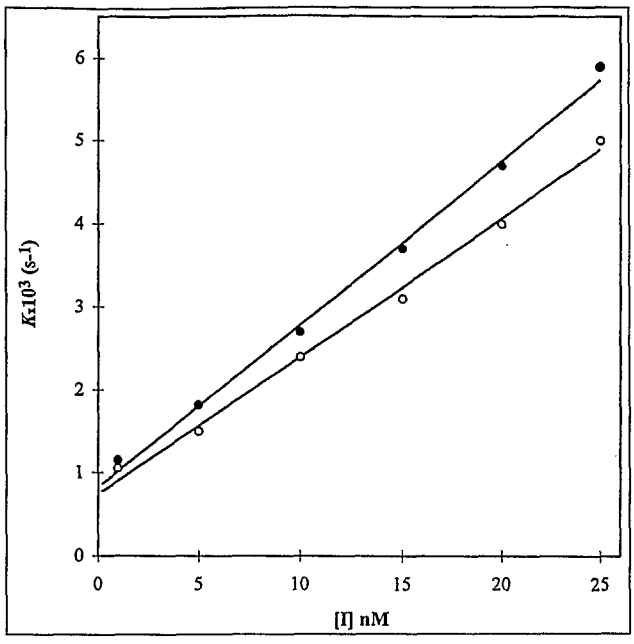

Figure 3. ***Plots of the apparent first-order rate constant*** **k** ***for the exponential phase of the inhibition of substrate amidolysis*** **versus** ***iela melst concentrations.*** k *values were determined as indicated in* Results. *Reactions were initiated by addition of elastase (final concentration 5 nM). Substrates concentrations were 750 µM (top) and 1,250 µM (bottom).*

Discussion

Iela melst is a novel elastase inhibitor produced by the gorgonian *Melithea cf. stormii*. 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 *Anemonia sulcata*, iela 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 iela anesu, a non-classical Kazal-type elastase inhibitor [5]. As for all inhibitors of that type, both proteins presented a Cys II-Cys III segment of 7 residues whereas only 3 residues were found between Cys I 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 Cys V and VI as well as to the position next to Cys IV were not identified. This situation does not allow for any prediction as to how the 6 putative gorgonian half cystines could link up.

Iela melst inhibited the amidolysis of $Suc(Ala)_3pNA$ by PPE at concentrations very close to those of the elastase. Kinetic 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 iela melst-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_i (1.5×10^{-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, ($K_i = 1 \times 10^{-9}$ M) [18], an antileuko-proteinase 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 extra-coelenteral 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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